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On the Centroides of Human Knee Joints using Photographic Method

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Abstract: The kinematics characteristics of the human knee joint can be represented by its centroides. The sequential X-ray radiographs of the moving knee can provide the basic data for constructing the centroides if the cost and side effects of radiation are not in concern. This paper provides an alternative method to construct the centroides by using commercial digital camera to take the sequential pictures. In order to eliminate the undesired movements, a testing chair and a brace are specially designed. Two types of curve fitting methods are introduced to smooth the measured data of marked points on the lower leg. The differential method is applied to construct the centroides of knee joint from the measured data. This paper provides a non-expensive, non-invasion, and non-radiation way to study the centroides of human knee joint.

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Keywords Human Knee Joint, Centroides, Polodes, Curve fitting, Photograph

1. Introduction

Total knee arthroplasty is one of the best choices for treating the severe osteoarthritis of knee which has been approved by the orthopedic community. The researches of knee arthroplasty have been focused on several topics like materials of artificial knee, design of artificial knee, manufacturing of artificial knee, surgical operation, and rehabilitation.

Nwoye et al. derived a model to calculate the concentration of upgraded iron for production of stainless steel based biomedical devices used in Orthopaedics^[1]. The kinematics theories have been applied to study the biomechanics of human knee joints^[2-3] and the performance of knee prosthesis^[4]. Centroides of four-bar linkage type knee prosthesis were studied by Menschik^[5] and Van de Vee^[6]. The centroides have been also applied to study the characteristic of injured knee joints^[7]. Lee and Chang developed the custom-made femoral stem by integrated investigation of CAD/CAM^[8]. Lee et al. studied the rapid prototyping for the femoral component of knee prosthesis and by multi-axis NC Machining^[9]. Shereif and Hassanin made a comparison between uses of therapeutic exercise and heat application on relieve pain, stiffness and improvement of physical function for patient with knee Osteoarthritis^[10]. Tien and Yu found the Tai chi exercise affected the isokinetic torque but not changed hamstrings^[11].

The centroides of human joint can be constructed from a sequential X-ray radiographs of the moving knee. But the cost and side effects of radiation are bothersome. This paper aims to develop a non-expensive, non-invasion, and non-radiation way for people to study the centroides of human knee joint.

2. Equipment Setup and Calibration

A Sony digital camera $\alpha 55$ equipped with 50~200mm lens is used for taking pictures of the human knee joints. It can take 10 high resolution pictures, 16.2 Mega pixels, per second. Since there exists the distortion problem in most of the commercial camera lenses, the accuracy checking and compensation processes of the photographs are required. A 1200mm x 900mm reference grid paper (Figure 1) is used for making correction of the photographs. In figure 1, the space between horizontal grid lines or vertical grid lines are 100mm and the increments of radii of the circles are 100mm.

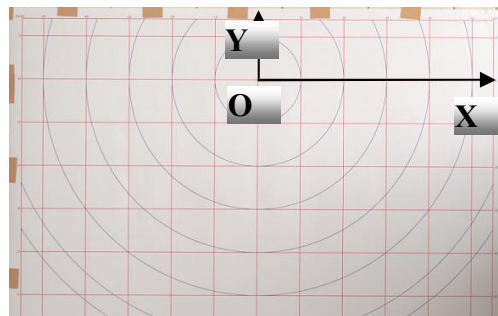


Figure 1. Reference grid for lens correction

The camera is set on a tripod aimed the center point of circles at right angle at distance 5,000mm away from the reference grid. The differences among the actual data and measured data from the captured photograph are shown in tables 1 and 2. Therefore the coordinates of a point in the photograph can be calibrated by using these tables.

Table 1. Correction data for X coordinates

Unit: mm

Dislocated coordinates of X	Actual Coordinates of X				
		-300	-200	-100	0
Actual Coordinates of Y	+100	-298.9	-199.2	-99.5	0.1
	0	-298.9	-199.4	-99.6	0.0
	-100	-299.0	-199.3	-99.5	0.0
	-200	-298.9	-199.3	-99.7	0.0
	-300	-299.0	-199.3	-99.6	0.0
	-400	-298.9	-199.3	-99.5	0.0
	-500	-298.7	-199.2	-99.6	0.0
	-600	-298.5	-199.2	-99.5	0.0
X ave		-298.8	-199.3	-99.6	0.0
average error		1.2	0.7	0.4	0.0

Table 2. Correction data for Y coordinates

Unit: mm

Dislocated coordinates of Y	Actual Coord. of X				Ave. error
		-300	-200	-100	
Actual Coordinates of Y	+100	100.0	100.1	100.1	0.2
	0	-0.1	0.0	-0.1	0.1
	-100	-100.2	-100.1	-100.2	0.0
	-200	-200.3	-200.2	-200.3	-0.1
	-300	-300.3	-300.3	-300.5	-0.2
	-400	-400.3	-400.2	-400.4	-0.1
	-500	-500.1	-500.2	-500.2	0.1
	-600	-599.7	-599.7	-599.8	0.4

3. Measurement of the Knee Motion

In order to eliminate the errors from the undesired movements, the upper leg is firmly wrapped on a special designed rigid chair as shown in figure 2. The foot and lower leg are firmly wrapped on a rectangular brace made of extruded aluminum beam. Three markers C, D, and E are set on the rectangular brace for tracing the movements of the lower leg, although from the kinematics point of view only the consequence coordinates of two points on a moving rigid body is required to determine the centrodes of this rigid body. There are two reasons for using three points. One can easily apply the homogenous coordinate techniques to calculate the displacement matrix. The other one is for double checking the experimental results. The loci of point C, D, and E are shown in figure 3.

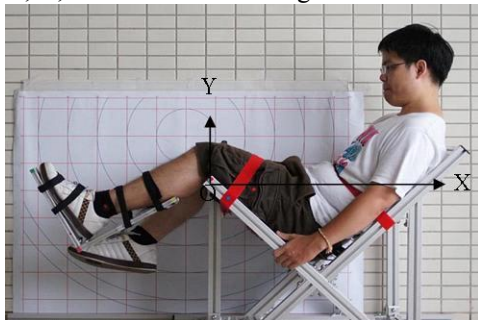


Figure 2. The testing chair and rectangular brace

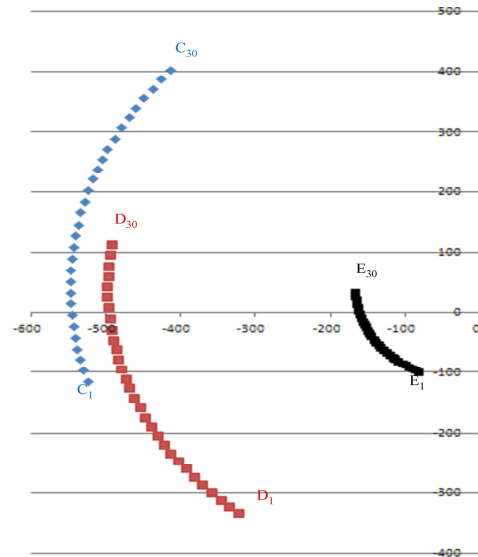


Figure 3. Loci of point C, D, and E

4. Determination of the centrodes

Two methods for determining the centrodes are demonstrated in this paper for comparison.

4.1 Finitely separated position method

The finitely separated position method is one of the commonly used methods in kinematics and industry practices. Draw the perpendicular bisectors of lines C1C2 and D1D2. The intersection point of these two perpendicular bisectors is a pole for position 1 and 2 as shown in figure 4. The loci of poles is called the polodes or called centrodes if the consequence points are very much closed to one others. This method is very easy to carry out, but it is very sensitive to the accuracy of coordinates of measurement points. The centrodes obtained by these methods is shown in figure 5. Obviously, the result is scattered and not acceptable. Therefore this method is not suitable in this study for determining the centrodes of knee joints.

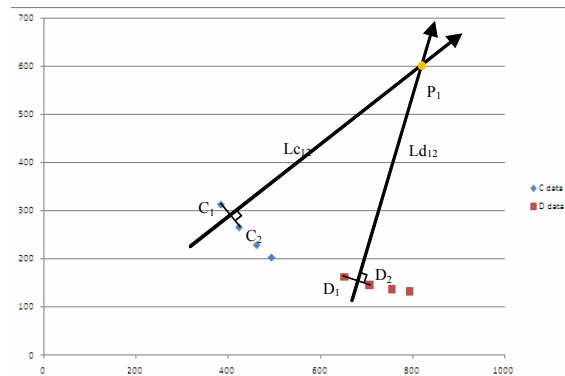


Figure 4. Finitely separated position method for finding the centrodes

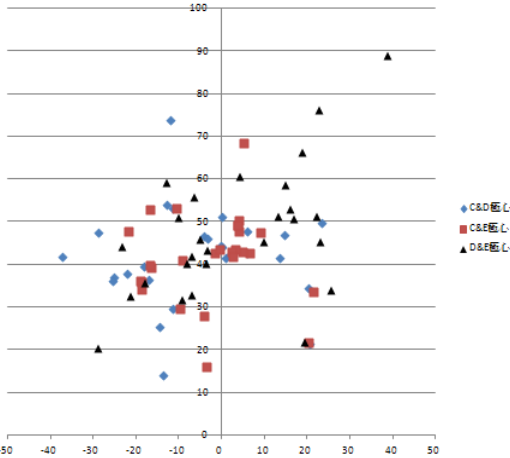


Figure 5. Centroides using finitely separated position method

4.2 Differential Method

The loci of the marked points C, D, and E can be described as continuous functions by using curve fitting techniques. Although there are many types of curve fitting methods, only the polynomial function type and exponential function type are chosen for this study. For a planar curve described as a function $y=f(x)$, the normal of this curve at point P (x_i, y_i) on the curve can be expressed as:

$$y = -x/y' (x_i, y_i) + k_i \tag{1}$$

Where $y' (x_i, y_i)$ is the derivative of y respective to x at point P(x_i, y_i), and k_i is a constant.

At any instance, the intersection of the normal of loci curve C at $C_i(x_{ci}, y_{ci})$ and of loci curve D at $D_i(x_{di}, y_{di})$ is the instantaneous center as shown in figure 6. The loci of instantaneous center is the centroides

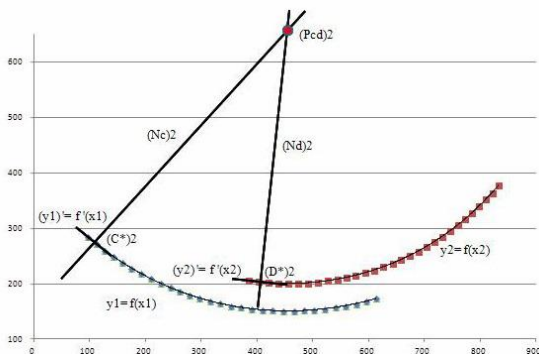


Figure 6. Differential method for finding the centroides

Since the exponential function is applied to fit the measured data of marked point C, D, and E, no negative coordinates are allowed. Therefore before the curve fitting, all of the measured coordinates have

to make a translation (-600, 500) followed by a rotation of 90° CCW. The parameters of fitting equations are shown in table 3 and table 4 for the polynomial type and exponential type respectively. Although the obtained curves by both methods fit the measured data very well, the one obtained by the exponential function is slightly better than the other one as shown in figure 7 and figure 8.

Theoretically from any two functions of lines of marked points C, D, and E can construct a centroides. Practically, the combination of points D and E is not suggested, because the connecting line of C and D almost parallel to the tibia. Figure 9 and figure 10 show the centroides constructed from curves C-D and C-E fitted by using polynomial function and exponential function respectively. Figure 11 is the comparison of all centroides from both methods. Since it is very sensitive to the accuracy of measured data, there exists a variation around 5mm. This phenomenon may be improved by increasing the accuracy of measured data. as indicated by previous study as shown in figure 12 [2]. In order to give an idea of the actual dimensions of the centroides, they are shown on the picture of a human knee joint in figure 13.

Table 3. Parameters of polynomial function

$$y = ax^4 + bx^3 + cx^2 + dx + e$$

	a	b	c	d	e
C	8.00E-10	1.58E-06	2.05E-03	1.18	3.81E+02
D	2.91E-09	-5.95E-06	5.59E-03	-2.49	6.12E+02
E	2.78E-07	-5.64E-04	4.33E-01	-1.48E+02	1.97E+04

Table 4. Parameters of exponential function

$$y = a \exp(bx) + c \exp(dx)$$

	a	b	c	d
C	367	-0.003525	16.87	0.003343
D	370.4	-0.002402	13.47	0.003827
E	590.1	-0.0002757	0.008603	0.01591

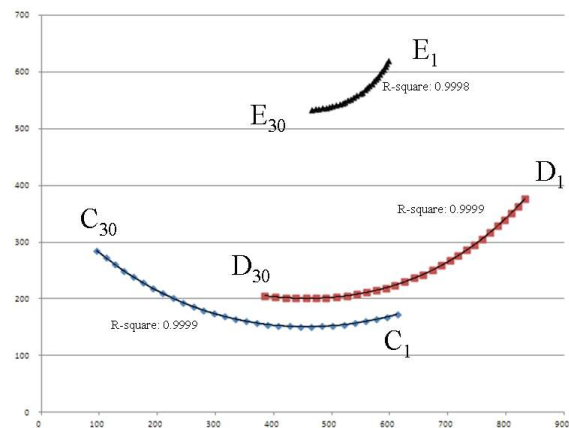


Figure 7. Curve fitting by polynomial function

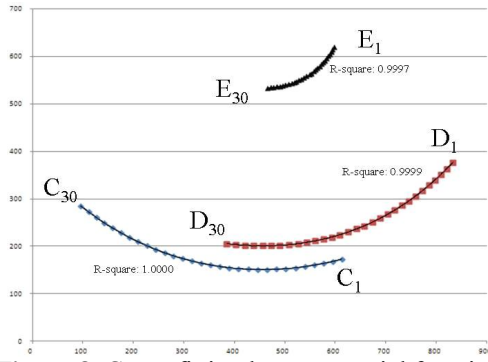


Figure 8. Curve fitting by exponential function

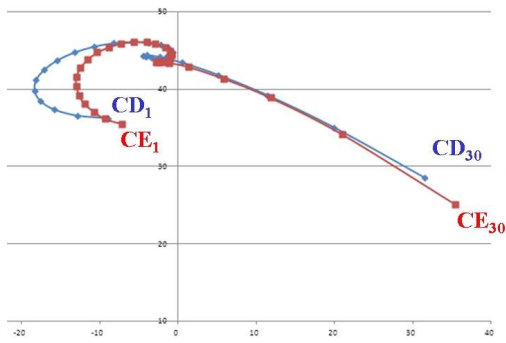


Figure 9. Centroides using polynomial function

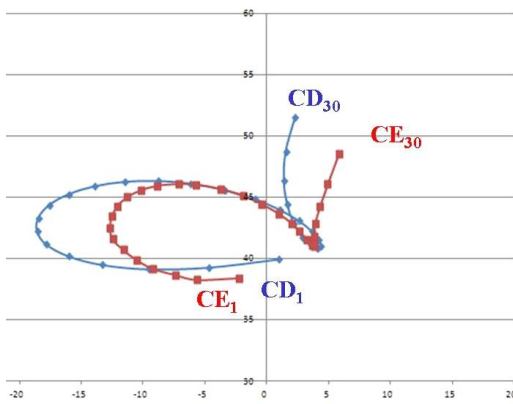


Figure 10. Centroides using exponential function

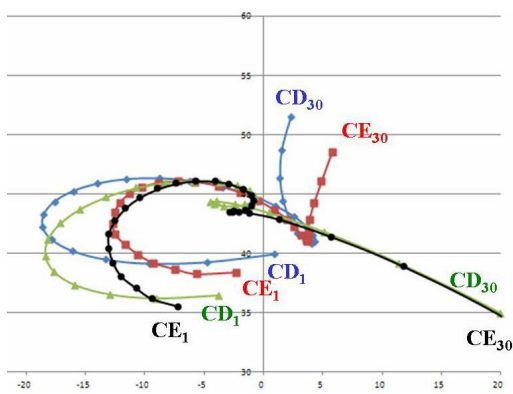


Figure 11. Comparison of centroides



Figure 5(a1). Reference knee motion 1 (Denver report)

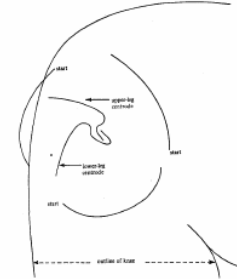


Figure 5(a2). Reference knee motion 2 (Denver report)

Fig. 12 Centroides of knee joint from the X-ray data [2]



(a) Moving range of lower leg



(b) Using polynomial curve fitting method



(c) Using exponential curve fitting method

Figure 13. Centroides of knee joint

5 Conclusion

Although the sequential X-ray radiographs of the moving knee provide an ideal data base for constructing the centrodes of knee joint, the cost and side effects of radiation may be bothersome. This paper provides an alternative method to construct the centrodes by using commercial digital camera to take the sequential digital photographs. In order to eliminate the undesired movements, a testing chair and a brace are specially designed. Two types of curve fitting methods are introduced to smooth the measured data of three marked points on the lower leg. Since it is very sensitive to the accuracy of the measured data, the finitely separated position method is not an ideal one for determining the centrodes of knee joints. Therefore the differential method is applied to construct the centrodes of knee joint from the measured data. Although the accuracy of centrodes obtained in this study is around 5mm due to the errors of measured data, this paper still provides a non-expensive, non-invasion, and non-radiation way for people to study the centrodes of human knee joint.

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References

1. Nwoye, C.I., Obasi G.C., Nwoye U.C., Okeke K., Nwakwuo C.C. and Onyemaobi O.O., "Model for calculating the concentration of upgraded iron designated for production of stainless steel based biomedical devices used in Orthopaedics," *Life Science Journal*, Vol. 7, Issue 4, 2010, pp. 1-6.
2. F. Freudenstein, L. S. Woo, "Kinematics of the Human Knee Joint," *Bulletin of Mathematical Biophysics*, Vol. 31, 1969.
3. Frankel, V.H., Burstein, A.H., Brooks, D.B., "Biomechanics of internal derangement of the knee: pathomechanics as determined by analysis of the instant centers of motion," *The Journal of Bone and Joint Surgery*, 1971, 53: 945-977.
4. Hobson, D.A. and Torfason, L.E., "Optimization of four-bar knee mechanisms-A computerized approach," *Journal of Biomechanics*, Volume 7, Issue 4, 1974, pp. 371-376.
5. Menschik, A., "Prosthetic knee joint for attachment to a natural or prosthetic leg," US Patent 3969773, 1976.
6. Van de Vee, P. G., "Swivel connection between two parts of an orthopedic technical aid," US Patent 5181931, 1993.
7. Hollman, J. H., Deusinger, R.H., Van Dillen, L.R., Matava, M.J., "Knee Joint Movements in Subjects Without Knee Pathology and Subjects With Injured Anterior Cruciate Ligaments," *Physical Therapy*, Volume 82, Number 10, 2002, pp. 960-972.
8. Lee, J. M. and Chang K. Y., "An integrated investigation of CAD/CAM for the development of custom-made femoral stem," *Life Science Journal*, Vol 7, No 1, 2009, pp. 56-61
9. Lee J.N., Chen, H.S., Luo, C.W. and Chang K.Y., "Rapid prototyping and multi-axis NC machining for the femoral component of knee prosthesis," *Life Science Journal*, Vol. 7, no. 1, 2010; pp. 79-83.
10. Shereif, W.I. and Hassanin, A.A., "Comparison between uses of therapeutic exercise and heat application on relieve pain, stiffness and improvement of physical function for patient with knee Osteoarthritis," *Life Science Journal*, Vol. 8, No. 3, 2011, pp. 388-396.
11. Tien, Y.K. and Yu, C.K., "Tai chi exercise affects the isokinetic torque but not changes hamstrings: quadriceps ratios," *Life Science Journal*, Vol. 6, No. 4, 2009, pp. 50-55.
12. Green, M.P., Radcliffe, C.W., "Technical report on the TKG-5PS0SLK, TKG-5PS0 and the TKG-50S0 PC Knee Units," DAW Industries Inc. technical report, 2003.
13. Muggleton, J. M., Allen, R., "Insights into the measurement of vertebral translation in the sagittal plane," *Medical Engineering & Physics*, 20, 1998, 21-32.
14. Rousseau, M., Bradford, D.S., Bertagnoli, R., Hu, S.S., Lotz, J.C., "Disc arthroplasty design influences intervertebral kinematics and facet forces," *The Spine Journal* 6, 2006, 258-266.
15. Stark, G., "Overview of Knee Disarticulation," *Journal of Prosthetics and Orthotics*, Vol. 16, Number 4, 2004, pp. 130-137.
16. Vujić, D., Radojković, S., "Synthesis procedure of planar bar linkages in infinitesimally close position," *FACTA UNIVERSITATIS Series: Mechanical Engineering*, Vol.1, No 7, 2000, pp. 835-848.
17. Wu, T.-M., Chen, C.-K., "Computer-aided curvature analyses of planar four-bar linkage mechanism," *Applied Mathematics and Computation* 168, 2005, 1175-1188.
18. Hall, A.S., *Kinematics and Linkage Design*, Waveland Press, 1986.

Custom-made Biomechanical Model of the Knee Joints

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Abstract: This paper obtained the data of custom-made tibia, femoral joints and meniscus morphology by studying relating researches and constructed several sagittal section of the knee joint. Based on the 2D bone morphology of femoral condyle and the contact radius relationship of the tibial plateau, it created the design of the sphere curvature of femoral condyle to conform to the demands of the patients. To simulate the various meshing curved surface between bones, this paper considered using sphere to fit femoral condyle, using femur image data to fit the femur into spheres and using the Hertz's elastic contact theory to construct a femur-tibia and femur-meniscus biomechanical model. Lastly, using the contact stress and contact deformation obtained by solving the numeric examples under specific cases can be used as a reference in the design and production of future prosthesis.

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Keywords Elastic Contact, Biomechanical Model, Femur, tibia

1. Introduction

Recently, the quality of computer medical image for artificial prosthesis greatly improved. Due to these improvements, the patient who can only use crutches or canes to walk or even the patients who are amputated in the past can walk like normal people and this improved the quality of their life greatly. Not only that knee joint is the most complicated joint constructed and the biggest joint of a human body, it has high expectations in its movement function. Every year, a great number of patients have to undergo artificial knee arthroplasty. At present, the people that underwent artificial knee arthroplasty, the limbs of the patients can load, stretch, abduce and rotate in a very stable manner.

Most of the artificial knee prosthesis being clinically used should be imported because there are very few companies, such as United Orthopedic Corporation, who design and develop artificial knee products. Importing prosthesis is quite expensive and the products are designed based on knee joint parameters of Americans and Europeans which sometimes are incompatible to Asian knees. To recover the complex activities of the knees, the artificial knee prosthesis designed should be compatible in anatomy, biomechanical and kinematics. Stress analysis allows the stress morphology of the prosthesis and the human skeleton to be more compatible to improve the long-term stability of the prosthesis and avoid unreasonable stress peak and contact area morphology. Moreover, because the model of the reconstructed bones can be rotated and cut at any angles, it provides a good method to the complex surface geometric morphology researches and simulation surgeries of the knee. To design a better knee prosthesis

compatible to Taiwanese, custom-made normal knees underwent bone morphology measurement and kinematic researches to create multi-segmented meshing curved surface and Hertz theory was used to derive the biomechanical model of the femur and tibia in different meshing sections. In addition, the model is used to derive the average contact stress, contact area in the meshing area. These results can serve as a reference to clinical diagnosis and custom-made prosthesis manufacture.

The studies relating to the femur and tibia contour morphology are as follows:

David Siu measured the medial femoral condyle and its distal condyle, radius of the patellar surface and arc angle and the lateral femoral condyle and its distal condyle, radius of the patellar surface and arc angle of 5 cadaver's knee joints after CT scanning a 3D reconstruction^[1]. The result of the study of Elias where 10 samples of cadavers with normal knees are dissected and compared to 6 normal adult knees showed patients of different races, different sizes or due to curve fitting results to different arcs for different femoral condyle joints^[2]. Zhou's research shows that the width of the medial and lateral femoral condyle and the width of the medial and lateral tibia plateau are very similar which proves that the width of femoral condyle and tibia plateau is related. Greater femoral condyle width shows greater tibia plateau width. Thus, the value of femoral condyle and tibia plateau can be used as the index to represent the size of the knees. His simplified model shows that sagittal femoral condyle profile can be fitted into three sections arc curves. Moeinzadeh et al^[3]. researched on the biomechanics of the femur tibia of knee joints where they are the first to create an artificial knee dynamic model human knee joint

and fixed the femur. When the model is used to measure the fixed femur, the tibia is relative to the motion of the femur and can compute for the contact stress of the knee joints. Wongchaisuwat et al^[4]. created another knee dynamic model where the femur is fixed and the tibia is simulated as a simple pendulum to analyze the control strategy which the femur is relative to tibia's sliding and rolling. Komistek et al^[5]. created a model by applying Kane's method where the peak of the contact stress between femur and tibia was obtained by analyzing different walking speeds^[6,7]. Wang released femur and created a two-dimensional biodynamic model of femur and tibia^[8-11]. Tumer and Engin created a femur-tibia-patellar dynamic model of human knee. The model simulated that when the femur is fixed, tibia is relative to the relative motion of femur and patellar^[12]. Wang released femur again and created a femur-tibia-patellar three knee joint 2D meshing motion mathematical model^[13]. Jia created a lower limb 3D model to compute for the force and torque of the femur and tibia during gait cycle^[14]. Please refer to^[15,16,17] for other related researches.

The main task of this paper is to discuss the sagittal plane section near femoral condyle and tibial plateau under the 3D geometry of the obtained femur and tibia through geometric analysis software to obtain the bone contour shape of the knee joint. Numerical method was applied on the contour to fit the arc with different curvature and the contour is also fitted into several spheres with different diameter by setting the length of an appropriate Z axle. Similar cross section was also applied to proximal tibia to explore the contact stress of the femoral and tibial meshing.

The results include:

1. applying the elastic theory to simplify the biomechanical model of femur, tibia and meniscus can compute the contact stress of the femur, tibia and meniscus.
2. through image processing, use the femoral condyle and tibial plateau meshing to fit different spheres and calculate the contact stress and contact area according to different patients.

Through the sagittal section of the femoral condyle and tibial plateau stated above, we can create sphere radius of contact morphology in any section to use as the knee stress analysis foundation which can be used as the design and production of custom-made artificial knee joints.

2. Research Methodology

The research method of this paper include construction and analysis of the contact morphology of profile fitting of the femur and tibia under the

same section and solve the problem by using the meshing surface as a multiple sphere radius according to the bone morphology of the different patients.

2.1 Femur fitting calculation

Figure 1 shows the femur/tibia meshing 3D geometry. By the use of the software, we can obtain the bone morphology of the sagittal plane and the bone meshing surface as shown in Figure 1 and 2.

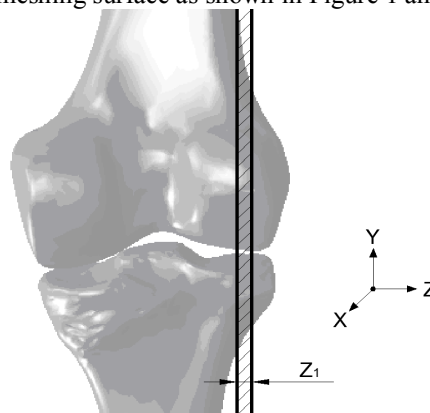


Figure 1. Geometric model operation

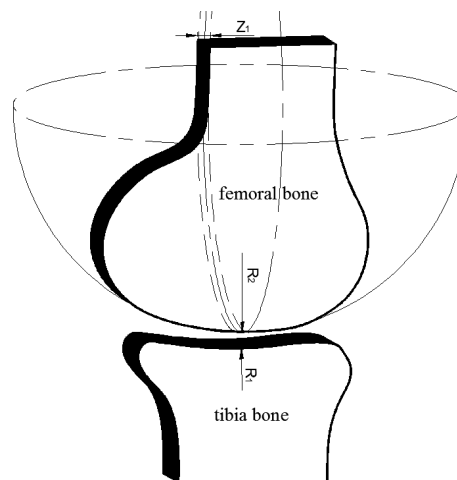


Figure 2. Sagittal Skeletal Profile

2.2 The sphere fitting explanation using femur is as follows

Consider the Z-direction length on Figure 1 and with C given, the sphere equation is established.

$$R^2 = (x - A)^2 + (y - B)^2 + (z - C)^2 \quad (1)$$

$$R^2 = x^2 - 2Ax + A^2 - 2By + B^2 + z^2 - 2Cz + C^2 \quad (2)$$

where (A,B,C) are the coordinates of the center and R is the radius of the sphere.

When $a=-2A$, $B=-2B$, $c=-2C$, $d=A^2+B^2+C^2-R^2$, another form of sphere formula can be derived as shown in equation (3)

$$x^2+y^2+z^2+ax+by+cz+d=0 \quad (3)$$

As long as a, b,c and d are derived, we can obtain the center and radius parameters:

$$\begin{cases} A = -\frac{a}{2} \\ B = -\frac{b}{2} \\ C = -\frac{c}{2} \\ D = \frac{1}{2}\sqrt{a^2 + b^2 + c^2 - 4d} \end{cases} \quad (4)$$

In ball point set $(X_i, Y_i, Z_i), i \in (1, 2, 3 \dots N)$, D_i is the distance between midpoint and the center.

$$D_i^2 = (X_i - A)^2 + (Y_i - B)^2 + (Z_i - C)^2 \quad (5)$$

Thus, we can obtain the distance between the center to points (X_i, Y_i, Z_i) and square difference of the length of radius (δ):

$$\delta = D_i^2 - R^2 \quad (6)$$

$\Phi(a, b, c)$ can be obtained as the square least of δ :

$$\Phi(a, b, c, d) = \sum \delta_i^2 = \sum (X_i^2 + Y_i^2 + Z_i^2 + aX_i + bY_i + cZ_i + d)^2 \quad (7)$$

Use partial derivatives to obtain a,b,c,d values and the value of $\Phi(a, b, c, d)$ is the smallest, thus

$$\begin{cases} \frac{\partial \Phi(a, b, c, d)}{\partial a} = \sum 2(X_i^2 + Y_i^2 + Z_i^2 + aX_i + bY_i + cZ_i + d)X_i = 0 \\ \frac{\partial \Phi(a, b, c, d)}{\partial b} = \sum 2(X_i^2 + Y_i^2 + Z_i^2 + aX_i + bY_i + cZ_i + d)Y_i = 0 \\ \frac{\partial \Phi(a, b, c, d)}{\partial c} = \sum 2(X_i^2 + Y_i^2 + Z_i^2 + aX_i + bY_i + cZ_i + d)Z_i = 0 \\ \frac{\partial \Phi(a, b, c, d)}{\partial d} = \sum 2(X_i^2 + Y_i^2 + Z_i^2 + aX_i + bY_i + cZ_i + d) = 0 \end{cases} \quad (8)$$

From equation (8), the values of a,b,c,d can be derived. Equation (4) derived the center and radius which completed the feature extraction of the spatial sphere.

2.3 Axis line alignment

The design method of the tibial plateau is similar as the one above. The difference is that the diameter of the tibial plateau is bigger and it can be flat, concave or convex after fitting according to the different elastic model. In obtaining tibia and the tibia cross-section, several important axis need to be fit into one straight line, thus the vertical fitting algorithm is needed.

The axis line equation is $Ax + by + 1 = 0$ (9)

In using the least square fit, the deviation (ℓ) of equation (10) should be the smallest.

$$\ell = \sum (ax_i + by_i + 1)^2 \quad (10)$$

Equation (10) separately solves for the partial derivatives of a and b setting the partial derivatives to 0.

$$\begin{cases} \frac{\partial \ell}{\partial a} = 2 \sum (ax_i + by_i + 1)x_i = 0 \\ \frac{\partial \ell}{\partial b} = 2 \sum (ax_i + by_i + 1)y_i = 0 \end{cases} \quad (11)$$

Equation (11) can obtained the smallest a and b values of the deviation (ℓ) to fit the required straight line.

2.4 Bone profile morphology related authentication

Several researchers conducted studies regarding the curvature design of distal femur femoral condyle among which Zhang measured three fitting image as shown in Figure 3 and 4. Similar study^[18], Chou has the curvature design of the 3 blocks of the femoral condyle. Table 1 describes the measurement results and errors of the sample patients in the statistics^[3].



Figure 3. The X-ray shows the 3 arcs of the distal femur articular surface (ref. 16)

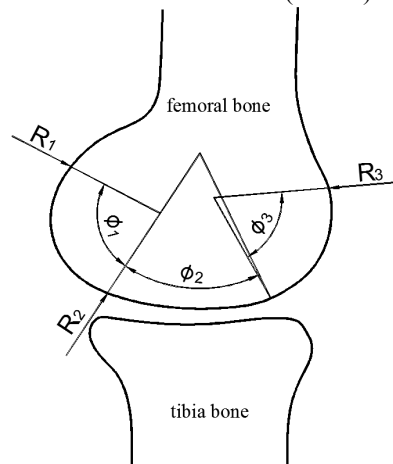


Figure 4. The measured parameters of distal femur

Table 1. Parameters of distal femur (ref. 4)

Geometric dimension	Values
r ₁ (RPMFC)	20.62 ± 3.59
φ ₁ (APMFC)	114.72 ± 6.17
r ₂ (RDMFC)	37.94 ± 6.79
φ ₂ (ADMFC)	52.62 ± 7.68
r ₃ (RPaMFC)	25.71 ± 2.45
φ ₃ (APaMFC)	42.59 ± 5.43

2.5 Joint elastic contact model

The organizational form of the joint of femur and tibia is shown in Figure 5. The distal femur and tibial plateau don't have a pure rolling meshing relationship which affects the flexing posture and stretching ligaments and because the meniscus roll buffer is usually a sliding movement. The motion range of every bone has its limit. Thus, the curvature geometric design and the construction of elastic mechanical model helps simulate contact stress and contact area morphology through different flexing needs.

The E_f, μ_f is set as the elastic modulus and Poisson's ratio of knee femoral elastic meshing, respectively. E_t, μ_t is the occlusion of tibial elastic modulus and Poisson's ratio, respectively. R_f, R'_f is the two main curvature radius of the elastic femur contact surface, respectively. R_t, R'_t is the two main curvature radius of the elastic tibia contact surface, respectively. a and b are the long and short axis the tibial-femoral joint contact spnere surface. δ is the relative displacement of the femur and tibia elastic center. q_0 is the average stress of the tibial-femoral joint contact elastic surface. P is the axial static pressure. Based from Hertz theory, the maximum stress, elastic occlusal contact area and the deformation of the solution between the meshing contact surface of the knee, shown below, among which the average stress is $q_0 = \frac{3P}{2\pi ab}$ and a and

$$a = \alpha \cdot \sqrt[3]{\frac{3}{4} \frac{P}{A} \left(\frac{1 - \mu_f^2}{E_f} + \frac{1 - \mu_t^2}{E_t} \right)} \tag{12}$$

$$b = \beta \cdot \sqrt[3]{\frac{3}{4} \frac{P}{A} \left(\frac{1 - \mu_f^2}{E_f} + \frac{1 - \mu_t^2}{E_t} \right)} \tag{13}$$

For sphere, the coefficient a=b.

$$q_0 = \frac{3P}{2\pi ab} \tag{14}$$

$$q = q_0 \sqrt{1 - \frac{x^2}{a^2} - \frac{y^2}{b^2}} \tag{15}$$

$$\delta = \lambda \sqrt[3]{\frac{9}{128} AP^3 \left(\frac{1 - \mu_f^2}{E_f} + \frac{1 - \mu_t^2}{E_t} \right)} \tag{16}$$

$$A = \frac{1}{2} \left(\frac{1}{R_f} + \frac{1}{R'_f} + \frac{1}{R_t} + \frac{1}{R'_t} \right) \tag{17}$$

$$B = \frac{1}{2} \left[\left(\frac{1}{R_f} - \frac{1}{R'_f} \right)^2 + \left(\frac{1}{R_t} - \frac{1}{R'_t} \right)^2 + 2 \left(\frac{1}{R_f} - \frac{1}{R'_f} \right) \left(\frac{1}{R_t} - \frac{1}{R'_t} \right) \cos 2\Phi \right]^{\frac{1}{2}} \tag{18}$$

$$\cos \theta = \frac{B}{A} \tag{19}$$

In the equation, Φ is the two curvature angle of

R_f and R_t in the between planes.

Moreover, α, β, λ is used as the parameters where the values can be based on the θ value derived from equation (19) where the results is shown in Table 2. If the two equations of the meshing surface of the knee joint is known, the maximum contact stress, contact area and meshing deformation of the tibial-femoral surface of the knee can be solved according to equations (12)~(19).

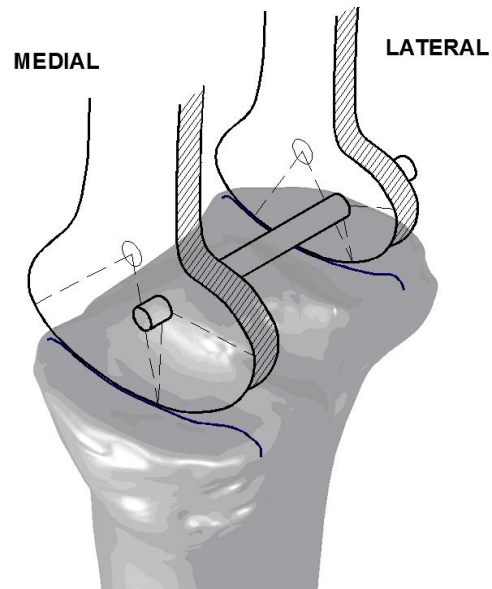


Figure 5. The intra-articular organizational profile

Table 2. The coefficient of equations

θ	0°	10°	20°	30°	35°	40°	45°	50°
α	∞	6.612	3.778	2.731	2.397	2.135	1.926	1.754
β	0	0.319	0.408	0.493	0.530	0.567	0.604	0.604
λ		0.851	1.220	1.453	1.550	1.637	1.709	1.772
θ	55°	60°	65°	70°	75°	80°	85°	90°
α	1.611	1.486	1.378	1.284	1.202	1.128	1.061	1.000
β	0.678	0.717	0.759	0.802	0.846	0.893	0.944	1.000
λ	1.828	1.875	1.912	1.944	1.967	1.985	1.996	2.000

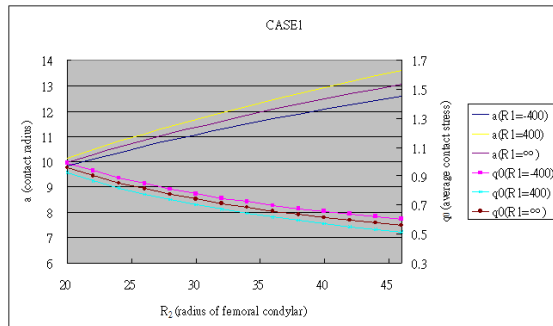
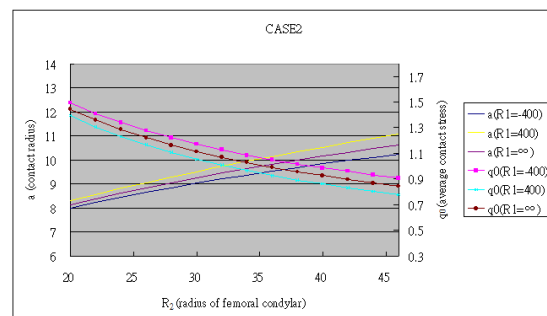
3. Results and Discussion

Combining the results of literatures based on femoral condylar morphology, the hypothesize tibia and femoral condyle software contact of this study separately considered tibia plateau radius as -400mm and plateau as 400mm where the contact radius (R) and average pressure (q_0) are shown in Figure 6 and the parameters are shown in Table 3.

If the contact between meniscus and femur is considered, the meniscus coefficient is $E1=59\text{MPa}$ and $\nu1=0.49$. The meniscus and femur contact radius considers the meniscus as -400mm, plane as +400mm and the femur condyle changes 2° each time from 20° to 40°. The contact radius and average pressure is shown in Figure 7.

Table 3. The coefficient of two cases

Case1	Case2
$E_r = E_f = 5\text{MPa}$	$E_r = 59\text{Mpa} ; E_t = 5\text{Mpa}$
$\nu_r = \nu_f = 0.46$	$\nu_r = 0.46 ; \nu_t = 0.49$
$h_t = h_f$ (cartilage thickness) = 1mm	$h_f = 1\text{mm}$
F: bearing capacity	F: bearing capacity

**Figure 6.** Contact of femur and tibia**Figure 7.** Contact of femur and meniscus

4. Conclusion

The demand for artificial knee in the market and the quality of its demand rapidly works toward the direction of custom-made. According to the human skeleton contour modeling techniques and then according to the patient's weight, different lifestyle and habits and movement patterns of the knee, designing and constructing knee prosthesis is quite complex and challenging. With a main direction of custom-made and with the help of the results medical images, this paper designed a femur condyle sphere and sphere or concave sphere of tibia plateau biomechanical model through change curvature value of the bone processing technique and with the elastic theory of Hertz between balls to calculate the contact stress, contact area and deformation in the meshing of the joints to serve as a reference in the design, production and analysis of custom-made prosthesis.

References

- David Siu, et al. Femoral articular shape and geometry-A three-dimensional computerized analysis of the knee. *Journal of Arthroplasty* 1996; 11(2): 166-173.

- Elias SG, Freeman MAR, Gokcay EI: A correlative study of the geometry and anatomy of the distal femur. *Clinical Orthopaedic* 1990; 260: 98-103.
- Zhou, Feihu, Measurement and related research of the three-dimension morphology of the healthy Chinese knee, *The Orthopedic Journal of China*, 2004, 12(8).
- M.H Moeinzadeh, A.E Engin, N. Akkas, Two-dimensional dynamic modeling of human knee joint, *Journal of Biomechanics*, 1983 vol.16,253-264.
- C. Wongchaisuwat, H. Hemami and H.J. Buchner, Control of sliding and rolling at natural joints, *ASME, Journal of Biomechanical Engineering*, 1984, vol.106,368-375.
- R.D. Komistek, Mathematical modeling of the human arm: an aid in the investigation of the role of muscle forces in the development of lateral epicondylitis, [PH.D. Dissertation], University of Memphis, 1992.
- R.D. Komistek, J.B.Stiehl, D.A. Dennis, Mathematical model of the lower extremity joint reaction forces using Kane's method of dynamics, *Journal of Biomechanics*, 1992, vol. 31, 185-189.
- X.S. Wang, N. Akkas, Two-dimensional biodynamic model of the human femur-knee joint-tibia system, *French: The 14th International Conference on Biomechanics*, 1993.
- X.S Wang, Two-dimensional biodynamic model of human lower extremity [Ph D] Dissertation, Ankara, Turkey: Middle East Technical University, 1994.
- S.T. Tumer, X.S. Wang, N. Akkas, A planar dynamic anatomical model of the human lower limb, *Biomedical Engineering*, 1995, vol. 7, 365-378.
- Wang, Xishi, Bai, Ruipu, S.T. Tumer, The mathematical model of a human knee joint and its biomechanical response, *Medical Biomechanics*, 1998, vol. 13(1), 10-16.
- S.T. Tumer, A.E. Engin, Three-body segment dynamic model human knee, *ASME, Journal of Biomechanical Engineering*, 1993, vol. 115, 350-356.
- Wang, Xishi, Bai, Ruipu, S. Turgut Tumer, et al., Femur-tibia-patellar three knee joint meshing motion mathematical model, *Biomedical Engineering*, 1998, vol. 18(4), 360-363.
- X.H. Jia, M. Zhang, W.C.C. Lee, Load transfer mechanics between trans-tibial prosthetic socket and residual limb-dynamic effects, *Journal of Biomechanics*, 2004, vol. 37, 1371-1377.
- Bo Wun Huang, Huang Kuang Kung, Kuan-Yu Chang, Po Kai Hsu, Jung-Ge Tseng, "Human Cranium Dynamic Analysis," *Life Science Journal*, 2009; 6(4): 15- 22.
- Chang Kuan Yu, Chang Hong Chang, Yu Pu Ping, Yen Ke Tien and Huang Bo Wun, "Natural Properties in a Micro Drill Cutting into Bones," *Life Science Journal*, 2009; 6(4): 28 - 33.
- Jeng-Nan Lee, Hung-Shyong Chen, Chih-Wen Luo and Kuan-Yu Chang, "Rapid Prototyping and Multi-axis NC Machining for The Femoral Component of Knee Prosthesis," *Life Science Journal*, 2010; 7(1): 79 - 83.
- Zhang, Xiuli, Liu, Yong, The correlation between the geometry and anatomy in the distance of the distal end of femur, *Hebei Medical Journal*, Jan 2008, vol. 30, No.1.

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Electrically-Controllable Fresnel Lens Based on Liquid Crystal

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Abstract: This study proposes an electrically-controllable Fresnel lens based on liquid crystals. A special design patterned electrode type and a homogeneous alignment sample cell has been fabricated to form a liquid crystals lens. The transmission light can be focused or defocused by varying the applied voltage. The focal length in this Fresnel lens is around 300 mm.

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<http://www.lifesciencesite.com>. 71

Keywords: Fresnel lens, liquid crystals

1. Introduction

Fresnel lens is highly appreciated because of its well known properties of long distance optical communication, large collecting aperture, optical interconnection, ease of replication, and additional degrees of freedom in correcting aberrations and three dimensional display systems [1-7]

Conventional Fresnel lens, fabricated by electron beam writing, has some limitations, such as a fixed focusing efficiency and narrow fabrication tolerance. However, liquid crystals (LCs) lens is a good candidate for a variety of applications in optics due to large birefringence and electrically-controllable focal length [1-3]. Because of simple fabrication processes and tunable optical properties, switchable LC Fresnel lenses have attracted considerable attention in research [4-7].

In this study, we demonstrated Fresnel lens based on liquid crystal. The diffraction efficiency of a liquid crystal Fresnel lens is electrically controllable, the focusing and defocusing states can be tuned by varying the applied voltage, and the focal length in this Fresnel lens is around 300 mm.

2. Experimental Device Fabrication

Figure 1 schematically shows the procedure for fabricating the Fresnel zone plate in a homogenous-aligned LC cell. The zone plate photomask plays a key role in this work; it has transparent odd zones and opaque even zones. The concentric rings photomask within a diameter of 1

cm was used in this study as shown in Figure 1(a). Figure 1 (b) illustrates the schematic fabrication of the liquid crystal Fresnel lens. The radius r_m of the m th zone can be expressed as $r_m^2 = m r_1^2$, where r_1 is the radius of the inner zone. The primary focal length f is related to the inner radius r_1 as $f = r_1^2 / \lambda$, where λ is the wavelength of the incident beam. The LC E7 (Merck, $n_o=1.5216$, $n_e=1.7462$), are injected into the homogenous empty cell and the thickness of the cell is $12\mu\text{m}$, forming a LC Fresnel lens.

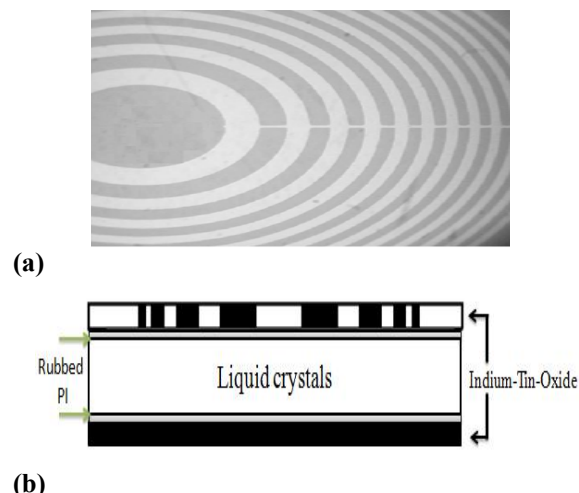


Figure 1 (a) the concentric rings photomask (b) the schematic fabrication of the liquid crystal Fresnel lens.

Setup

Figure 2 shows the experimental setup for the focusing features of the liquid crystal Fresnel lens. The incident linearly polarized He-Ne laser beam of the wavelength 633 nm is expanded and then passes through a diaphragm and the LC Fresnel lens with an external applied voltage V . A photodiode is linked to a computer to measure the transmission intensity, in which another diaphragm D2 is placed in front of the photodiode.

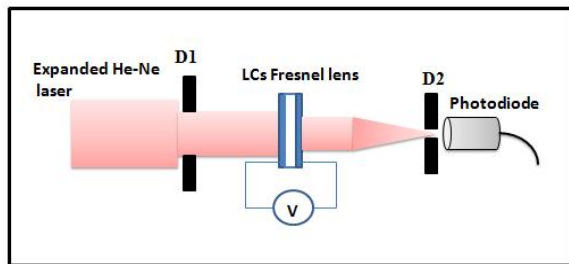


Figure 2 Experimental setup. D1, D2 are diaphragms

3. Results and Discussions

Figure 3 plots the variation of the transmission intensities at various applied voltage-controlled at 5 V, 7 V, 10 V, and 12 V. According to theoretical calculations, the theoretical focal length of approximately 300 mm, the transmission intensity can be significantly changed in the vicinity of this location as the voltage changes. From Figures 3(a)-(b), the maximum transmission intensity maintains a stable level, the transmission intensity gradually decays as the applied voltage is larger than ~10V as shown in Figures 3(c)-(d). The fringing effect at a higher voltage causes the bent electric field partially to reorient the LC molecules, and degrades the Fresnel lens effect.

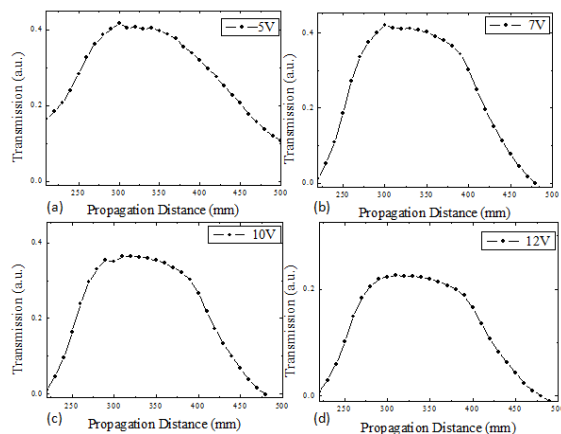


Figure 3 The variation of the transmission intensities at various applied voltage-controlled at (a) 5 V (b) 7 V (c) 10 V (d) 12 V

Figure 4 plots the variation of the transmission intensities with varying the applied voltage at position 300 mm. The result shows the transmission light can be focused with a lower applied voltage, and gradually defocused as the applied voltage exceeds 7 V. In the appropriate voltage, Fresnel lens can be electrically tuned the focusing or defocusing state.

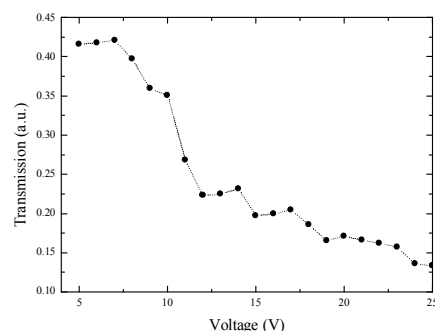


Figure 4 The variation of the transmission intensities with varying the applied voltage at position 300 mm.

4. Conclusion

In this study, we demonstrated a simple method to fabricate liquid crystal Fresnel lens. The focusing and defocusing states can be tuned by varying the applied voltage, and the focal length in this Fresnel lens is around 300 mm.

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References

1. J.A. Jordan, P.M. Hirsch, L.B. Lesem, and D.L. Van Rooy, "Kinoform lenses," *Appl. Opt.* **9**, 1883-1887 (1970).
2. K. Miyamoto, "The phase Fresnel lens," *J. Opt. Soc. Am.* **51**, 17-20 (1961).
3. R.A. Hyde, "Eyeglass. I. Very large aperture diffractive telescopes," *Appl. Opt.* **38**, 4198-4212 (1999).
4. D. Faklis, G.M. Morris, "Broadband imaging with holographic lenses," *Opt. Eng.* **28**, 592-598 (1989).
5. P. Andrés, V. Climent, J. Lancis, G. Mínguez-

- Vega, E. Tajahuerce, and A.W.130(5): 769-74.
6. T. Fujita, H. Nishihara, and J. Koyama, "Fabrication of micro lenses using electron-beam lithography," *Opt.Lett.* **6**, 613-615 (1981).
 7. J. Jahns and S. J. Walker, "Two-dimensional array of diffractive microlenses fabricated by thin film deposition," *Appl. Opt.* **29**, 931-936 (1990).

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The Influence of Low-powered Family LED Lighting on Eyes in Mice Experimental Model

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Abstract: Ocular tissue damage because of exposure to visible light has been demonstrated by the results of human and animal studies. The short-wavelength visible light between 430 nm to 500 nm (blue light) is especially associated with retina damage. Recently, new powerful sources and relatively inexpensive blue energy of LED (light emitting diodes) family lamps in home illumination are available. The aim of this study is to investigate the effects of illumination source from the low-powered and the conscious spectrum source of LED family lamps on retina tissues. The illumination source of LED family lamps was analyzed from 300 nm to 800 nm using an UV-visible spectrophotometer. In animal experiments, young adult mice were assigned to expose to family LED light for 2h every day ranging 2 to 4 weeks or light environment using LED family lamps for 39 weeks. After LED light treatment, sections of eyes were stained with hematoxylin and examined using histopathology. The data clearly demonstrated irradiation of the white LED is above 400 nm and is not within the ultraviolet light region. However, the analysis of spectrum distribution demonstrated that the family LED lighting exhibited power-peak at 450 nm is within the blue light region. Histological results showed that the photoreceptor layer is significantly reduced in thickness after 4 weeks of LED exposure 2h every day or LED illuminated environment. This study provides important data regarding the efficacy and safety of LED light in family illumination. It is impossible to consider these degenerative changes are related unavoidably part of their mechanism of action or an avoidable toxic effect.

[Mei-Lin Peng, Cheng-Yu Tsai, Chung-Liang Chien, John Ching-Jen Hsiao, Shuan-Yu Huang, Ching-Ju Lee, Hsiang-Yin Lin, Yang-Cheng Wen, Kuang-Wen Tseng. **The Influence of Low-powered Family LED Lighting on Eyes in Mice Experimental Model.** Life Science Journal. 2012;9(1):477-482] (ISSN:1097-8135). <http://www.lifesciencesite.com>. 72

Keywords: Light emitting diodes, Photoreceptor, Blue light

1. Introduction

Optical radiation includes ultraviolet light (UV) (100-400 nm), visible light (400-750 nm), and infrared radiation (750-10,000 nm). Visible light is referred to as short- (blue), medium- (green), and long-wavelength (red) radiation. Irradiation below 286 nm is absorbed by the stratospheric ozone layer on earth. Thus, we are exposed to wavelengths above 286 nm, most of which fall within the visual light spectrum [1].

Although the primary function of the retina is to receive and is to transduction light from the environment, excessive exposure to light is risky to vision [2]. In addition, excess light has a role in the progression of photoreceptor degeneration, age-related macular degeneration (AMD) and retinitis pigmentosa as determined in a human public-health study [3-4]. Photoreceptor death is an unalterable damage and can cause loss of visual field and night

blindness. UV light does not provide useful vision, instead of harm the retina in acute intense exposures [5-6]. The cornea blocks UV radiation in wavelengths below 300 nm, and the crystalline lens blocks most UV radiation between 300 nm and 400 nm [7]. The absorption properties of the cornea and the crystalline lens contribute to the protection of the retina against the hazards of UV light exposure.

Besides UV light, the visible blue light can cause damage to the eye. Short-wave length blue radiation is believed to cause retinal damage or to contribute to the development of age-related macular degeneration [8-9]. Animal experiments indicate photochemical damage of photoreceptor and retinal pigment epithelial cells after retinal exposure to extreme levels of blue light [10-11]. The high-energy photons create reactive oxygen species, which are deleterious to DNA and to a variety of cellular

organelles, particularly the mitochondria [12-13]. Shorter wavelength light is the most hazardous component of the visible spectrum, and is known to generate reactive oxygen species in the retina [14-15].

Considered the ultimate lamp, the longevity and efficiency of light emitting diodes (LEDs) make them optimal for conserving energy. Recently, advancement in materials and in manufacture has resulted in the commercial availability of LEDs with high luminance. LEDs are used in digital monitors and in illuminating alarm clocks, coffee makers, traffic lights, billboards, and more, may one day be used in lieu of fluorescent lighting in offices and in homes.

With the development of high-efficiency and high-powered LEDs, it has grown possible to use LEDs in lighting. Replacement light bulbs have been made. New powerful sources and relatively inexpensive blue energy of LED lamps in family illumination are available. However, at present, there are no uniform standards at this time for low LED power emissions, and the retinal damage thresholds have not been directly tested. Therefore, this study set up the LED-irradiated animal model to test if any potential risk on the retina of eyes.

2. Material and Methods

Mice

The common black strain of mice, C57BL/6, was used in this experiment. A total of 40 six-week-old mice were purchased from National Laboratory Animal Center, Taipei, Taiwan. All mice were raised under a 12-hr light/12-hr dark cycle.

LED exposure and study groups

The 40 mice were randomly split into four groups (each contained 10 mice), including (1) white family LED irradiation for a consecutive 2-week period (2 hr/day), (2) white LED irradiation for a consecutive 4-week period (2 hr/day), (3) environmental light source with white family LED lamps (the illuminated light of 12-hr light/ 12-hr dark cycle with white family LED lamps), and (4) blank control (no LED exposure), as summarized in figure 1. All experiments were reviewed and approved by the Animal Care and Use Committee in Chung Shan Medical University.

Spectrophotometry

Spectrum distribution of LED family lamps was analyzed using an UV-visible spectrophotometer (Ultrospec 3000, Pharmacia Biotech, Cambridge, UK) from 300 nm to 800 nm.

Histopathology evaluation

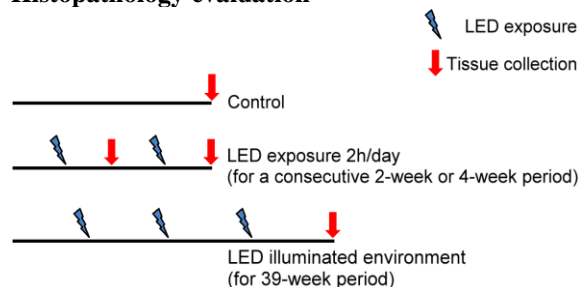


Fig. 1: Schematic summary of the protocol used for analyzing effects of white family LED irradiation on eyes. The response of retinal photoreceptor degeneration after LED exposure was studied by exposed mice to low-powered LED for 2 hr/day (a consecutive 2-weeks and 4-week period) and to illuminated environment of 12-hr light/12-hr dark cycle with white family LED lamps for 39 weeks.

The entire enucleated eyes from light-exposure and control mice were perfused intracardially with a fixative containing 4% paraformaldehyde in phosphate-buffered saline (PBS, pH7.4). Tissues were dehydrated through a graded series of ethanol, and then embedded in paraffin. Sections (70 μm -thick) were collected and stained with hematoxylin. Subsequently, sections were mounted and examined under a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Measurement of the outer nuclear layer thickness and area

The retinal sections obtained after light exposure and unexposed controls were stained with hematoxylin. Using Image Pro Plus image analysis system (Media Cybernetics, Silver Spring, MD, USA), the outer nuclear layer (ONL) thickness and area were measured at 0.5, 1, 1.5, 2, 2.5 and 3.0 mm superior and inferior to the optic nerve disc.

Statistical Analysis

Data are presented as the mean \pm SEM. Statistical comparisons were made using a one-way analysis of variance (ANOVA) followed by a Student's t test. The statistical significance was assessed at $p < 0.05$.

3. Results

3.1 The spectral irradiance profile of LED source

The emission spectrum of UV/visible lighting ranging from 300 to 800 nm was recorded (Fig. 2). The wavelength within the UV range was not detectable from the lamp of LED source. The spectrum distribution of lighting began to increase at 300 nm and reached sustained maximum transmission at 450 nm. Major peak of the spectrum

distributes was about 0.35 mW/cm² within the visible wavelength of the blue light. Spectral irradiance profile of LED source showed that the main optical radiation ranging from 430nm to 500 nm.

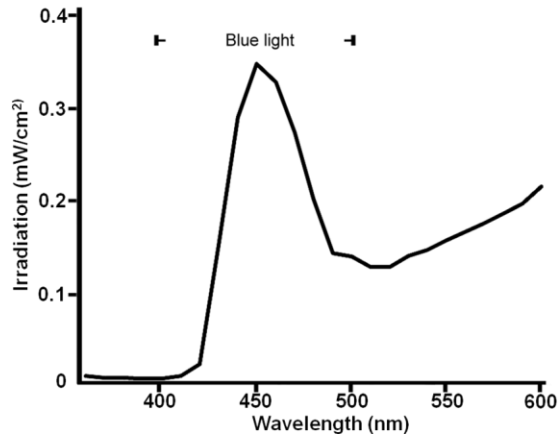


Fig. 2: Spectrum distribution of family LED

Spectrum distribution of low-powered LED is analyzed from 360 nm to 600 nm. Spectra show the peak wavelength of LED lamp is at 450 nm wavelength of blue light region.

3.2 The changes of retinal histology after light exposure from white family LED lamps

We assessed retinal images after LED exposure using morphological methods with hematoxylin staining. In control retinas, well-ordered photoreceptor layer could be seen (Figs 3A and 3B). The ONL thickness was remarkably thinned in LED exposure group compared to control mice (Fig. 3C-3H). For 2 week LED exposure 2 hr every day (Figs 3C and 3D), the light-induced changes in the retinal morphology were less severe than those eyes after changed were significantly severest after 38 weeks exposure (Figs 3G and 3H). In sections from LED exposure mice for 2 weeks and 39 weeks, the ONL thickness was significantly smaller than from control mice (Figs 3E-3H).

3.3 Effects of the LED exposure on light-induced thinning of ONL

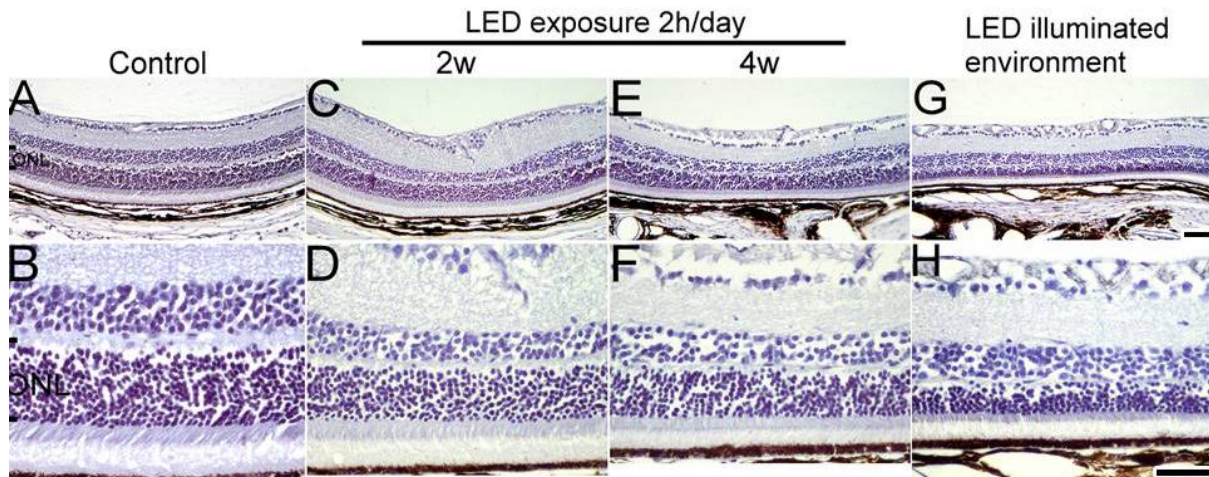


Fig. 3: Representative images of hematoxylin staining for retina section in control and LED-exposure mice

Light micrographs were taken from the mouse retinas. Sections of control and light-exposed retinas stained with hematoxylin. In control retinas (A and B), the outer nuclear layer (ONL) shows the photoreceptor nuclei normally (B). The arrangement of photoreceptor cells in the outer nuclear layer was slightly distorted and the thickness of the outer nuclear layer was decreased after 2 weeks (C and D) and 4 weeks (E and F) exposure. At 39 weeks after light exposure (G and H), with a significant reduction in the thickness of the outer nuclear layer (G), and the photoreceptor cell loss is evident (H). After light exposure, noted that the outer nuclear layer becomes thinner over time. ONL, outer nuclear layer. Scale bars=50 μ m.

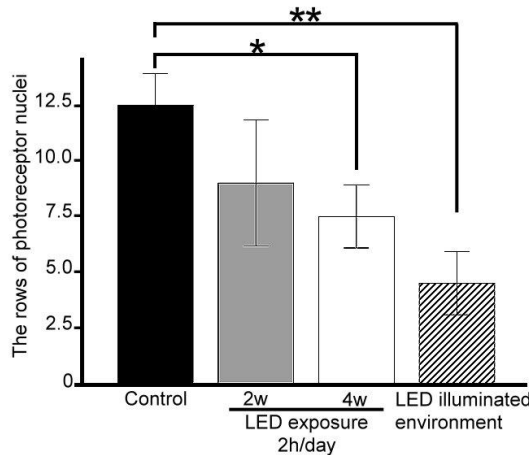


Fig. 4: Quantitative analysis the rows of photoreceptor nuclei in outer nuclear layer after low-powered LED exposure.

In control retinas, the outer nuclear layer shows 12 to 13 rows of photoreceptor nuclei. However, after 2 to 4 weeks period of light exposure, the thickness of outer nuclear layer was become thinner over time. At 39 weeks after light exposure, photoreceptor cell loss is evident, with a reduction in the outer nuclear layer thickness in some region to 4 to 5 rows of photoreceptor nuclei. Compare with control, after 4 weeks exposure and continuous LED illuminated environment, the rows of photoreceptor nuclei were significantly decreased. Data are expressed as means (\pm S.D.); ** indicate a value statistically different ($p < 0.01$) from the control, and * indicate a value statistically different ($p < 0.05$) from the control.

In control mice, the ONL thickness was identical in the entire retina. In LED-exposed for 2 weeks mice, the rows of ONL was decreased, whereas there was no significant different between in 2 LED-exposed for 2 week mice and in control mice (Fig. 4). After LED exposure for 4 week period, the number of row of photoreceptor cells was scarce in retina (about 7 rows) compared to the control eyes (about 12 rows), and the difference reached statistical significance. The decreased rows of photoreceptor cells with statistical significance were also detectable in the mice after LED illumination for 39 week. These results show that LED exposure decrease the rows of ONL in the retina, and ONL became thinner over time (Fig. 4). Thus, histogram photoreceptor cell rows clearly showed the effect of retina by the LED exposure.

3.4 Effects of the LED exposure on light-induced cell death of the photoreceptor cells

Sections showed that retinas subjected LED exposure were thinner than control eyes. The retinal

thinning was mainly due to the decrease in size of ONL as a consequence of a fall in the number of photoreceptors. In control retinas, the ONL showed more photoreceptors nuclei. After LED exposure 2 hr every day for 2 week and 4 week period, the number of ONL became fewer over time. Moreover, photoreceptor cell loss with a reduction in ONL thickness after LED illuminated environment for 39 week. To determine if the lighting have any damaging effect, the number of photoreceptor nuclei from LED-exposed eyes were compared to those from controls (Fig. 5). By quantitative analysis, the number of photoreceptor cells in ONL from LED exposure 2 hr every day for 2 week was not different from control eyes ($p > 0.05$). However, eyes from LED exposure 2 hr every day for 4 week period and from LED illuminated environment for 39 week, the number of photoreceptor nuclei was significantly fewer than that from control eyes ($p < 0.05$).

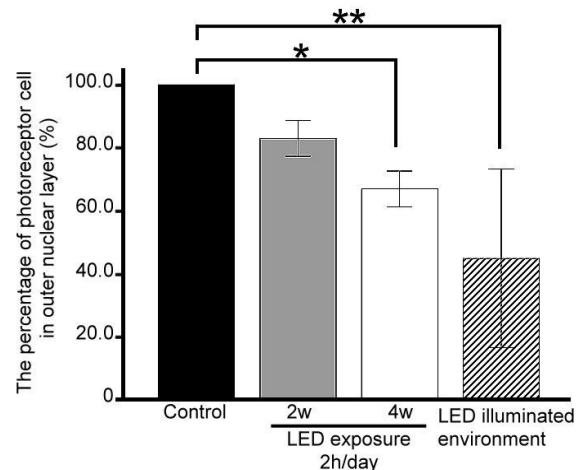


Fig. 5: Quantitative analysis the percentage of the photoreceptor cell number in outer nuclear layer after low-powered LED exposure

In control retinas, the outer nuclear layers were normally observed the photoreceptor cells in outer nuclear layer. However, the number of photoreceptor cells was decreased about 13% and 23% in the outer nuclear layer from 2 weeks exposure and maintain decreased for 4 weeks exposure. A significant decrease about 45% in the number of photoreceptor cells is observed from LED exposure environment for 39 weeks. Data are expressed as means (\pm S.D.); ** indicate a value statistically different ($p < 0.01$) from the control, and * indicate a value statistically different ($p < 0.05$) from the control.

4. Discussion

It was well known that visible light produce damage to photoreceptor cells of retinal tissues [16]. Histopathology examinations 30 days after lighting indicated that blue LED exposure greater than 60 J/cm² caused a significant disruption of photoreceptor cells of retina [17]. It also demonstrated that the continuous spectrum, blue light from LED is a danger to retinas in normal young rhesus monkeys [18]. In our study, the light from low-powered white house LED output mainly falls greater than 400 nm (Fig. 2). Moreover, as showed in the spectrum distribution, it exhibited a power-peak of at 450 nm within the wavelength range of the blue light. Consequently, these data indicate that white house LED lamps might have additional risk of degenerative factors on photoreceptor cells of retinal tissues after lighting.

As higher-powered blue light from LED is known to induce retinal damage and dysfunction [17], we demonstrated the effect of photoreceptor cells on light-induced photoreceptor degeneration by irradiation from low-powered white family LED in mice with hematoxylin staining. Several studies were indicated that the thickness of the ONL in retina decrease after 1 day [19]. In this study, histopathology study showed significant atrophy of the ONL thickness after 4 week period of LED exposure 2 hr/day, and more serious atrophy over time. Overall, the findings of the present study demonstrate that the thickness of ONL is decrease by low-powered white family LED exposure over time.

Previous studies revealed the action spectrum of retinal phototoxicity increases logarithmically as the wavelength as the wavelength of exposure decrease. The irradiation near 410 nm may contribute to degenerate of the retina [16-18]. However, other results indicated that the ONL area was significant difference after exposure to not only short blue light with peak wavelength 420 nm (ranging 380-500 nm), but also long blue light with peak wavelength 446 nm (ranging 400-540 nm) [20]. In our current study, abnormal thickness of ONL was found after lighting with peak wavelength about 450 nm from white family LED. It suggested that the long blue light may induce the retinal injury in eyes, even if the lighting of low-powered output from white house LED. The findings of the present study also demonstrate that the thickness of ONL is decrease by long blue light exposure over time.

Although the primary function of the retina is to receive and transduce light from the environment, excess exposure to light is hazardous to vision [3-4]. Shorter wavelength light is the most hazardous component of the visible spectrum [8-9], and it is known to generate reactive oxygen species (ROS) in the retina [21]. The RPE is especially susceptible to oxidative stress because of its high light, oxygene,

fluorophore (e.g. lipofuscin) and membrane lipid (e.g. polyunsaturated fatty acids) levels [22]. Several molecules in the retinal cell are implicated in mediating the phototoxicity of light.

Removing the lens by cataract surgery increases the amount of light exposure that approaches the retina [23]. To compensate for reduced blue light filtering intraocular lens, blue light- and UV-absorbing yellow-tinted intraocular lens were introduced in the 1990s and made with rigid polymethylmethacrylate material. More recently, these intraocular lens were product with foldable silicone or soft acrylic material. However, the significantly protective effects of yellow intraocular lens filter through its blocking of the transmission of light against shorter (420 nm) peak wavelength blue light, but not against longer (446 nm) peak wavelength blue light. The light source from white house LED was detected the 450 nm peak wavelength of longer blue light (Fig. 2). Accordingly, the longer blue light from the LED lamps was not filtered through the yellow intraocular lens and might be hazardous to the photoreceptor cells of retina.

5. Conclusion

The present data clearly demonstrated irradiation of the white LED is above 400 nm and is not within the ultraviolet light region. However, the exposure of eye in LED illuminated environment was related to the development of photoreceptor loss. It must be noted that the light illuminations used in the present study as an experimental tool were not fully similar to normal condition that which would impinge upon the retina.

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References

1. Bass AM, Paur RJ. (1984) Ultraviolet absorption cross-sections of ozone: measurements, results, and analysis, in: Quadrennial Ozone Symposium, Reidel, Halkidiki, Greece: 606-10.
2. Siu TL, Morley JW, Coroneo MT. (2008) Toxicology of the retina: advances in understanding the defence mechanisms and

- pathogenesis of drug- and light-induced retinopathy. *Clin. Exp. Ophthalmol*, 36(2): 176-85.
3. Hirakawa M, Tanaka M, Tanaka Y, Okubo A, Koriyama C, Tsuji M, Akiba S, Miyamoto K, Hillebrand G, Yamashita T, Sakamoto T. (2008) Age-related maculopathy and sunlight exposure evaluated by objective measurement. *Br J Ophthalmol*, 92(5): 630-4.
 4. Klein R, Klein BE, Tomany SC, Wong TY. (2004) The relation of retinal microvascular characteristics to age-related eye disease: the Beaver Dam eye study. *Am J Ophthalmol*, 137(3): 435-44.
 5. Ham WT Jr, Mueller HA, Sliney DH. (1976) Retinal sensitivity to damage from short wavelength light. *Nature*, 260(5547): 153-5.
 6. Mainster MA, Ham WT Jr, Delori FC. (1983) Potential retinal hazards. Instrument and environmental light sources. *Ophthalmology*, 90(8): 927-32.
 7. Norren DV, Vos JJ. (1974) Spectral transmission of the human ocular media. *Vision Res*, 14(1): 1237-44.
 8. Taylor HR, West S, Munoz B, Rosenthal FS, Bressler SB, Bressler NM. (1992) The long-term effects of visible light on the eye. *Arch. Ophthalmol*, 110(1): 99-104.
 9. Braunstein RE, Sparrow JR. (2005) A blue-blocking intraocular lens should be used in cataract surgery. *Arch. Ophthalmol*, 123(4): 547-9.
 10. Hafezi F, Marti A, Munz K, Reme CE. (1997) Light-induced apoptosis: differential timing in the retina and pigment epithelium. *Exp. Eye Res*, 64(6): 963-70.
 11. Suter M, Reme C, Grimm C, Wenzel A, Jaattela M, Esser P, Kociok N, Leist M, Richter C. (2000) Age-related macular degeneration. The lipofusion component N-retinyl- N-retinylidene ethanolamine detaches proapoptotic proteins from mitochondria and induces apoptosis in mammalian retinal pigment epithelial cells. *J. Biol. Chem*, 275(50): 39625-30.
 12. Boulton M, Dontsov A, Jarvis-Evans J, Ostrovsky M, Svistunenko D. (1993) Lipofuscin is a photoinducible free radical generator. *J. Photochem. Photobiol*, 19(3): 201-4.
 13. Rozanowska M, Pawlak A, Rozanowski B, Skumatz C, Zareba M, Boulton ME, Burke JM, Sarna T, Simon JD. (2004) Age-related changes in the photoreactivity of retinal lipofuscin granules: role of chloroform-insoluble components. *Invest. Ophthalmol. Vis. Sci*, 45(4): 1052-1060.
 14. Crockett RS, Lawwill T. (1984) Oxygen dependence of damage by 435 nm light in cultured retinal epithelium. *Curr. Eye Res*, 3(1): 209-15.
 15. Ruffolo JJ, Ham WT, Mueller HA, Millen JE. (1984) Photochemical lesions in the primate retina under conditions of elevated blood oxygen. *Invest. Ophthalmol. Vis. Sci*, 25(8): 893-8.
 16. Lawwill T. (1982) Three major pathologic processes caused by light in the primate retina: a search for mechanisms. *Trans Am Ophthalmol Soc*, 80:517-79.
 17. Koide R, Ueda TN, Dawson WW, Hope GM, Ellis A, Somuelsen D, Ueda T, Iwabuchi S, Fukuda S, Matsuishi M, Yasuhara H, Ozawa T, Armstrong D. (2001) Retinal hazard from blue light emitting diode, *Nihon Ganka Gakkai Zasshi*, 105(10): 687-95.
 18. Ueda T, Nakanishi-Ueda T, Yasuhara H, Koide R, Dawson WW. (2009) Eye damage control by reduced blue illumination. *Exp Eye Res*, 89(6): 863-8.
 19. Santos AM, Martín-Oliva D, Ferrer-Martín RM, Tassi M, Calvente R, Sierra A, Carrasco MC, Marín-Teva JL, Navascués J, Cuadros MA. (2010) Microglial response to light-induced photoreceptor degeneration in the mouse retina. *J Comp Neurol*, 518(4): 477-92.
 20. Tanito M, Kaidzu S, Anderson RE. (2006) Protective effects of soft acrylic yellow filter against blue light-induced retinal damage in rats. *Exp Eye Res*, 83(6): 1493-504.
 21. King A, Gottlieb E, Brooks DG, Murphy MP, Dunaief JL. (2004) Mitochondria-derived reactive oxygen species mediate blue light-induced death of retinal pigment epithelial cells. *Photochem Photobiol*, 79(5): 470-5.
 22. Radu RA, Hu J, Yuan Q, Welch DL, Makshanoff J, Lloyd M, McMullen S, Travis GH, Bok D. (2011) Complement system dysregulation and inflammation in the retinal pigment epithelium of a mouse model for Stargardt macular degeneration. *J Biol Chem*, 286(21): 18593-601.
 23. Mainster MA, Sparrow JR. (2003) How much blue light should an IOL transmit? *Br J Ophthalmol*, 87(12): 1523-9.

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The expression of plasma lysophosphatidic acid in patients with epithelial ovarian cancer at advanced stage before and after interventional therapy

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Abstract: The objective is to evaluate the clinical effect of artery chemotherapy infusion and embolism in patients with epithelial ovarian cancer at an advanced stage by investigating the expression of plasma lysophosphatidic acid (LPA) and the tumor volume before and after the interventional therapy. Uterine artery and ovarian artery chemotherapy infusion and embolism were performed on 22 patients with epithelial ovarian cancer at an advanced stage. The level of plasma LPA in patients with benign epithelial ovarian tumor (22 cases) and epithelial ovarian cancer (22 cases, before and after interventional therapy) was measured by biochemical method. At the same time, the tumor volume of patients with epithelial ovarian cancer was observed both before and after interventional therapy. The Results were that the average levels of plasma LPA of 20 benign cases were within the normal range. The average levels of plasma LPA in patients with epithelial ovarian cancer at an advanced stage were significantly higher than the levels in the benign cases ($P < 0.05$). Following interventional therapy, the levels of plasma LPA and the tumor volume both declined, albeit at different degrees. There was also a significant difference between the levels before and after interventional therapy ($P < 0.05$). So we found that the application of artery chemotherapy infusion and embolism can decrease the levels of plasma LPA, reduce the volume of the tumor, and improve the resection rate for the patients with epithelial ovarian cancer at an advanced stage. Interventional therapy is an effective method to treat epithelial ovarian cancer at an advanced stage.

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【Key words】 interventional chemotherapy infusion and embolism; advanced stage epithelial ovarian cancer; lysophosphatidic acid; tumor volume

1. Introduction

Ovarian cancer is a common type of gynecologic malignant tumor. Because of the position of the ovaries deep within the pelvis and because of a lack of early symptoms, ovarian cancer has the highest mortality rates of any gynecological malignancies. Approximately 75-80% of patients receive a late diagnosis, and the 5-year survival rate is below 30%. The related antigen CA125 has been used clinically to detect the biological index of ovarian cancer for many years. However, its sensitivity and specificity are low (72% and 73%, respectively) [1]. Lysophosphatidic acid (LPA) is a cell membrane lipid derivative, which is significantly elevated in the plasma and ascites of patients with ovarian cancer. This rise in LPA levels seems to be specific for ovarian cancer since a similar increase is not seen in patients with other cancer types, such as leukemia and breast cancer [2]. Eder et al reported that LPA levels produced by normal ovarian epithelial cells is far below the levels produced by ovarian epithelial cancer cells [3]. Additionally, LPA

can stimulate the cancer cells to produce more LPA, thus forming an autocrine loop [4]. The use of LPA levels to diagnose ovarian cancer provides greater sensitivity and specificity compared to the common clinical tumor marker CA125. In vitro studies show that LPA can restrain ovarian tumor cell proliferation and promote apoptosis [5]. Therefore, it is inferred that LPA is related to the occurrence, development, infiltration, and transfer of ovarian cancer [6]. This study explores the plasma LPA levels and the tumor volume changes of late stage epithelial ovarian cancer patients before and after interventional treatments. The goal is to understand the therapeutic value of interventional therapy for late stage epithelial ovarian cancer.

2. Materials and methods

1. Case choice: We selected 42 patients with epithelial ovarian cancer for our study. Patients were recruited between October 2008 and December 2011 from the first affiliated hospital of Zhengzhou

University. 22 patients had malignant disease (15 cases of serous cystadenocarcinoma and 7 cases of mucinous adenocarcinoma of the capsule) and were classified as III ~ IV period patients. 20 patients had benign cases. These included 14 cases of serous cystadenoma, 5 cases of mucinous cystadenoma, and 1 case of an ovaries endomembrane tumor. All patients between the ages of 38 and 70 years old were seeing the doctor for the first time. Benign cases were separated from both ovarian tumors and from the one disaster side ovariectomy. Malignant cases were characterized by adhesion to surrounding organs. Thus, there was poor mobility within the pelvic cavity, and abdominal transfer was also exist. All cases underwent CT or ultrasound examination, which was better able to identify cases of ovarian cancer. Because of the specific condition of these patients, doctors were unable to perform surgery. Instead, they tried to reduce and destroy tumor cells via intervention treatment. This helped to cure epithelial ovarian cancer cases.

2. Methods

2.1 LPA determination: We drew approximately 4ml of venous blood from fasting patients with benign ovarian epithelial neoplasms. Blood was drawn from patients both before and after intervention treatment and also 15-20 days before surgery. According to the LPA determination protocol, we placed blood samples in anticoagulation tubes. Samples were centrifuged for 10 min at 8000 r/min. From 1ml of the supernatant, we extracted phospholipids. The phospholipid composition was also enriched and separated. As a final step, samples were placed in a 90°C water bath for 5min after adding a specific color-developing agent. After 35min from removing samples from the greenhouse, we measured the samples colors at a wavelength of 636nm. The LPA reagent was produced by Beijing's technology development corporation by following the biochemical method. All readings were taken on the 722 spectrophotometer instrument. Reference range: normal < 2.9 u mol/L, critical value 2.9 ~ 3.2 u mol/L, abnormal > 3.2 u mol/L.

2.2 Malignant cases were performed right femoral artery puncturing. A 5F Pigtail catheter was placed by using Seldingers technology. We then performed abdominal aortography at the 12 thoracic vertebrae level in order to observe the blood supply of the arteria ovarica or inferior mesenteric artery. We also determined the position of the bilateral iliac artery bifurcate and the distribution of blood supply of the internal iliac artery. We performed the bilateral internal iliac artery angiography. After exchanging, the 5 F Cobra catheter is placed in the internal iliac artery to observe the blood supply of the internal iliac artery. The catheter was placed in the uterine artery, and we used the micro-catheter to choose the ovarian artery.

We performed infusion chemotherapy after diluting the anticarcinogen cisplatin with physiological saline. We used gelatin sponge particles to embolize until the mainblood flow was blocked. The 5 FCobra catheter was used for inferior mesenteric artery radiography. Five cases can be seen with tumor stain. For these cases, it is considered that the intestinal tract has been infringed upon, thus, cisplatin chemotherapy perfusion is adopted. The total cisplatin given is 80 mg/m² according to the blood supply. The ovary tumor rebulking operation was adopted after 15 to 20 days.

2.3 tumor size: We ultrasonically determined the tumor diameter line before and after intervention treatment and calculated the tumor size according to the formula $(4 \pi abc / 3)$ cm³ tumor.

2.4 efficacy judgment standard: It is the change of LPA and the tumor size of the epithelial ovarian cancer before and after the intervention treatment (two weeks after the vaginal B ultrasound to check).

2.5 statistics management: We adopted the count material related analysis method and, managed the statistical data with the statistical software SPSS 17.0. Data is represented as mean ± standard deviation. We compared differences between groups, and took $P < 0.05$ to be statistically significant.

3. Results

3.1 interventional treatment results: Only 2 out of 20 patients in the benign ovarian epithelial neoplasm group had levels of plasma LPA greater than 3.2umol/L. All others were in the normal range. The 20 cases of late stage epithelial ovarian cancer displayed plasma LPA levels that were significantly higher than normal before interventional therapy. After interventional treatment, the levels dropped (Table 1) and tumor size shrank to a significant degree ($P < 0.05$) (Table 2).

22 patients with cases of late epithelial ovarian cancer had DSA radiography that showed that the bilateral iliac artery (mainly for uterine artery) and the ovarian artery were thick. The pelvic tumor was stained with dye. The intestinal tract of some cases were violated, so tumor color dyeing was also seen here. After two weeks of performing gynecologic B ultrasound examinations, we detected mass reduction, a softer mass, and improved mobility. These effects can be seen in table 1 after two weeks. After interventional treatment, the 20 patients displayed reduced levels of ovarian cancer cells after 20 days. However, for 2 cases, we could not perform surgery because of extensive abdominal transfer. It can be seen from the operation that the tumor size decreases, the mass becomes softer, the mobility is improved, it is easier to strip, the lymph nodes undergo necrosis, and there is less bleeding among the 19 patients.

3.2 adverse reactions and complications

Common adverse reactions include embolization reaction after interventional treatment, such as fever, pain, nausea, and vomiting. Additionally, there is a decrease in white blood cells down and liver function. 5 cases displayed intestinal invasion. One had intestinal obstruction after the operation and was fully recovered

after being disposed of for one week. All patients experienced ventosity to varying degrees because the tumor tissue undergoes ischemia, anoxia, and necrosis after chemotherapy embolization. The patients were advised to breathe gradually to alleviate symptoms, and this does not appear to be a serious complication.

Table 1 The plasma LPA test results of the benign ovarian epithelial neoplasm, epithelial ovarian cancer group before and after interventional treatment

Tissue types	numbers (n)	lysophospholipids acid (μ mol/L)
Benign epithelial ovarian tumor	20	2.877 \pm 0.354
Epithelial ovarian cancer group		
before intervention	22	6.286 \pm 1.033 *
after intervention	22	4.919 \pm 1.404 #

* : benign group, epithelial ovarian cancer before the intervention group plasma LPA results show: $t = 14.435$, $p < 0.05$, a statistically significant difference.

: epithelial ovarian cancer groups before and after interventional treatment plasma LPA results show: $t = 4.437$, $p < 0.05$, a statistically significant difference.

Table 2 the ovarian epithelial cancer tumor size changes before and after interventional treatment

Before and after intervention treatment	numbers	tumor size (cm3)
before interventional treatment	22	1463.617 \pm 1408.069
after interventional treatment	22	807.362 \pm 759.195

The epithelial ovarian cancer groups' tumor size before and after intervention treatment : $t = 4.437$, $p < 0.05$, a statistically significant difference.

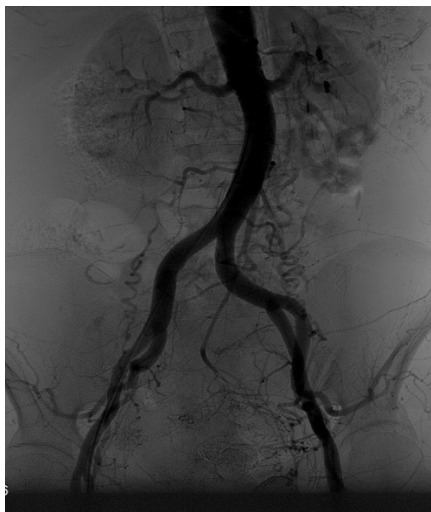


Figure 1. Abdominal aortography



Figure 2. Inferior mesenteric arteriography

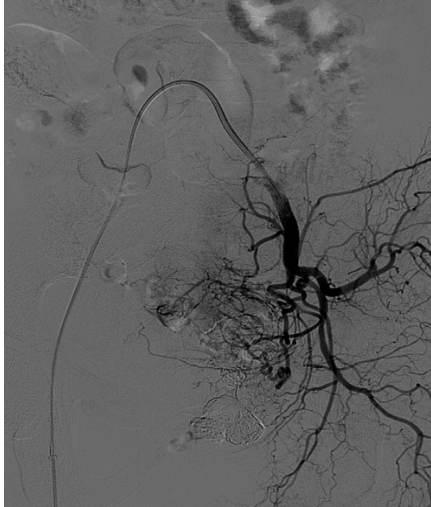


Figure 3. Before left uterine artery embolism



Figure 4. After left uterine artery embolism

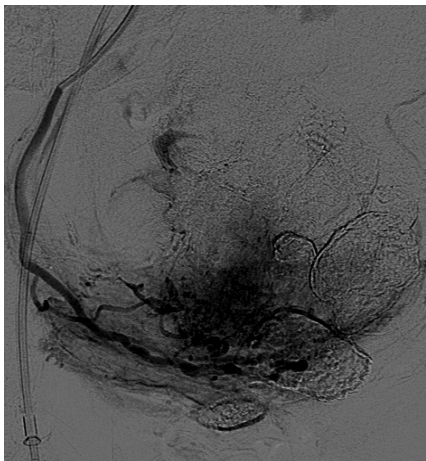


Figure 5. Before right uterine artery embolism

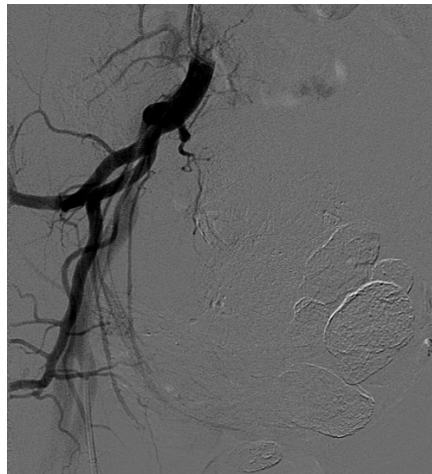


Figure 6. After right uterine artery embolism

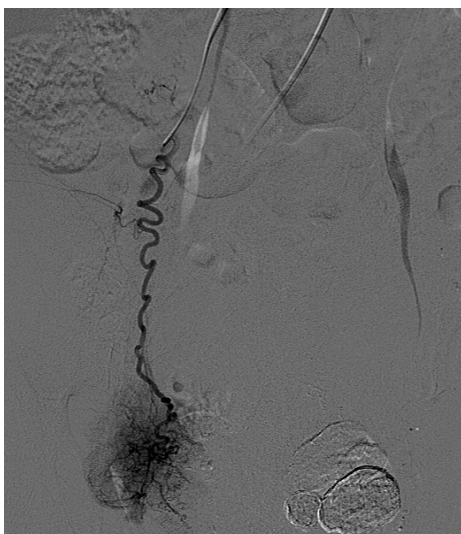


Figure 7 before left ovarian embolism

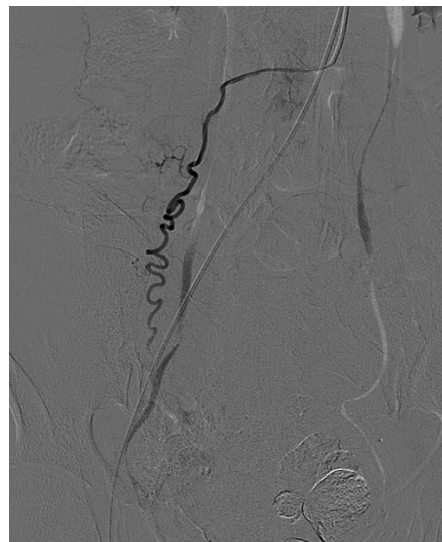


Figure 8 after left ovarian artery embolism



Figure 9 before right ovarian artery embolism

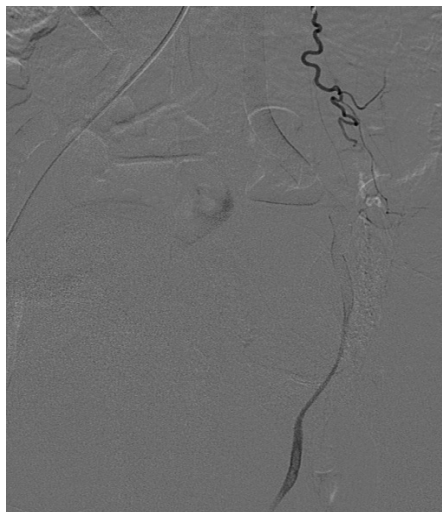


Figure 10 after right ovarian artery embolism

4. Discussions

LPA is a multi-functional phospholipid that induces intercellular signal transduction and elicits a number of biological effects via signaling through G protein coupled receptors [7]. Xu and Sutphen [8] found that nearly 90% of patients with stage I ovarian cancer have plasma LPA levels that are increased. Furthermore, these levels increase with increasing tumor grade. Thus, plasma LPA levels can be used as a biomarker for ovarian cancer diagnosis. LPA is related to the cancer occurrence, progression, infiltration, and transfer. LPA can promote ovarian cancer cell proliferation and survival. It can also promote production of proteases and other factors that promote angiogenesis. In addition, LPA can inhibit cancer cell apoptosis through inducing expression of vascular endothelial growth factor (VEGF) [9].

Presently, the most common treatment options for ovarian cancer are surgery and chemotherapy. However, surgical resection for advanced patients (stage III and above) is difficult and accompanied by a poor prognosis. For advanced patients, primary cytoreductive surgery and postoperative platinum chemotherapy are the most common treatment methods. With the development of interventional therapy technology, arterial infusion chemotherapy and embolism have gradually become part of the treatment regimen for gynecologic malignancies. The combination of chemotherapy and embolism can significantly enhance the clinical effect. Many studies show that artery infusion chemotherapy can shrink the tumor volume, reduce tumor stage, and create the opportunities for successful surgery [10, 11].

The blood supply to the ovary mainly comes from the ovarian branch of uterine arteries and the ovarian

artery. The interventional therapies for ovarian cancer include intra-ovarian arterial chemotherapy perfusion and uterine arterial embolization. Research shows that even a single increase in local drug concentration results in an increased number of the cancer cells being killed by almost 10-fold. Selective iliac artery intubation can deliver chemotherapy drugs directly into the tumor blood supply artery to increase the local drug concentration to a greater extent than systemic venous chemotherapy. This can improve the effect of the treatment. In patients with advanced ovarian cancer, the tumor cells are widely transferred in the pelvic cavity and the peritoneal cavity. As a result, operation is not possible. In this case, the intervention therapies include laparotomy and cytoreductive surgery. They are effective at increasing patient life span. The role of LPA and its receptor in tumor formation and transfer has been confirmed. In this study, 22 cases showed high plasma LPA levels that decreased after the intervention. Focal cancers decreased and the disease was controlled to some extent. 2 cases did not undergo cytoreductive surgery because tumor cells were too widely transferred. Artery perfusion chemotherapy and embolism are effective treatments for ovarian cancer, especially in the case of advanced disease. Monitoring plasma LPA levels in ovarian cancer has clinical value.

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Reference

1. Obeidat B, Latimer J, Crawford R. Can optimal primary cytoreduction be predicted in advanced stage epithelial ovarian cancer? Role of preoperative serum CA125 level. *Gynecol Obstet Invest*, 2004 57 (3): 153-156
2. Shen Z, Wu M, Elson P, et al. Fatty acid composition of lysophosphatidic acid and lysophosphatidylinositol in plasma from patients with ovarian cancer and other gynecological diseases. *Gynecol Oncol*. 2001, 83(1): 25-30
3. Eder AM, Sasagawa T, Mao M, et al. Constitutive and lysophosphatidic acid (LPA)-induced LPA production: role of phospholipase D and phospholipase A2 [J]. *Clin Cancer Res*, 2000, 6(6):2482-2491
4. Sengupta S, Xiao YJ, Xu Y. A novel laminin-induced LPA autocrine loop in the migration of ovarian cancer cells [J] *FASEB J*, 2003, 17:1570-1572
5. Xu Y, Shen Z, Wiper DW, et al. Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers *JAMA*, 1998, 280: 719-723.
6. Ren J, Xiao YJ, Singh LS, et al. Lysophosphatidic acid is constitutively produced by human peritoneal mesothelial cells and enhances adhesion, migration, and invasion of ovarian cancer cells. *Cancer Res*. 2006 Mar 15; 66(6): 3006-3014
7. Sakamoto S, Yokoyama M, Zhang X, et al. Increased expression of CYR61, an extracellular matrix signaling p-rotein in human benign prostatic hyperplasia and its regulation by lysophosphatidic acid. *Endocrinology*, 2004, 145(6): 2929-2940.
8. Sutphen R, Xu Y, Wilbanks GD, et al. Lysophospholipids are potential biomarkers of ovarian cancer. *Cancer Epidemiol Biomarkers Prev*. 2004, 13(7): 1185-1191
9. Hu X, Mendoza FJ, Sun J, et al. Lysophosphatidic acid (LPA) induces the expression of VEGF leading to protection against apoptosis in B-cell derived malignancies [J]. *Cell Signal*, 2008, 20: 1198—1208
10. Minagawa Y, Kigawa J, Irie T. et al. Radical surgery following neoadjuvant chemotherapy for patients with stage mB cervical cancer[J]. *Ann Surg Oncol*. 2004, 5: 539-543
11. Minkarah AR. The better of lymphnode debukiing in metastatic cervical cancer: A research bias or an actual effect[EJ]. *Gynecol Oncol*, 2002, 87(2): 161-162

2/20/2012

Studies on Hematological Parameters and DNA Structure in Newborn Rats Exposed to Extremely Low Frequency Magnetic Fields

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Abstract: The aim of this study was to evaluate the possible effects of *in vivo* exposure to extremely low frequency magnetic fields (ELF-MF) on some hematological parameters, pathological variations and DNA structure in newborn rats. Six female pregnant Wistar rats were obtained from the National Research centre in Egypt and gave birth to 30 rats at the animal house of Cairo University. The newborn rats were divided into two separate groups: one exposed group (50 Hz, 0.5 mT, 30 days, 24 h/day) and one control (sham). Red blood cells (RBCs), hemoglobin and hematocrit levels decreased significantly ($P < 0.02$) while white blood cells (WBCs) and platelets levels significantly increased ($P < 0.04$) in newborn rats that were exposed to ELF-MF. There was no significant difference in mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV) levels and DNA structure between the exposed and sham-exposed groups. ELF-MF induced a marked necro-degenerative change in kidney tissue and peri-portal fibrosis in liver tissues. Our results indicate that the applied ELF-MF exposure may induce statistically significant alterations in some hematological parameters, kidney and liver tissues of newborn rats.

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Key Words: Electromagnetic field, hematological parameters, DNA, pathological tissues.

1. Introduction

Results of experimental *in vitro* and *in vivo* studies carried out in recent years have given more attention to the biological effects of electromagnetic fields. Previous data suggest the association between electromagnetic field exposure and the increased incidence of certain types of tumor, particularly childhood and adult leukemia, childhood and adult brain tumors and increased risk of the neurodegenerative diseases [1, 2].

It is now known that ELF-MF can change cell behaviors and activations by affecting the biochemical and/or biophysical processes. Chemical and physical processes at the atomic levels are the bases of reactions between biomolecules in an electromagnetic field (EMF), since the field can magnetically affect chemical bonds between adjacent atoms with consequent production of free radicals [3-5].

The blood is one of the major homeostatic systems of the body in humans and animals, supporting normal viability, integrity and adaptive responses. The functional state of the blood systems changes dynamically according to the nature strength and duration of exposure to external factors. **Rogers et al.**, [6] reported that exposure to EMF changes the level of melatonin in blood.

Several studies have been reported concerning the effect of ELF-MF on cellular DNA damage. **Lai and Singh** [7] reported that acute (2hrs) exposure of rats to a 60 Hz magnetic field (flux densities 0.1, 0.25 and 0.5 mT) caused a dose- dependent increase in

DNA strand breaks in brain cells. On the other hand, there are several reports which demonstrate that ELF-MF does not cause DNA damage, especially DNA strand breaks [8-10]. The results of **Ivancsits et al.**, [11-13] indicate that the interaction of MF with DNA is quite complicated and apparently depends on many factors.

2. Materials and Methods

Animals

Animals were housed and maintained according to guidelines for the Care and Use of Laboratory Animals' [14] and approved by the animal Ethics Committee at Cairo University. Briefly, timed pregnant Wistar rats were purchased from the National Center of Researches in Egypt and gave birth at the animal house of Cairo University. They were maintained for one week in the laboratory for adaptation. Adult female rats with their newborns were housed in plastic cages with free access to drinking water and standard chow diet. They were also maintained in a controlled environment with 12h light cycle. All animal groups were housed in clean first hand cages under standard condition in a separate laboratory which belongs to animal care unit.

Magnetic Field Exposure

The exposure was performed by a magnet with a fixed magnetic field value of $0.5 \text{ mT} \pm 0.025$. The magnetic field (MF) was generated by a solenoid carrying current of 18 A (ampere) at 50 Hz from the

main supply (220-230 Volt) via a variac (made in Yugoslavia). The magnet consisted of a coil with 320 turns made of electrically insulated 0.8 mm copper wire. The coil was wound around a copper cylinder of 2mm thickness, 40cm diameter and 40cm length. The cylinder wall was earthed to eliminate the electric field. The magnetic field was measured at different locations to find out the most homogenous zone inside the solenoid core. This was done using Gauss/ Tesla meter model 4048 with probe T-4048 manufactured by Bell Technologies Inc. (Orlando-Florida USA). Plastic cages containing six adult female rats (Wistar) and their newborns were placed in the middle of the exposure chamber prior to MF exposure. When the newborns reached 10- days of age, they were randomly divided into two groups of 15 each: one control (sham) and one exposed. The latter group has been exposed to 50 Hz, 0.5 mT \pm 0.025 MF for 30 days 24 hrs per day. The control (sham) group was treated like the exposed group with the sole difference that it was not exposed to magnetic field. The two groups were treated equally considering light and food. The temperature and humidity were monitored continuously throughout the experimental period. This ensures that the control and the exposed animals were maintained in the same condition. During the experimental period, cleaning and changing water and food were done to all animals two times daily. The field was switched off during cleaning the cage. After 30 days of exposure, the two groups of newborn rats were sacrificed by decapitation. Five newborn rats from each group were used for DNA analysis. Another 5 newborn rats were used for hematological parameters and lastly 5 newborn rats were maintained for histopathological examination.

Analysis of DNA Fragmentation

Analysis of DNA fragmentation was measured using agarose gel electrophoresis, according to the protocol developed by **Kasibhatla et al.** [15]. 0.5 gm homogenized liver and spleen were transferred to 1.5 ml sterile micro centrifuge tubes. Centrifugation occurred at 200xg in an Eppendorf tube, centrifugation for 5min at 4°C and then the supernatant was removed. 20 μ l of TES (20mM EDTA (ethylenediaminetetraacetic acid), 100 mM Tris(hydroxymethylaminomethane), pH8.0, 0.8% (w/v) Sodium dodecyl sulfate) lysis buffer were added and mixed with cell pellet. 10 μ l of RNase Cocktail were added, mixed well and incubated for 30-120min at 37 °C. 10 μ l of proteinase K, were added and incubated at 50 °C for at least 90min. 5 μ l of 6x DNA loading buffer were added and DNA samples were loaded into dry wells of a 1-1.5% agarose gel in TAE (242g Tris base, 57.1 ml Acetic acid, 100 ml of 0.5 M EDTA, pH 8.0) buffer containing 0.5 μ g/ml

ethidium bromide. The gel was run at low voltage (i.e., 35V for 4 hours or until loading dye has run two-thirds of the way down the gel). DNA ladders are finally visualized by a ultra-violet (UV) light source and documented by photography. The gels were analyzed using the software: Gel-Pro Analyzer 3.1. The used chemicals were purchased from Sigma chemical co. (St. Louis MO, USA).

Histopathological Examination

The liver and kidney of newborn rats from control and exposed groups were dissected, removed and fixed in 10% neutral formalin. Then, they were embedded in paraffin blocks, sectioned and stained with hematoxylin and eosine (H&E).

Liver and kidney tissue sections were examined using light microscope (CX31 Olympus microscope) connected with digital camera (Canon) at a power x400.

Hematological Parameters

The blood is drawn from the control and exposed groups in test tubes containing anticoagulant (EDTA). White blood cells (WBC), red blood cells (RBC) indexes (hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC)) and platelets were counted and determined as methods described by (Dacie and Lewis)[16].

Statistical Analysis

All data are expressed as mean \pm standard error. The significance of difference in the results was evaluated with one-way ANOVA test.

3. Results

The results from DNA fragmentation using agarose gel electrophoresis technique are shown in figures (1, 2). Figure (1) shows that, lane (1) represents marker of standard molecular weight, lane(2) represents DNA isolated from the spleen of the control group, lanes (3,4,5) represent DNA isolated from the spleen of the exposed group, lane (6) represents DNA isolated from the liver of the control group, lanes (7,8,9) represent DNA isolated from the liver of the exposed group. Figure (2) shows the densitometry scan of figure (1). The results show that the DNA from viable cells stayed on the top of the gel as a high molecular weight band. The DNA from apoptotic cells formed as a distinct DNA ladder. The results do not show any difference between the DNA isolated from the spleen and liver of the control and exposed groups.

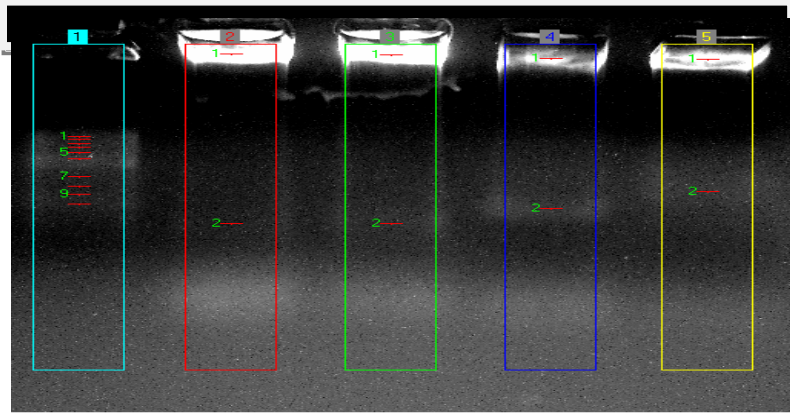


Figure (1): Gel electrophoresis of DNA. Lane(1) marker of standard molecular weight, lane(2) DNA isolated from spleen of control group, lanes(3) DNA isolated from spleen of exposed group, lane(4) DNA isolated from liver of control group, lanes(5) DNA isolated from liver of exposed.

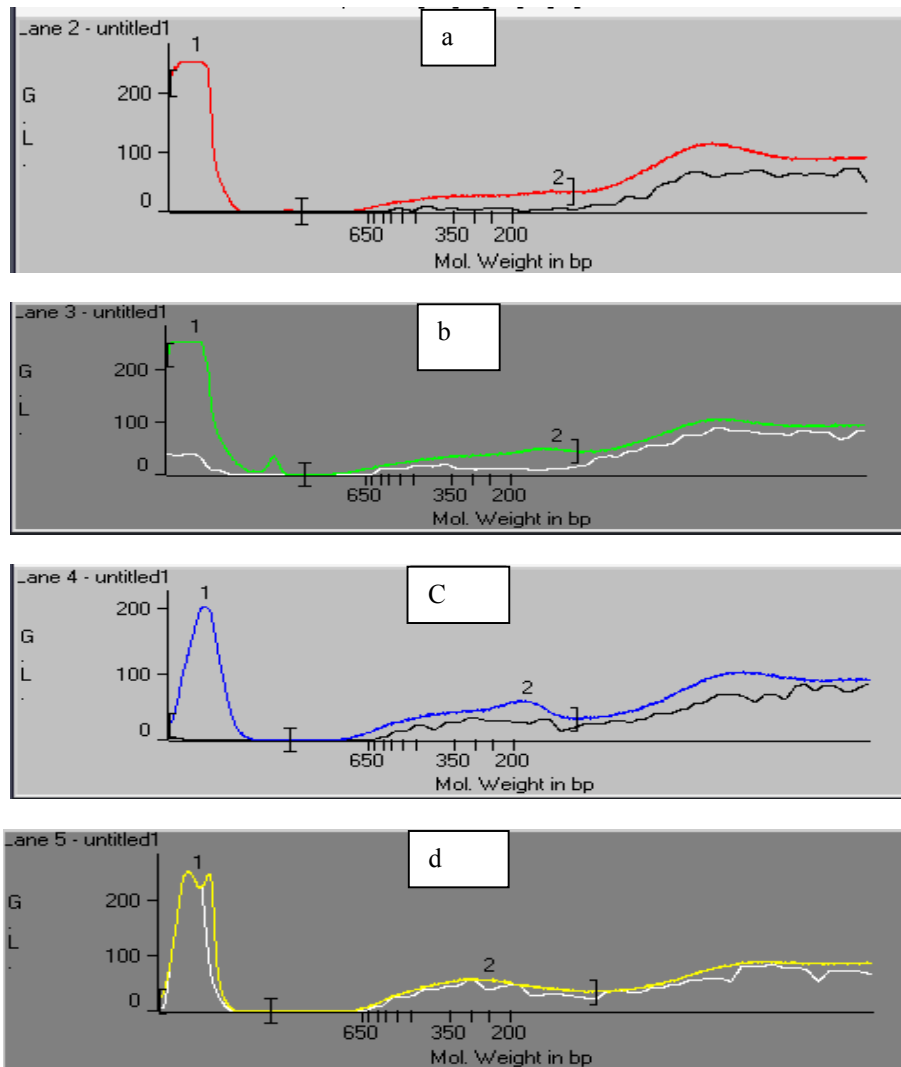


Figure (2 a-d): Densitometry scan of fragmented DNA samples.(a) DNA isolated from spleen of control group, (b) DNA isolated from spleen of exposed group, (c) DNA isolated from liver of control group, (d) DNA isolated from liver of exposed.

Figure (3a,b) represents liver sections from control and exposed groups respectively. Liver section of control group showed the portal tract intact triad of bile duct, hepatic artery and portal vein. On the one hand, no necro-inflammatory or degenerative changes, fibrosis, cirrhosis, or abnormal deposits

occurred. On the other hand liver section of the exposed group showed that portal tract is enlarged with peri-portal fibrosis. The hepatic artery showed mild congestion, the hepatocytes diffuse macro and micro vesicular steatosis and no established cirrhosis.

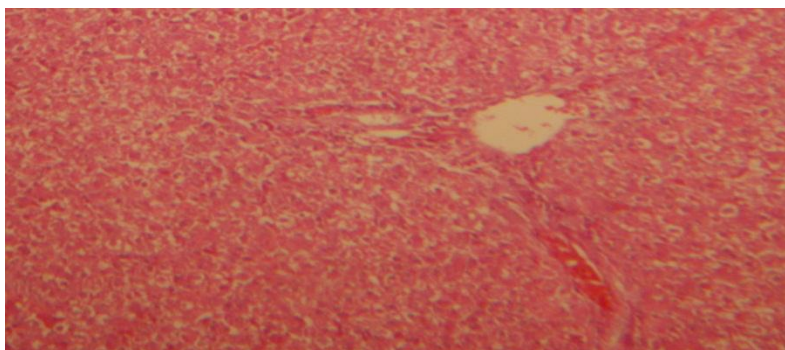


Figure (3a) : Liver, the control group: The portal tract shows intact triad of bile duct, hepatic artery & portal vein. No necro-inflammatory or degenerative changes, fibrosis, cirrhosis, or abnormal deposits. H&E, x200.

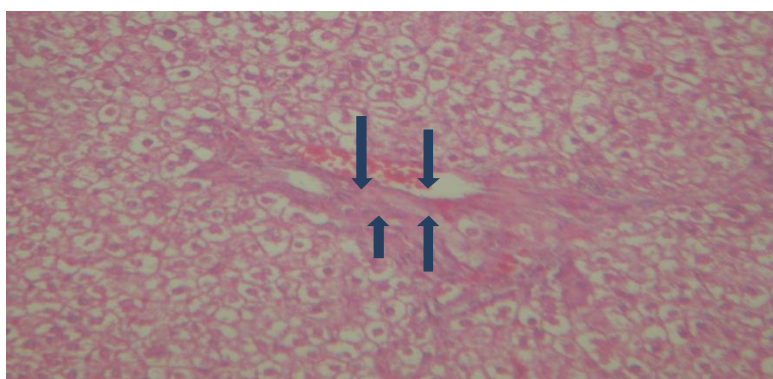
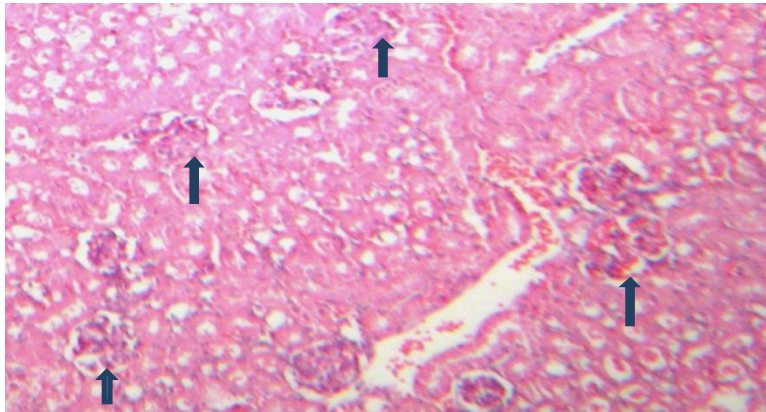


Figure (3b): Liver, the exposed group: The portal tract is enlarged with peri-portal fibrosis & early fibrous bridges (arrows) attempts (ie. Incomplete bridge formation) The hepatic artery shows mild congestion. The hepatocytes show diffuse macro & microvesicular steatosis. No established cirrhosis. H&E, x200.

Figure (4a,b) represents kidney sections from control and exposed groups respectively. Kidney section of control group showed renal tubules that are intact with patent lumens, free of cellular or hyaline casts. The cell lining is viable, free of necro-degenerative changes. The interstitium is free of inflammatory exudates, granulomas, fibrosis, abnormal deposits or vascular injury. Also renal glomeruli are intact with normo-cellularity, free of necro-degenerative changes, inflammatory exudates, atrophy, sclerosis, abnormal deposits, mesangioproliferative activity, or vascular injury. Meanwhile kidney section of exposed group showed renal tubules with necro-degenerative change, swollen edematous cell lining. There is focal partial luminal

obliteration by hyaline casts. Also renal glomerulus showed fibrous atrophy, necrosis and hyaline degeneration.

Table (1) shows the level of hematological parameters in control and exposed groups. Results of the analysis were given as mean \pm standard deviation. The level of hemoglobin, RBCs and hematocrit in exposed group decreased compared to the control group ($p < 0.02$). The level of WBCs and platelets in exposed group increased compared to the control group ($p < 0.04$). No significant difference in MCV, MCH and MCHC were found between exposed and control groups.



Figure(4a) : Kidney, the control group: Renal tubules are intact with patent lumens, free of cellular or hyaline casts. The cell lining is viable, free of necro-degenerative changes. The interstitium is free of inflammatory exudates, granulomas, fibrosis, abnormal deposits or vascular injury. Renal glomeruli are intact (arrows) with normo-cellularity, free of necro-degenerative changes, inflammatory exudates, atrophy, sclerosis, abnormal deposits, mesangioproliferative activity, or vascular injury. H&E, x100.

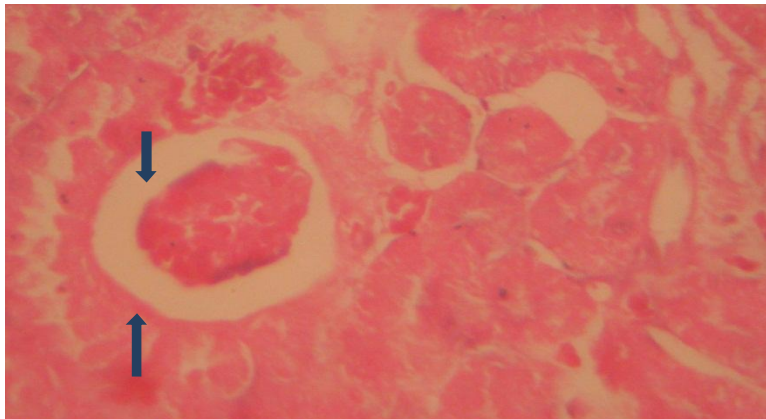


Figure (4b): Kidney, the exposed group: Renal tubules at the Rt. Of the field show necro-degenerative change, swollen edematous cell lining. There is focal partial luminal obliteration by hyaline casts (thin arrows). The glomerulus (bold arrows) show fibrous atrophy, necrosis & hyaline degeneration. H&E x250.

Table (1): Hematological parameters of newborn rates after whole body exposure to (50 Hz, 0.5 mT MF) for 30 days/24 h (Mean± S.D.)

Parameters	Control	Exposed	<i>P</i> -value
WBCx10 ³ cells/μl	5.2±0.17	5.97±0.18	0.04
RBCx10 ⁶ cells/μl	5±0.23	4.07±0.03	0.02
Hemoglobin g/dl	14.47±0.66	11.73±0.12	0.02
Hematocrit	44.81±2.15	36.3±0.36	0.02
MCV	89.6±0.2	89.2667±0.26	0.374
MCH pg	28.9±0.0577	28.8667±0.066	0.725
MCHC g/dl	32.3±0.05774	32.3±0	1.0
Platelets x10 ³ μl	196.7	231.7	0.04

4. Discussion

The present study is concerned with the effects of ELF-MF on the structure of DNA (isolated from liver and spleen tissues), pathological changes in liver and kidney tissues and some hematological parameters. The data indicated that 50 Hz, ELF-MF at a flux density of 0.5 mT exposure does not produce enough energy to induce DNA damage figures (1,2), this result is in consistent with the studies of **Milena et al.** [17] and **McNamee et al.** [18] who reported that there was no significant effect on DNA strand breaks upon ELF-MF exposure. In consistence this dose causes pathological changes which might be due to ELF-MF induced oxygen free radicals which can evoke an interaction with membrane lipids, proteins and nucleic acids leading to extensive tissue damage that was confirmed by histopathological examination (Fig. 3,4). Accordingly the liver is a hematopoietic organ and any anatomic changes within this organ, cause alterations in hematological parameters.

The present data demonstrated that ELF-MF significantly decreased the level of RBCs, hemoglobin and hematocrit. This was in agreement with **Cakir et al.** [19]. Additionally the field significantly increased the value of WBCs and platelets and also no significant changes in MCV, MCH and MCHC were observed (Table 1). **Lino et al.**, and **Chater et al.**, [20,21] stated that hemoglobin is sensitive to EMF, **Higashi et al.**, [22] reported that RBCs orient with the applied MF and **Atef et al.** [23] hypothesized the change in conformation of Hb under MF action. **Cabrales et al.** [24] observed that hematocrit and hemoglobin levels decreased significantly compared to the control group after ELF-EMF exposure. **Regan et al.** [25] explained the reason for the observed hematological variability in response to ELF-EMF exposure as spleen hyper function. **Oroza et al.** [26] observed that for exposed rats to magnetic fields for 1,2 and 4 weeks, only the 4 week exposure induced an increase in WBCs count. By contrast **Fiorani et al.** and **Selmsoui et al.** [27,28] indicated that MF had no significant effect on RBCs, WBCs and Hb. **Osbakken et al.** [29] observed that spleen hyper function increases the rate of distraction of red blood cells, white blood cells and platelets. The reasons for these conflicting results probably stem from differences in exposure setups and experimental conditions.

Conclusion:

In conclusion the present work show that ELF-MF might possess a capability under specific conditions to influence some hematological and pathological parameters in newborn rats at significant values.

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References

- 1-Wertheimer N, Savitz DA, Leeper E., 1995. Childhood cancer in relation to indicators of magnetic fields from ground current sources. *Bioelectromagnetics*, 16;86-96.
- 2-Aldrich TE, Andrews KW, Liboff AR., 2001. Brain cancer risk and electromagnetic fields (EMFs): assessing the geomagnetic component. *Arch Environ Health*; 56(4):314-9.
- 3- Till U., Timmel C.R., Brocklerhurst B., and Hore P. J., 1998. The influence of very small magnetic fields on radical recombination reactions in the limit of slow recombination. *Chem. Phys. Lett.*, 208: 7-14.
- 4-Simko M. and Mattsson M. O., 2004. Extremely Low Frequency Electromagnetic fields as effectors of cellular responses *in vitro*: Possible Immune Cell Activation. *J. Cell Biochem.*, 93: 83-92.
- 5-Rollwitz J. Lupke M. and Simko M., 2004. Fifty-hertz magnetic fields induce free radical formation in mouse bone marrow-derived promonocytes and macrophages. *Biochim. Biophys. Acta.*, 1674: 231-238.
- 6-Rogers WR, Reiter RJ, Smith HD, Barlow-Walden L, 1995. Rapid onset/offset, variably scheduled 60 Hz electric and magnetic field exposure reduces nocturnal serum melatonin concentration in nonhuman primates. *Bioelectromagnetics Supplement*, 3:119-122.
- 7- Lai H. and Singh P. N., 1997. Acute exposure to a 60 Hz magnetic field increase DNA strand breaks in rat brain cells. *Bioelectromagnetics*, 18: 156-165.
- 8- Reese J.A., Jostes R.F. and Frazier M.E., 1988. Exposure of mammalian cells to 60 Hz magnetic or electric fields: analysis for DNA single-strand breaks. *Bioelectromagnetics*, 9:237-247.
- 9-Frazier M.E., Reese J.A., Morris J.E. Jostes R.F and Miller D.L., 1990. Exposure of mammalian cells to 60 Hz magnetic or electric fields: analysis of DNA repair of induced single-strand breaks. *Bioelectromagnetics*, 11:229-234.
- 10- Fairbairn D.W. and O'Neill K.L., 1994. The effect of electromagnetic exposure on the formation of single-strand breaks in human cells. *Cell. Mol. Biol.*, 40:561-567.
- 11-Ivancsits S, Diem E, Pilger A, Rudiger HW, Jahn O., 2002. Induction of DNA strand breaks by intermittent exposure to extremely-low-frequency

- electromagnetic fields in human diploid fibroblasts. *Mutat Res.*, 519:1–13.
- 12-Ivancsits S, Diem E, Jahn O, Rudiger HW. 2003a. Intermittent extremely low frequency electromagnetic fields cause DNA damage in a dose-dependent way. *Int Arch Occup Environ Health*, 76:431–436.
- 13-Ivancsits S, Diem E, Jahn O, Rudiger HW., 2003b. Age-related effects on induction of DNA strand breaks by intermittent exposure to electromagnetic fields. *Mech Ageing Dev.*, 124:847–850.
- 14-National Research Council., 1996. Guide for the care and use of laboratory animals: National Academy Press, Washington DC
- 15-Kasibhatla S, Gustava P, Mendes A, Finucane D, Brunner T, Wetzal E, and Green DR, 2006. Analysis of DNA Fragmentation Using Agarose Gel Electrophoresis, Cite as: Cold spring Hrb. Protocol; doi:10.1101/pdb.prot4429.
- 16-Dacie SJ and Lewis SM, 1991. Practical Hematology. Churchill livingstone 7thed, 179-200
- 17- Milena V, Massimo M, Giuseppina S, Bruno B and Rossana P., 2006. Effects of co-exposure to extremely low frequency (50 Hz) magnetic fields and xenobiotics determined *in vitro* by the alkaline comet assay. *Science of the Total Environmental.*; 361:208-219.
- 18-McNamee JP, Bellier PV, McLean JRN., Marro L, Gajda GB and Thansandote A., 2002. DNA damage and apoptosis in the immature mouse cerebellum after acute exposure to a 1mT, 60 Hz magnetic field. *Mutation Research*, 513: 121-123.
- 19-Cakir D, Yokus B, Zulkuf M, Sert C and Mete N., 2009. Alterations of hematological variations in rats exposed to extremely low frequency magnetic fields (50 Hz). *Archives of Medical Research*, 40: 352-356.
- 20-Lino M., 1997. Effects of a homogeneous magnetic field on erythrocyte sedimentation and aggregation. *Bioelectromagnetics*; 18:215-222.
- 21-Sihem Chater S, Abdelmelek H, Pequignot JM, *et al.*, 2006. Effects of subacute exposure to static magnetic field on hematologic and biochemical parameters in pregnant rats. *Electromagn Biol Med.*; 25:135-144.
- 22-Higashi T, Yamagishi A, Takeuchi T, *et al.*, 1993. Orientation of erythrocytes in a strong static magnetic field. *Blood*; 82:1328-1334.
23. Atef MM, Abd el-Baset MS, El-Kareem A., 1995. Effects of a static magnetic field on hemoglobin structure and function. *Int J Biol Macromol.*; 2:105e111.
- 24-Cabrales LB, Ciria HC, Bruzon RP., 2001. ELF magnetic field effects on some hematological and biochemical parameters of peripheral blood in mice. *Electro-Magnetobiology*; 20:185-191.
- 25- Regan HA, Buscbom RL, Pipes MJ, *et al.*, 1983. Hematologic and serum chemistry studies in rats exposed to 60 Hz electric fields. *Bioelectromagnetics*; 4:79-90
- 26-Oroza MA, Calcicedo L, Sanchez-Franco F, *et al.*, 1987. Hormonal, hematological and serum chemistry effects of weak pulsed electromagnetic fields on rats. *J Bioelectricity*; 6:139-151.
- 27-Fiorani M, Biagiarelli B, Vetrano F, *et al.*, 1997. *In vitro* effects of 50 Hz magnetic fields on oxidatively damaged rabbit red blood cells. *Bioelectromagnetics*; 18:125-131.
- 28-Selmaoui B, Bogdan A, Auzeby A, *et al.*, 1996. Acute exposure to 50 Hz Magnetic field does not affect hematologic or immunologic functions in healthy young men. *Bioelectromagnetics*; 17:364-372.
- 29-Osbakken M, Griffith J, Taczanowsky P. A., 1986. Gross morphologic, histologic, hematologic, and blood chemistry study of adult and neonatal mice chronically exposed to high magnetic fields. *Magnet Reson Med.*; 3:502-517.

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Synergism Effect of Nisin Peptide in Reducing Chemical Preservatives in Food Industry

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Abstract: Due to increasing interest to natural preservatives, many studies have been performed in recent years. Nisin peptide as a natural preservative is very interesting for the control of food pathogens and microorganisms of food spoilage. In this study antibacterial activity of nisin and its synergism in decreasing concentrations of common chemical preservatives like sodium nitrite and benzoic acid against standard bacteria of *Staphylococcus aureus* and *Listeria monocytogenes* has been investigated. For this purpose, the MIC (minimum inhibitory concentration) of nisin peptide, sodium nitrite and benzoic acid were evaluated at different pH based on the dilution method. Results showed that these compounds have antibacterial activity against *Staphylococcus aureus* and *Listeria monocytogenes*. MIC for nisin peptide, sodium nitrite and benzoic acid in *Staphylococcus aureus*, was 350, 200, 25 (ppm) respectively. These values were 100, 200, 10 (ppm) in *Listeria monocytogenes*. It has been also shown that simultaneous use of nisin with chemical preservatives sodium nitrite and benzoic acid reduced MIC of these compounds against two strains of bacteria. This synergistic effect of nisin could reduce the use of chemical preservatives in food industry.

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Keywords: Nisin, Cancer, Sodium nitrite, Benzoic acid, Bacteria.

Introduction:

In food industry achieving a healthy product with a proper maintenance can be one of the main indexes in order to food producing. Therefore variety of methods has been evaluated. Nowadays, preservatives are among the methods that used commonly in food processing. To prevent spoiling of food that created by microbial growth or chemical alterations, preservatives that either natural or artificial, are added to food products. Dietary antimicrobial preservatives are one of the most important groups of conservative that improve food quality, extend the shelf life, waste reduction and processing cost. In recent years, nitrate and sodium nitrite as a chemical preservatives have been widely used in meat and dairy processing with a suitable antimicrobial effects. (Goldstein 1). But it should be noted that application of these compounds in 1960 following liver toxicity of domestic animals fed canned fish meal content large amounts of sodium nitrite was confronted with challenges. Studies have shown these compounds in gastrointestinal tract are converted to carcinogenic compound called nitrous amine, which is effective in appearance of malignant tumors. The presence of nitrates in food may cause Met-hemoglobin phenomenon, created by oxidation of Oxy-hemoglobin to Ferry-hemoglobin. This can be fatal, especially in newborn infants, since reduced met-hemoglobin has low oxygen-carrying capacity that may eventually lead

to anemia and Blue Baby Syndrome. Other adverse effects of these compounds are its deterrent effect on nutrient absorption at intestine (Fan & Steinberg 2).

Benzoic acid and its salts (sodium, calcium, potassium) are used such other chemicals in food industry in order to keep jam, initiators, beverages, salads and olives. There are also concerns about the use of benzoic acid and its derivatives including reaction possibility of these compounds with ascorbic acid (vitamin C) and formation of small amounts of benzene, which is a carcinogenic substance. Professor Peter Piper of the University of Sheffield claims that sodium benzoate by itself can damage and inactivate vital parts of DNA in a cell's mitochondria. Mitochondria consume oxygen to generate ATP, the body's energy currency. If they are damaged due to disease, the cell malfunctions and may enter apoptosis. Research published in 2007 for the UK's Food Standards Agency (FSA) suggests that certain artificial colours, when paired with sodium benzoate may be linked to Attention Deficit-Hyperactivity Disorder (ADHD). (McCann D. 3). The results of this study indicate that certain combination of artificial food colors and sodium benzoate preservatives is associated with increasing hyperactivity behavior in children. Therefore the adverse effects of chemical substances in food preservation considering to natural and biological preservatives with inhibitory effects on wide spectrum

of pathogenic microorganisms, which having no side effects and improve the smell and taste of food, have expanded in recent years. Accordingly, Hurdle technology as a new method for producing safe, sustainable, nutritious, tasty and economical food is developed. Combination use of natural preservatives such as herbal essential oils and probiotic agents are important in order to achieve a high level of health, hygiene and shelf life of food products. According to Hurdle technology, more than 60 affordable and safe options are presented to improve health and quality of foods that use of bacteriocin as probiotic products is one of the options. Maximum allowable concentration of nitrites in meat products is 200 ppm. European Union in 1985 announced 50mg/lit permitted level of Nitrites in drinking water (Ivanov 4).

In 1969, nisin peptide was approved by F.A.O & W.H.O (Food and Agriculture Organization / World Health Organization) to use as a preservative in food issues. (Noonpakdee et al 5). Nisin peptide as a bacteriocin agent has been operating since 1987 as a formal additive in food and dairy products. This peptide is composed of 34 amino acids, which produced by the dairy starter culture *Lactococcus lactis* subsp. *lactis*. (Liu, W., N. Hansen 6). Nisin was not toxic and is rapidly inactivated by digestive enzymes. Today's, this bacteriocin has a relatively broad spectrum of inhibitory activity against gram-positive bacteria and is commonly used as a preservative in many countries around the world in foods such as canned goods, dairy products, pasteurized cheeses, dairy desserts, , frozen meats and sea foods. (Hurst, A, 7, Ross 8).

Due to complications of chemical preservatives like sodium nitrite and benzoic acid compounds, the objective of this research was to evaluate effect of nisin bacteriocin as a natural and harmless preservative, and its capability on MIC and MBC reduction of the chemical preservatives.

Materials and Methods:

1- Solution preparation of chemical preservatives ;(sodium nitrite, Benzoic acid) and nisin peptide: Suitable solvents for Benzoic acid and sodium nitrite (Merck) are 90% ethanol and distilled water respectively. In case of nisin peptide, 2% HCL (High Media) are applied. Benzoic acid and sodium nitrite stock solution equal to 30mg/ml combined with 10mg/ml nisin peptide was prepared and each of solution was sterilized separately by millipore filter with a diameter of 0.22 micrometers.

2- Microbial strains: Strains of *Staphylococcus aureus* ATCC 1112 and *Lysteria Monocytogenes* ATCC 1301 as standard samples used in experiments. After confirming above strains according to laboratory criteria, primary cultures of bacteria were prepared

with TPB (Tryptose phosphate broth) medium at proper temperature of 37 C °.

3- MIC and MBC tests: at this study MIC and MBC experiments were done based on macro dilution in *Mueller Hinton Broth* medium with initial bacteria amount of $\sim 5 \times 10^5$ CFU / ml (prepared based on 0.5 Mac Farland) were conducted according to NCCLS protocol in two stages.

In the first stage MIC and MBC of sodium nitrite, Benzoic acid and nisin compounds separately at three different pH for each strain were evaluated to determine the best effectiveness of compounds at the optimum pH. So culture medium tubes containing *Mueller Hinton Broth* (MHB) individually prepared with pH of 7, 6 and 5.5 in association of bacterial suspension and dilution series of preservatives with concentrations of 4500, 3500, 2500, 1500, 750, 500, 350, 200, 100, 50, 25, 10 (ppm) were made and the lowest concentration that inhibits bacterial growth was detected. MBC evaluations were carried out in *Mueller Hinton Agar* (MHA) medium based on the lowest concentration that inhibits bacterial growth rate up to 99.9%.

In the second stage, MIC and MBC of sodium nitrite, Benzoic acid in combination of nisin peptide were evaluated at the optimum pH for each strain. accordingly in a separate tubes containing MHB medium and bacterial suspension, dilution series of benzoic acid and sodium nitrite with a concentration of 5, 25, 50, 100, 200, 350, 500 (ppm) were prepared and then to the sample tubes, nisin peptide was added equal to its MIC concentration at optimum pH. Tubes were incubated for 18 hours at 37C° and the experiments were repeated three times.

Results:

First stage results of experiments showed sodium nitrite MIC for *S.aureus* at 7, 6 and 5.5 pH was 4500, 750 and 350 ppm respectively and these values in *L.monocytogenes* were 3500, 750 and 100 ppm respectively.

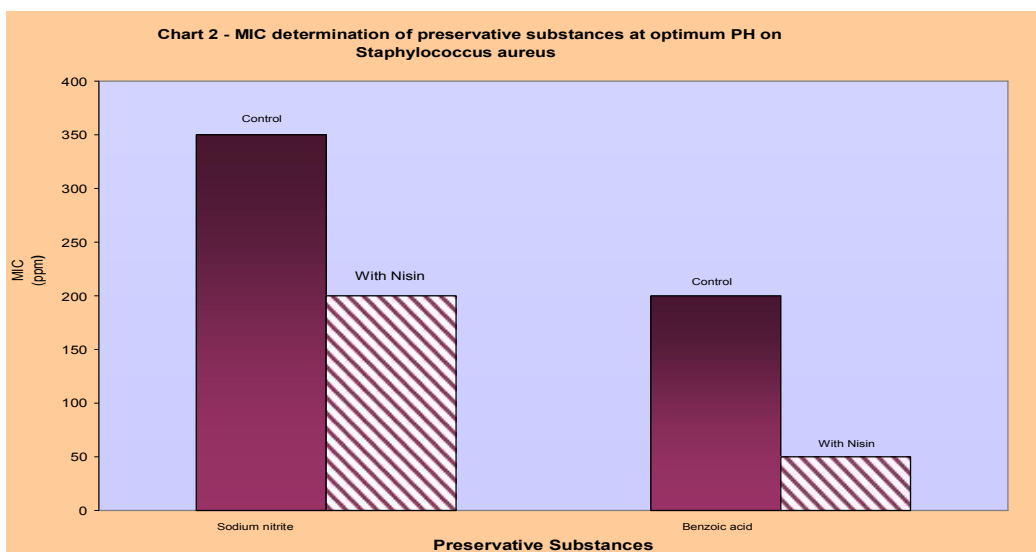
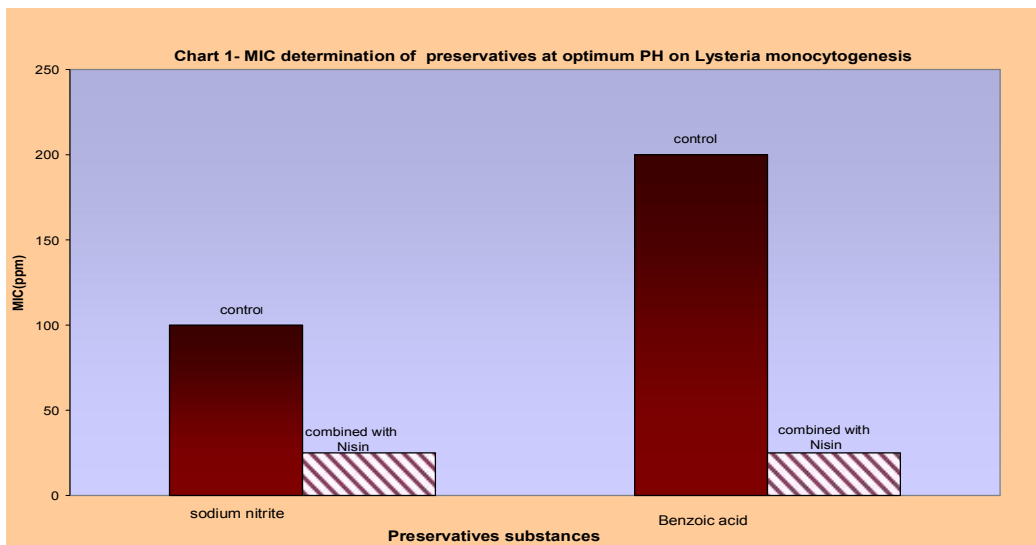
Benzoic acid MIC in *S.aureus* at 7, 6 and 5.5 pH was 750, 500 and 200 ppm respectively, and these values for *L.monocytogenes* were 750, 500 and 200 ppm. MIC of Nisin also at 7, 6 and 5.5 pH in *S.aureus* and *L.monocytogenes* was 100, 50, 25 ppm and 50, 25, 10 respectively.

Therefore, the results can be concluded that optimum pH of three compositions for highest activity and lowest effective concentration of MIC and MBC are equal to 5.5.

The second stage of experiment, at the optimum pH when chemical preservatives, sodium nitrite and benzoic acid, were applied in association of nisin peptide showed the MIC of compounds against two strains of bacteria were reduced significantly so that

sodium nitrite and benzoic acid MIC is reduced to 200, 50 ppm respectively for *Staphylococcus aureus* and these values in *Lysteria Monocytogenes* were 25, 25 ppm. (Figure1, 2).

Therefore, according to results, presence of nisin as a safe preservative has reduced significantly MIC and MBC of chemical preservatives like sodium nitrite and Benzoic acid.



Discussion:

The effects of preservatives on microorganisms are carried out with different mechanisms. Nitrite in acidic pH can be converted to nitrous acid, and has been shown this compound is capable of reaction with various materials, such as myoglobins, ascorbic acid, phenols, secondary structure of amines, compounds with amine agent groups, metalloporphyrins and iron-sulfur cluster, and inhibits the growth of microorganisms. (Ferric C. Fang 9). Benzoic acid also at low pH has inhibitory activity against

microorganisms, in this condition due to increased membrane permeability, uncatalyzed form of compound can pass freely through the cytoplasmic membrane and following the cell influence shows its effect. (Booth & Kroll 10).

Nisin peptide with bacteriocin activity has a relatively broad spectrum of inhibitory activity against gram-positive bacteria and bacterial spores. (Blackburn et. al, P 11). This peptide binds to specific protein receptor on the cell wall and destabilizes the cell membrane function so increases permeability of the

cytoplasmic membrane to ions, especially cations. Producer strains and many resistant gram-positive bacteria adsorb nisin to the cell wall with no consequences. These protein receptors may also function as receptors for other metabolites which are transported into the cell. The release of important cations, such as K^+ , from nisin-treated cells results in a dissipation of the proton motive force potential. This force serves to control many membrane functions such as the active transport of amino acids and sugars across the cellular membrane. These and other cell membrane functions that depend on the existence of the proton motive force are lost following nisin peptide treatment. A further consequence is losses of cellular ATP reserves are expended in an attempt to maintain the membrane potential. The decrease in the intracellular concentration of ATP inhibits many energy dependent reactions in the cells. (Hurst and Hoover, 12). Finally, this process reduces the pH equilibrium of cells and collapses the proton motive force (PMF), so that eventually suppress all biosynthetic processes. (Chen & Hoover 13).

In this research, synergism and effect of simultaneous use of nisin peptide with chemical preservatives of sodium nitrite and benzoic acid and MIC reduction of these compounds were studied. Results showed nisin could reduce significantly sodium nitrite MIC at the studied bacteria so that the amount of nitrite was reduced to below 200 ppm, which is the limit of nitrite consumption. These studies also showed nisin could reduce Benzoic acid MIC in *Staphylococcus aureus* and *Listeria monocytogenes* from 200ppm to below 50ppm. The allowable amounts of nitrate are 120 to 200ppm, but rarely may exceed this amount from 150ppm. Food and Drug Administration of America (F.D.A) pointed out the amount of nitrite and nitrate that should not be more than 200ppm and 500 ppm respectively (Federal Register 14). In general, to prevent the activity of pathogenic bacteria, 80 to 150ppm of the compounds is necessary. Benzoic acid and its sodium salt derivatives in concentration of 50-500ppm inhibit activity and growth of poisoning and pathogenic bacteria. There are many reports about combined effect of preservatives and nisin peptide on various microorganisms. For example, Karim et al (2010) reported that the combination of nisin peptide in association of chemical inhibitors such as EDTA, citrate and phosphate may be more effective against *Escherichia coli* and *Salmonella*. (15). Chung & Hancock also showed the effects of lysozyme with nisin could be more effective against *Lactic bacteria*. Synergism effects of nisin in association of Monolaurin and lauric acid have also observed against *Streptococcus agalactic* and *Lactobacillus plantarum* respectively. Chung & Hancock and in 2000 showed there are synergism

between Lysozyme and Nisin against *Lactic bacteria* (Chung & Hancock 16) Synergism effects of nisin in association of Monolaurin and lauric acid have also observed against *Streptococcus agalactic* and *Lactobacillus plantarum* respectively. (Stefania & Loredana 17). Alexander O. Gill, Richard A. Holley (2002) observed the combination of EDTA and Nitrite can effect on *E.coli* and *Salmonella* (18, 19). Rayman and colleagues(20) also in 2006 showed combination of 40ppm nitrite with 75-100ppm nisin peptide can inhibits the growth of bacteria such as *Clostridium sporogenes*, confirming the results of synergism between nisin and nitrite combination. It is clear that the low level of nitrite with the least adverse effect is sufficient for meat maintenance. This study has also shown sodium nitrite and Benzoic acid combined with nisin peptide has synergism effect, so that in *Staphylococcus aureus*, active concentrations of sodium nitrite and benzoic acid based on MIC, have been reduced 40 and 75 percent respectively. Also concentrations of sodium nitrite and benzoic acid in *Listeria Monocytogenes* were significantly reduced to 75 and 87 percent. Reduction of minimum effective concentrations of chemical compounds in association of nisin peptide is due to permeability of cell membrane by function of nisin peptide, so that this peptide penetrates and intruded into the lipopolysaccharide of cell membrane causing instability in biophysical connections, then by generating pores, increases cell permeability of compounds such as sodium (Chung, K. T, et. al, 21) nitrite and benzoic acid, which results enhanced sensitivity of bacteria to these compounds and thus reduces minimum active concentrations against two experimented strains. Mechanism modality of nitrite effect on bacteria are still not entirely clear, but is thought that, next to the production of nitrous amine, which was discussed, nitrite may impact on sulfhydryl compounds of microorganisms causes changing in their metabolism and eventually disrupt the bacterial growth and proliferation. Antimicrobial effect of nitrite is depended on pH of products, so by falling of pH equal to one unit, uncatalyzed part of nitro acid (HNO_2), which is operating as an antimicrobial agent, increases its effect up to 10 fold, and therefore is recommended to add small amount of edible acids to products intended to reduce level of pH. (Foegeding, P. M. et. al, 22) Antimicrobial and anti bacterial activity of Benzoic acid also has a direct relation with pH, so that at the low pH shows more potency. Accordingly, it was also observed that at three investigated pH, MIC and MBC of chemical preservatives, sodium nitrite and benzoic acid, had minimum active concentration in the lowest pH, which indicate the influence of pH on maximum antimicrobial activity. Functionally Benzoic acid interferes in cellular energy transferring system and

inhibits activity of enzymes that associate in metabolism of acetic acid and Krebs cycle, so that after uptake of acid by cells, intracellular pH may come down to 5 or lesser and anaerobic fermentation of glucose by Phosphofructokinase is reduced up to 95%, which leads to bacterial disruption. (Kristo E, 23, Parmar & Forestry 24, Jin et al 25).

As mentioned, nisin peptide next to the increasing of bacterial cell membrane permeability, disrupt proton pumps in the membrane structure that results in disturbance of proton gradient and intracellular pH of bacteria. (Klaenhammer, T. K.26). Therefore besides increasing in membrane permeability, pH changes also cause to increase activity and ability of compounds such as sodium nitrite and benzoic acid, which result to reduce the minimum active concentration.

Based on the findings obtained in laboratory conditions, other factors also may be effective on the MIC and MBC of preservatives, so it is necessary to assess the results at industrial conditions.

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References:

- Goldstein, David. "FDA finds benzene in soft drinks", 2006, 03-03.
- Fan.AM and SteinbergV.E, Health implications of nitrate and nitrite in drinking water: an update on methemoglobinemia occurrence and reproductive and developmental toxicity, 1996, pp. 35-43.
- McCann D, Barrett A, Cooper A, Crumpler D, Dalen L, Grimshaw K, Kitchin E, Lok K, Porteous L, Prince E, Sonuga-Barke E, Warner JO, Stevenson J. Lancet; 370(9598):1560-7. Food additives and hyperactive behavior in 3-year-old and 8/9-year-old children in the community: a randomised, double-blinded, placebo-controlled trial, 2007.
- Ivanov. VMJournal of Analytical Chemistry, Vol. 59, No. 10, pp. 1002 to 1005. Translated from Zhurnal Analiticheskoi Khimii, 2004, Vol. 59, No.10, pp.1109-1112.
- Noonpakdee, W., Snativarangekna, C., Jumriangrit, P., Sonomot, K. and Panyim, S. Isolation of nisin - producing *Lactococcus lactis* WNC20 from Nham, 2003.
- Liu, W., and N. Hansen. Some chemical and physical properties of nisin, a small protein antibiotic produced by *Lactococcus lactis*. *Appl. Environ. Microbiol.* 1990, 56: 2551-2558.
- Hurst, A. Nisin. *Adv. Appl. Microbiol.* 1981, 27: 85-122.
- Ross, R. P., Morgan, S. and Hill, C., Preservation and fermentation: past, present and future, 2002.
- Ferric C. Fang, Mechanism of Nitric Oxide-related to Antimicrobial activity, The American Society for Clinical Investigation, 1997, Vol.99, No.12; pp.2818-2825.
- Booth,I.R.and Kroll, RG, The preservation of foods by low pH. In: Gould, G.W. Editor, Mechanisms of Action of Food Preservation Procedures Elsevier, London, 2006, pp. 119-160.
- Blackburn, P., J. Polak, S. Gusik, and S. D. Rubino. Nisin composition for use as enhanced, broad range bacteriocins. International Patent Application Number PCT/US89/02625; International Publication Number WO89/12399. Applied Microbiology, Inc., New York, 1989.
- Hurst, A. and D. G. Hoover. Nisin, In P. M. Davidson, and A. L. Bronen(ed.), Antimicrobials in foods. 2nd ed, Marcel Dekker, New York, NY, 1993.
- Chen, H. and Hoover, D.G. Bacteriocins and their Food Applications. Comprehensive reviews in food science and food safety. 2003, 2: 82-100.
- Federal Register. Nisin preparation: affirmation of GRAS status as a direct human food ingredient. Fed. Reg. , 1988, 58: 11247-11250.
- Karim Naghmouchi, Djamel Drider, John Baah, Ron Teather, Nisin A and Polymyxin B as Synergistic Inhibitors of Gram-positive and Gram-negative Bacteria. *Probiotics & Antimicro.* 2010, Prot. 2:98-103, DOI 10.1007/s12602-009-9033-8.
- Chung, W. and Hancock, REW, Action of Lysozyme and Nisin mixtures against lactic acid bacteria. *Int. J. Food Microbiol.* , 2010, 60; pp. 25-32.
- Stefania Quintavalla, Loredana Vicini, "Antimicrobial food packaging meat industry", *Meat science*, 2002, 62; pp. 373-380.
- Alexander O. Gill and Richard A. Holley., Interactive inhibition of meat spoilage and pathogenetic bacteria by lysosyme, nisin and EDTA in the presence of nitrite and sodium chloride at 24 ° C. *International Journal of Food Microbiology.*; 2003, pp. 251-259.
- H.S. Lade, M.P. Chitanand, G. Gyananth, T.A. Kadam, Studies on some properties of bacterocins produced by *Lactobacillus* species isolated from agro-based waste, *Internet J. Microbiol.* 2, 2006.
- Rayman.MK, Aris. B and Hurst. A Nisin: a possible alternative or adjunct to nitrite in the preservation of meats. *Appl Environ Microbiol*; 2006, pp 375-380.
- Chung, K. T., J. S. Dickson, and J. D. Crouse. Effect of nisin on growth of bacteria attached to meat. *Appl. Environ. Microbiol.* , 1989, 55: 1329-1333.

22. Foegeding, P. M., A. B. Thomas, D. H. Pilkington, and T. K. Klaenhammer. Enhanced control of *Listeria monocytogenes* by situ-produced pediocin during dry fermented sausage production. *Appl. Environ. Microbiol.*, 1992, 58: 884- 890.
23. Kristo E, Koutsoumanis KP, Biliaderis CG. Thermal mechanical and water vapor barrier properties of sodium caseinate films containing antimicrobials and their inhibitory action on *Listeria monocytogenes*. *Food Hydrocolloids*, 2008, 22:373–86.
24. Dr. Y.S. Parmar and Forestry Nauni. Purification and Characterization of Bacteriocin Produced by *Bacillus subtilis* R75 Isolated from Fermented Chunks of Mung Bean (*Phaseolus radiatus*) Solan (H.P.) 173 230, India Received: November 17, 2009 Accepted: April 23, 2010.
25. Jin T, Liu LS, Zhang H, Hicks K. Antimicrobial activity of nisin incorporated in pectin and polylactic acid composite films against *Listeria monocytogenes*. *Int J. Food Sci Technol*, 2009, 44:322–9.
26. Klaenhammer, T. K. Bacteriocins of lactic acid bacteria. *Biochimie*, 1988, 70: 337-349.

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**Psychology of media and audience:
"A survey on Psychological tendency and attitude of Ardabil people to media"**

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Abstract: Today, in the era of vast media, attention to the characteristics, interests, demands and needs of the media audience is so important that the owners of the media must pay their special attention to. People in every area of human geography have special characteristics, behaviors and psychological states that attention to them can attract and keep audiences to be effective as a customer permanently otherwise people turn away from interior media and tend to Radio & TV channels of other countries. In Iran, local and national channels have been managed by government and therefore channels broadcast with a given policy from Iran government, also satellite use is prohibited under the constitution. However the use of satellite in Iran is growing and some statistics show that over 30 percent of people have satellite receivers. Iran is a multi-ethnic society with a variety of languages and religions. Indeed, Iran is a multicultural country, but anything e.g. media, education, schools, universities, offices, etc are in Persian language and other Iranian languages are not official. In this situation ethnics and their culture and languages are destroying. Government doesn't pay attention to ethnics and it is a multifaceted problem and we try to look at it from psychological aspects. This paper strives to study trends and tendency of Ardabil people in the cognitive, emotional and behavioral dimensions to satellite channels, especially Turkey and Republic of Azerbaijan from the perspective of psychology and describes and explains its findings based on psychological approaches.

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Key words: media, Ardabil province, satellite channels, media psychology, audience psychology, and media needs of audiences

Introduction

No doubt the media today in people's lives and their activities have been stirred. Many of these activities from entertainment to education and business surrounded most community areas. The media affect people willingly or unwillingly, but the audiences are interested in the topics which they experience or in those which happen in the surroundings. "On the contrary, the media acts in a way that once you achieve a particular piece of information, you take yourself as an expert at the transferred and intended information by the media. Let's supply you with an example: in political debates, the audiences regard themselves as politicians neglecting the fact that they have gained the huge body of their political know-how from the media" (Hakimara, 2010, p. 2)

Psychological impact of media is large and diverse. When an athlete's reputation goes up, with his presence in the ad, He becomes a billionaire, actor and maybe MP or politician. If the sign values of some products made by the media or are outstanding or if the people are satisfied with their government of a country invading other countries, those looking for media with special identity and those who turn away from the media in his country and tent to the satellite networks of other countries, all are the psychological

effects between media and audience. These effects are mainly taken as some of the wonders of the media indicating the fact that there are two kinds of influences: one referring to the communicative facet of the media and the other one being symbolic of the cognitive, emotional, and behavioral dimension.

Having Psychological perspective to the audience and the media means the interactive effects of scientific study, study of relationship between humans and media communication and understanding audience behavior in the range of individual and social behavior. What is more, people are aware of their requirements to media and also realize the reasons why they refer to media. Accordingly, the media should perceive how to exert their impact on the public in the process of this interplay and interaction.

Psychological analysis of the media is that media as one of the factors and influences that are driving through the influence of beliefs, attitudes and behavior have remained and the other trends and concerns of the audience in how to apply and use media, or lack of access to achieve interests, desires and needs of media in use or not use has a big impact. In fact, today audience isn't passive and actively takes steps toward the media. Audiences have their own belief and use media for strengthening of their belief.

If an opinion contrary to media contacts to be considered cannot expect the audience to use the media, the fact is that human behavior and belief systems are so complicated that changing them or creating new ideas and attitudes are difficult and the problem persisted and insisted and media contacts cause it to turn away. Audience following the use of media and to the satisfaction must partially achieve, otherwise the media will have more appeal to him, and given that today faced with many media and media in the competitive compact with the audience before major demands are counting Process so that the media will get the audience to put its axis will be lost.

This article with psychological approach studied media among the people of Ardabil. Ardabil is located in Azerbaijani region and part of Iran. The province is the neighborhood of Republic of Azerbaijan and has many cultural and linguistic commonalities with Turkey and Azerbaijan. All people speak Turkish, but all works and affairs are in Persian language. Persian language is the only official language in a multi-ethnic country, and such issues create a complex situation in this region and have caused the tendency of people to satellite channels and to find their channels that are in Turkish language. However, the use of satellite channels in Iran is prohibited under the constitution, the people of Ardabil use satellite channels hidden or even obviously. In the following section we will try to discuss approach of a psychological issue and audience media and psychological factors affecting people's use of media in Ardabil.

The purposes and questions

The present paper targets at the effects of the psychological variables in using the media and the attitudes of the people toward the domestic and foreign media. Based on the major tenets, the following research questions are to be tackled:

1. Which countries channels do you watch mostly?
2. How do you feel that the media meet your needs and demands?
3. How satisfactions do you have from Iran and foreign countries TV channels?
4. How effective is watching TV to changing your attitudes and behaviors?
5. While under the Iranian constitution prohibited the use of satellites, why people watch satellite networks?

The theoretical Approaches

Since the link with human behavior in the media make up the concept paper on the head. So, the audience and media related topics theories and

approaches are used within the psychological framework.

The concept of audience behavior consider with the media that as a primer uses the media and holds the initiative, so the audience is active and free to use the media that makes him popular and in the use of psychological, emotional and behavioral needs to satisfy his/her and achieves partial satisfaction. In an area such as the uses and gratification, media affiliation, and worth waiting theories have been used and these theories cover such subjects. In this context, we will discuss theoretical aspects of psychology of audience and media.

Media psychology focuses on understanding how information in print and electronic formats affects individuals, society and their behavior culture. Studies are in the field of media psychology and human relations and its impact on media on audience beliefs, attitudes and behavior.

Expectancy - value theory

This theory follows discussions about how attitude changes the behavior. According to this theory, two variables and social norms can become decisive attitude to be treated (Ajzen & Fishbein, 1980). In this context, people search that the programs that they watch on TV have been beneficial, for example, to make them laugh or to have information useful to them. On the other hand, it is important for people to know those around them about what comments they have. According to the Expectancy - value model, we see programs that expect to meet our needs and to see the high value we distinguish. After watching the programs, how they meet these needs have been assessed and we will weight. For example, consider whether the program was funny enough to see it or not. The assessment result is that we decided to see programs in the future to repeat or stop. This model of a theory called the action and reasonable uses within the public attitude toward the programs and uses them to explain the program. Theory of reasoned action proposed in the area of attitudes in social psychology and how the conversion approach explains the behavior. According to this approach into practice in the conduct of a respondent if the expectations be met, a pattern of media use habits and even the audience comes to see that program will be addicted. Here is what the audience expects from the media and evaluation program has been fulfilled or existing values are the same or a similar. It makes the audience use of media programs as an institutional behavior and his constant use of the media (Ajzen & Fishbein, 1980).

Dependency Theory

The theory considers long-term relationship between the audience and the media. As its audience targets represent an important part of why the media exposure he has exposed (Rokeach & Grube, 1984). According to this theory, the world rich in media, people who arrive here to find any information in any field depends heavily on the media (Defleur & Rokeach, 1976). An example of this dependency is weather news especially in areas where weather can change quickly. Dependency has other causes. For example, when people of a region seek to achieve media but the media don't give their media needs and interests, they go toward satellite channels to reach their aspirations. Defleure and Rokeach have raised three key ways to Dependence of people in the media: 1 - the media provide information that enables us to understand the world. In their understanding of the media provide information that will make your identity. Such as through making interpretations and compare their behavior with others is done that we've seen in the media. 2 - Might be dependent on the media that learn how to bias towards social issues. We act according to what is orientation like to vote in elections or be interactive (how we deal with social issues). 3 - The media need to play or provide entertainment. This can lull in the privacy of their own or in the form of social activity like going to the cinema (defleur & Rokeach, 1976).

Audience Psychology

Main topic in discussions of psychology audience is talk about how to shape cognition, emotion and behavior of people in contact streaming media. Here the face of the discussion is associated with human relationship between the media and society, so we are here faced with an ordinary human with all his characteristics. General human is with communication characteristics or attempting to communicate to resolve their needs. In these discussions, ideas such as the use and gratification and perception analysis pay respondent with psychological approaches:

The theory of uses and gratification

In this theory it is more or less assumed that people actively followed the concepts which gave them more gratification. The degree of this gratification depends on one's needs and interests. Whatever people sense the real concept meets their needs, more likely they would select that concept.

In 1974 Katz and Blumler simply reported that "we never ask what media do with people but conversely we ask what people do with media", this replacement show the mental challenge of the most mass communicational researchers which necessitated the existence of a special insight in mass

communicational process. For this subject, they (Windhal & Signiser, 1992) present a pattern which is a functional base perspective. In this pattern the sender element is a limited factor in mass communication process and generally being with the factors which have an effect on the selection of content by the audience. The following is a graphic representation of this pattern: The social, cultural and psychological roots cause the expectation from media and other sources which cause the diversity of media presentation patterns supplying of needs it has also other results (which are often unwished).

Figure 1: The pattern of uses and gratification theory (Windhal & Signiser, 1992)

Therefore, the reason for using media is to experience issues (such as data searching, social contact, deviation of attention, social learning and growth) which result from social, cultural and psychological condition and it is for solving them (supplying of needs) that the audience is an appeal to a media for help. If using media was not selective, it was not possible to consider it as a suitable tool for problem solving or even as a tool with a special meaning for the users. The conducted researches of the past 40 years show that the audiences state their using media within the framework of functionally - based terms (McQuail, 1988).

Perception theory

Cultural studies mainly focused on this issue that, how the groups with minimum power in practice consider the cultural products with their own method and employ them with the special forms for their amusement, resistance or identity formation. Also the audiences take only the media worthy using that in the way of coding and decoding between self and sender including cods that they like it.

This theory which was first founded by Stewart Hall's "coding and decoding of TV dialogue" indicates that in the media studies whatever recognized as a perception research is primarily accompanied by cultural studies. As (McQuail, 1977) writes:

The analysis of audience's perception from the media before being an independent research method is an effective agent of audiences researching in cotemporary cultural studies.

At the above mentioned essay, hall presents a four - step communicational theory: production, broadcasting, using and reproduction, which are "relatively independent". With relying on Philip Eliot he introduces the audiences as sender and receiver to TV message.

Hall Further has recognized 3 assumed stations on the base of which the TV discourse decoding is formed:

1. The dominant – hegemonic position: In this position, viewer perceives directly and completely the connotation of, for example, news or political program and decodes its messages according to the coding criteria of its resource. This is an ideal case or actually a transparent communication. 2. Negotiated position: most of the audiences understand according to their capacity of perception. What is defined as a dominant method and conveys its meaning in a professional manner. The interior decoding of this negotiation version is a mixture of compatible and incompatible elements. This decoding accepts the authenticity of hegemonic definition about the indications of real meaning, but in a limited and special manner established its basic principles. These coding positions are acted with some exceptions towards the principles. 3. Appositional position: hall believes that a viewer can understand both the connotation and denotation of a speech, but decode the message in a complete antithetical way. This viewer analyzes the message in a preferred position, to compound it in another source framework. This is more like to the viewer who listens to a conversation about the wage limitation, but each time when it refers to international interests, he takes it as a class interest. One of the most important and meaningful political moment for a TV channel is the time when the phenomena which is naturally decoded in appositional position and take some meaning, is read in an incompatible way. Here, we can observe the interior debate of a speech (During, 2001).

In the perception analysis, the fundamental prerequisite is that media texts is not of fixed or inherent meaning; but it is meaningful at the moment of its reception by an audience. It means that when an audience reads a text or views a program, he is not a mere receiver, but he produces some meanings too. The audience decodes and translates the media text with his social and cultural conditions and in a way which is related to his mental experience (Mehdizadeh, 2005). Another hypothesis of this theory is: activity of an audience, resistance of an audience against dominant and ideological meaning, the effect of social and cultural backgrounds, beliefs and attitudes of an audience in receiving of the texts among audiences.

The perception theory is in disagreement with the demagogic attitudes towards the mass audiences; this is rooted back to this belief that the attention of audience is not meant to have control on them. This theory aims to re-evaluate bestowing of power to the minority groups at the society margin.

In fact, the media consumption of these minorities and groups is not in agreement with any programs which are related to the control or social domination and also with media production priorities

which are acted within the framework of dominate culture.

Methodology

A method used in this study is a descriptive survey. Statistical Society is the city of Ardabil and Cochran formula which showed sample of 400. Sample through a proportional cluster sampling was chosen so that the population of Ardabil divided into three regions and compared to the rate of population questionnaire was devoted. To access validity and reliability of the questions, the designed questionnaire is controlled by some experts at the domain of audience and media and their professional views were practiced in preparing questionnaire. Therefore, the validity of the questions was approved and the qualified scale was external validity. Then primarily limited populations were tested. 25 questionnaires were completed and by means of **Cronbach's Alpha** test its reliability was tested. Kind of formal validity and reliability using Cronbach's formula was 82 percent. Due to the fact that the test's resulting measure must be or above the 0.7, therefore the obtaining results indicated the high level of reliability. Analyses of data obtained as a result of interviews with the people of Ardabil are presented as descriptive tables.

Results

To absorb audience, different components such as culture, language, ideology and psychological issues are effective. Topics in psychology, attention to psychological techniques to attract audience, including the requirement to identify psychological and behavioral desires, ways of media dependency are the issues which can be very important. Today, the use of psychological techniques to attract audiences to influence them should be emphasized in any media. Any media that can use these techniques will be more successful in attracting more audience. Today, various media competition has led to all the media are trying to use various psychological tricks to attract the audience and make them as permanent customers and also contract confidence in its audience. Especially on issues of cultural, social and political issues are more noticeable. Considering the growing levels and age-specific features are important factors to media. In this study, needs, desires and opinions of the audience were studied psychological perspective. What these results show that the satellite channels with various aspects of broadcast programs that can be effective in attracting the audience while the Iranian network audience demands less attention has been paid. This issue has caused the people of Ardabil to have less satisfaction from Persian channels and more from bringing

satellite channels and considering that in terms of language and culture with Turkey and Azerbaijan have common frequency channels, they watch most. Considering that the language of these two countries channels is Turkish nation and people, cultural commonalities with the people of both countries shall Ardebil is high. Also, various Turkish and Azerbaijani music (traditional, Mugham, khalgh Mahnisiy, modern, etc) broadcast from these two countries different network automatically moves a day is a deliberate reason which causes people to satisfy their needs, psychosocial emotional and more attention to these networks. The following tables describe the results and explain:

Rate satisfaction from Iran and foreign countries channels

According to research findings, satisfaction of the people of Ardabil from Persian channels compared with the satellite channels is lower. Only nine percent of respondents are satisfied with the Iranian channels, while 80 percent of them satisfied the satellite channels. Also, 25 percent of Iranian channels and 5 percent of satellite networks are not satisfied. 66 percent of Iranian channels are less satisfied and 15 percent of satellite channels are less satisfactory. (Table 1)

Table 1: Frequency distribution of respondents based on their rate satisfaction from Iran and foreign countries channels

row	Category	Iran Channels	Foreign countries channels
1	Very much	2	24
2	Much	7	56
3	Low	45	11
4	Very low	21	4
5	Not at all	25	5
total		100	100

Channels of which countries do people watch

People were asked for watching countries channels. Obtained results showed that 34 percent respondents watch Turkish and 33 percent Azerbaijan channels. This case indicates that Ardabil people tend to satellite channels and most people use satellite¹. Also, 23 percent of respondents watch Iran's channels. 6 percent of European and 4 percent of them watch American channels. (Table 2)

Ardabilians think how the media meet their media need

Ardebil respondents believe that the media meet their needs and wishes. 79 percent have said that part

¹. According to the Iranian constitution, Iranian people cannot use satellite and it is banned to use

of the psychological and emotional needs are met by the media to satisfy. 21 percent said the media needs are less resolved and only a half percent say their needs have not been resolved. (Table 3)

Table 2: Frequency distribution of respondents based on watching channels of which countries

row	countries	Frequency	percent
1	Turkey	246	34
2	Republic of Azerbaijan	239	33
3	Iran	167	23
4	European countries	46	6
5	The US	27	4
total		725 ²	100

Table 3: Frequency distribution of respondents based on their comments that they think how the media satisfy their media needs

row	Category	Frequency	percent
1	Very much	69	17/5
2	Much	244	61
3	Low	61	15
4	Very low	24	6
5	Not at all	2	0/5
total		400	100

Impact of media to change attitude and behavior of people

Media in changing people's attitudes and behavior have a lot of impacts. They can enhance a belief that is not strong or is weak or creates a new attitudes and behaviors in their audience. However, when they can do that, they recognize audience's thought, then they begin to manipulate. According to this research, 79 percent of respondents said that the media influence their attitudes and behaviors. 16 percent believe that the media have less effect on the audience, and 5 percent said the media can not in any way affect the respondent. (Table 4)

Table 4: Frequency distribution of respondents based on their comments on changing their attitudes and behavior by media

row	Category	Frequency	percent
1	Very much	74	19
2	Much	241	60
3	Low	29	7
4	Very low	35	9
5	Not at all	21	5
total		400	100

Reasons of watching satellite channels in Ardabil

Use of satellite channels in Iran are prohibited under the constitution. However, the tendency of satellite networks is high. Because of this tendency, when people were asked their reasons for this, they expressed: most replies indicate that the people of

². Each respondent could name three countries.

Ardabil tend to Turkey and Azerbaijan channels. 20 percent of replies showed that culture and language of the two countries is the same with ardabilain people. So, they get inclined to those channels. Nearly 20 percent Replies is related to Turkish and Azerbaijani music. 17 percent is because of lack of diversity and appeal of the channel programs of Iran, and 16 percent of respondents answered are included as having a variety of satellite channels. Also, 9 percent Replies is that there is lack of censorship and provide real news in the satellite and 6 percent Replies about attention to Persian nation, and the lack of attention to other ethnic groups in Iran. (Table 5)

Table 5: Frequency distribution of respondents based on their comments to Reasons of watching satellite channels in Ardabil

row	Category	Frequency	percent
1	Access to variety of Turkish and Azerbaijan music	96	20
2	The same culture and language	94	20
3	Lack of diversity and attractions of Iran channels	83	17
4	Diversity and attractions of satellite channels	79	16
5	Lack of censorship and provide real news	46	9
6	attending to Persian nation, and the lack of attention to other ethnic groups in Iran channels	27	6
7	Visual appeal	21	4
8	Being specialized channels	16	3
9	Educational channels	15	3
10	Others ³	12	2
	Total	489 ⁴	100

Conclusion

It is believed that if the audience through the use of media to reach a level of satisfaction and the media needs to satisfy itself will be raised again and again refer to the same media source. Such an approach in the media and audience interaction makes the relative dependence on the audience arise and according to her/his media learning and teaching, expectations and interests, access to recreation, etc, value to media by audience because the media will also influence the audience. So the audience's interests, needs and demands of its media meet will bring it on. As we witnessed in the research findings, most people tend to have channels of Turkey and Azerbaijan, and want to use the satellite channels. Also demand more varied and more attractive

programs and want to broadcast programs on Turkish language on Iranian channels. They believe that lack of diversity and distribution of all programs in Persian has caused people to be unhappy with the Iranian channels and search their interests and aspirations in Turkey and Azerbaijan channels. Because channels of these two countries are in Turkish and the same race and language and cultural commonalities between turkey, republic of Azerbaijan and Ardabil is very effective to trend in Ardabil people to these satellite channels.

Media audiences are affected when they feel that what is asked of them on television programs can be displayed. They use music, show, movies and series, sports, politics, etc that are consistent with culture, life experiences and thoughts and beliefs. So the media can contact the cognitive component in attracting audiences to broadcast programs and their impact will succeed. This research also shows people of Ardabil dissident from Iran channels and have less attendance to news, music, etc. they have the access to satellite channels they use.

Ardabil people speak Turkish and their language and culture and customs are like the people of Turkey and Azerbaijan. They like Turkish music and want to watch some channel programs that are in Turkish. Considering that all media are in Persian language and culture, so ethnics' cultures, languages, etc are in less attendance. For this, Ardebil people select Turkey and Azerbaijan channels. Their satisfactions with the both countries channels are high and there are more reasons to watch them like common culture and language and music. The diversity and appeal of their programs insist of the lack of variety and charms of Iranian channels are other factors to watch the satellite channels.

Taking into account the individual steps in the psychological aspects of cognitive, emotional and behavior can be observed that people in Ardebil in types of clothing, food, speech, music and other cultural issues, have communication with Turkey and Azerbaijan. Although limited in terms of clothing especially for women, nevertheless we can see clearly similarities and commonalities in the three regions in more issues.

Ultimately, this paper used to examine psychological factors for using media and cultural dimensions of the audience and conditions governing the media and according to existing theories that reinforce the conclusion reached by the media audience ideas, possible But to change and create new conditions it is very difficult and if the media wants to be stubborn, audiences turn away and turn to the media that are match with their culture, interests, needs and demands, etc.

³. 1. Learning foreign language 2. Familiarity with the culture of other countries 3. Networks exist for children 4. People trust the satellite channels 5. access to porno channels

⁴. Each respondent could say two cause

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References

1. Ajzen, I., & Fishbein, M. (1980). *Understanding the attitude and predicting social behaviour*. Enlewood Cliffs, New Jersey: Prentice - Hall Inc.
2. Ajzen, I., & Fishbein, M. (1980). *Understanding the attitude and predicting social behaviour*. Englewood Cliffs, New Jersey: Prentice - Hall Inc.
3. defleur, M., & Rokeach, S. J. (1976). A dependency Model of Mass - Media Effects: A sosiological framework. *Communication Research* , 500-510.
4. Defleur, M., & Rokeach, S. j. (1976). A dependency Model of Mass Media Effects: A sociological framework. *Communication Research* , 485-510.
5. During, S. (2001). *The cultural studies reader*. London: Routledge.
6. Hakimara, M. (2010). *Media Psychology: Emphasis on TV, child and young*. Tehran: IRIB Faculty Press.
7. McQuail, D. (1977). *Audiences Analysis*. London: Sage.
8. McQuail, D. (1988). *Mass Communication Theory: An introduction*. London: Sage.
9. Mehdizadeh, M. (2005). *The comprative study of cultivation and perception theories in communication*. Tehran: Center of Researches and Studies of Iran R & TV.
10. Rokeach, J. S., & Grube, J. W. (1984). *The great American value test*. New York: Free Press.
11. Windhal, S., & Signiser, O. (1992). *Using Communication Theory: an introduction to planned communication*. london: sage.
12. Windhal, S., & Signiser, O. (1992). *Using Communication Theory: An introduction to planned communication*. london: sage.

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Pharmacological properties of medicinal herbs by focus on secondary metabolites

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Abstract: Use of herbs based drugs and chemicals for treating various diseases are as old as human civilization. Herbs have vast ability to synthesize aromatic materials mainly secondary metabolites. Herbs and herbs-based therapies are the source of various modern pharmaceuticals. In many cases, these herbal materials serve as defensive molecules against microorganisms, insects, and herbivores. Further, some of which may involve in plant aroma (terpenoids), pigmentation (tannins and quinines), and flavor (ginger). The aim of this review was to study of secondary metabolites and bioactive chemical constituents of medicinal herbs and their pharmacological activity. Regarding our purpose we searched at Pub Med, MEDLINE, CNKI, EMBASE, Wiley Inter Science, Elsevier databases without language limitation. In this sense we applied different words related to herbal therapy, pharmacology, secondary metabolites and phytochemistry.

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Introduction:

Since ancient times peoples have applied herbs and its derivatives as therapeutic medicines (Thomson, 1978; Philipeon, 2003; Lesney, 2004; Newman, et al., 2003). It is an evolving practice recorded in both folklore and books of early practitioners. At present, despite the abundance and advancement of synthetic drugs, a significant proportion of the population of developing countries still depend on traditional medicines for their health care needs (Lesney, 2004; Okigbo and Mmeka, 2006). The aim of this literature review was to establish the current level of knowledge regarding the phytochemical specificity of medicinal herbs in the treatment of various diseases. This literature review starts with an overview of the historical and current use of traditional medicinal herbs and their products. This will then be followed by a discussion of most important secondary herbal metabolites with known medicinal and pharmacological activity. Next, will be an overview of general procedures used to bioprospect and to assess herbal materials for their pharmacological properties.

Methods:

We searched for papers published in Pub Med, MEDLINE, CNKI, EMBASE, Wiley Inter Science, Elsevier databases without language limit by retrieving key words "herb/herbal therapy, ancient medicines, human/patients, pharmacology, phytochemistry, secondary metabolites" to identify secondary metabolite of medicinal herbs that used in pharmacology applications. These searches were

collected and classified during 2011 and conducted by two independent examiners. The last date of searching and revising was January 4, 2012.

Results:

History of herbal medicine

Herbal medicine, also called botanical medicine or phytomedicine, refers to the use of a plant's seeds, berries, roots, leaves, bark, or flowers for medicinal uses. Peoples have been utilizing herbal medicine for the treatment, control and management of a variety of diseases since ancient times (Griggs, 1981; Kinghorn and Balandrin, 1993; Kong et al., 2003, Philipeon, 2003). There is plenty archeological evidence to support the fact that prehistoric man used plant and herbs for medicinal purposes. For instance, pollen analysis of numerous plants found in the grave the Neanderthal man buried 60000 years ago in Middle East, indicated that the plants buried with the corpse were all of medical value (Griggs, 1981; Kong, et al., 2003). In another example, medicinal herbs found in the individual belongings of the "Ice man" whose body was frozen in the Swiss Alps for more than 5,300 years, are thought to have been used to treat the parasites found in his intestines (Griggs, 1981; 1990; Kong et al., 2003). In written documentations, evidence that human have been applying plant and herbs for medicinal uses comes from several sources. Firstly, the Sumerian clay tablet (dating about 4000 years ago) recorded and described the medicinal use of plants such as laurel, caraway, and thyme by the ancient Sumerian of Mesopotamia (Kong et al., 2003; Philipeon, 2003). These plants are still employed all

over the world for medicinal uses. The Ebers papyrus, written about 3500 years points to the fact that ancient Egyptians applied plants such as mandrake for pain relief and garlic for the treatment of heart and circulatory disorders (Kong et al., 2003). Ancient China is also a source of information about the early application of medicinal herbs. "The Pun-tsao", a Chinese pharmacopoeia published around 1600 BC contain list several medicinal herbs and their applications including ma-Huang, the shrub that introduced the drug ephedrine to modern medicine (Kinghorn and Balandrin, 1993). In India, herbal medicines date back several thousand years to the Rig-Veda, a collection of Hindu sacred verses (Grover et al., 2002), system of health care known as Ayurvedic medicine, which is still widely practiced in India today. In the ancient Western world, the development of western medicine is believed to have been influenced by the writings of Greek philosophers, in particular, the writings of Hippocrates (460–377 BC) and Aristotle (384–322 BC), and the works of Dioscorides, who collected informations of more than 600 species of plants with medicinal value in his famous book "De Materia Medica". This book, which was written in the first century AD remained the standard medical reference in most of Europe for more than 1500 years (Goldman, 2001; Kong et al., 2001). During the Dark, Middle Ages and the Renaissance (476 -1500 A.D.), herbal medicine continued to play an important role in health care management throughout the world (Barter and Daly, 2000). At about the same time, Persians preserved much expertise, and expanded it to include the use of their own resources, together with those of Chinese and Indian herbs, till then unknown to the Greco-Roman world (Kalhor R. 1997, Cooper EL.2004, Saad B .et al., 2005, Philipeon, 2003). In this sense, Razes (860-930), a Persian physician, who approved treatment based on herbs and foods, and avoided synthesized medicine, except for necessary. Avicenna (980-1037) wrote many book on a wide range of subjects but he is perhaps most famous for his 'Law of Medicine' which includes divisions on the formulation of medicines in details(Kalhor R 1997, Saad B. et al., 2005, Cooper EL 2004) In the United States, herbal remedies handed down from European settlers and learned from Native Americans were a mainstay of medical care until the early 1900s when expansion of the pharmaceuticals industry, coupled with the advancement of technologies to isolate, purify and characterize natural products as well as increased knowledge of synthetic chemistry, led to a decline of herbal medicine in the United States and other developed countries (Kinghorn and Balandrin, 1993). Prior to the nineteenth century, herbal medicines were administered mostly in their crude forms as infusions

(herbal teas), tinctures (alcoholic extracts), decoctions (boiled extract of roots or bark), syrups (extracts of herbs made with syrup or honey) or applied externally as ointments (poultices, balms and essential oils) and herbal washes (Griggs, 1981; Gurib-Fakin, 2006). However, during the late nineteenth and early twentieth centuries, scientists began isolating purifying and identifying active ingredients from medicinal herb extracts. These efforts led to the discovery of some of the most important drugs that are still widely used in modern medicine (Goldman, 2001; Newman et al., 2003; Kong et al., 2003; Gupta et al., 2005). For example, morphine isolated from opium poppy (*Papaver somniferum*) is a powerful pain reliever and narcotic, quinine isolated from *Cinchona* plant species is an effective anti-malarial drug; taxol (isolated from *Taxus brevifolius*) and vincristine (isolated from *Catharanthus roseus*) are highly effective against certain types of cancer and serpentine (isolated from the root of the Indian plant *Rauwolfia serpentina*) is used in the treatment of hypertension (Newman et al., 2003; Lesney, 2004; Gupta et al., 2005; Gurib-Fakin, 2006). In addition to the biologically active plant-derived natural products mentioned above, many other plant derived natural products have served as "lead compounds" for the design, synthesis and development of novel drug compounds (Kinghorn and Balandrin, 1993; Newman et al., 2003; Lesney, 2004). In this context, some herb derived natural products have been modified slightly to render them more effective or less toxic in order to produce the so called "semi-synthetic drugs" (Kinghorn and Balandrin, 1993; Kong et al., 2003). As an example of this type of strategy, aspirin was developed in 1953 through structural modification of salicylic acid which was identified as the active ingredient in a number of plants known for their pain-relieving qualities (Kong et al., 2003; Lesney, 2004). In another example, the development of the current and popular oral hypoglycemic agent, metformin was based on the use of goat's rue (*Galega officinalis*) to treat diabetes (Kinghorn and Balandrin, 1993). The blood glucose lowering property of *Galega officinalis* has been attributed to the presence of a guanidine-type of alkaloid, galegine. Because galegine was found to be too toxic for human use, several structural analogs of this compound were synthesized and tested in clinical studies. These efforts culminated in the development and marketing of metformin as an effective antidiabetic drug (Kinghorn and Balandrin, 1993; Gupta et al., 2005).

Herbal medicine today

Although the direct use of herbal extracts in developed countries continued to decrease in the late nineteenth and early twentieth centuries, medicinal herbs still play a key role in health care system of

many parts of the world (Kong et al., 2003; Tapsell, 2006). According to World Health Organization (WHO, 2001) 60% of the world's population depend on traditional medicine, and 80% of the population in developing countries depend almost entirely on traditional medical practices, in particular, herbal medicine for their primary health care needs (Fransworth, 1994; Zhang, 2000). The long tradition of herbal medicine continues to the present day in China, India, and many other countries (Zhang, 2000; Kong et al., 2003; Tapsell, 2006). Medicinal herbs continue to contribute significantly to modern prescription drugs by providing lead compounds upon which the synthesis of new drugs can be made. According to Newman et al., (2003), 60% of the anticancer drugs and 75% of the anti-infectious disease drugs approved from 1981-2002, could be traced to natural origins. In addition, 61% of all new chemical entities introduced worldwide as drugs during the same period could be traced to or were inspired by natural products (Gupta et al., 2005). The use of, and search for, drugs and dietary supplements derived from plants have accelerated in recent years. Pharmacologists, microbiologists, biochemist, botanists, and natural-products chemists all over the world are currently investigating medicinal herbs for phytochemicals and lead compounds that could be developed for treatment of various diseases (Achaya and Shrivastava, 2008).

Secondary herbal metabolites with reported medicinal properties

The medicinal and pharmacological actions of medicinal herbs are often depended to presence of bioactive compounds called secondary herbal metabolites (Bruneton, 1999; Henrich et al., 2004). Unlike the ubiquitous macromolecules of primary metabolism (e.g. monosaccharides, polysaccharides, amino acids, proteins, nucleic acids, lipids) which are present in all plants, secondary metabolites with medicinal properties are found only in a few species of plants (Henrich et al., 2004). Some of these secondary metabolites serve as defensive compounds against herbivores and pathogens. Others function in mechanical support, in attracting pollinators and fruit dispersers, in absorbing harmful ultraviolet radiation, or reducing the growth of nearby competing plants. (Chynier, 2005; Gurib-Fakim, 2005) Secondary herbal metabolites with reported medicinal properties consist of waxes, fatty acids, alkaloids, terpenoids, phenolics (simple phenolics and flavonoids), glycosides and their derivatives. (Satyajit et al., 2006, Eloff, 2001; Cowan, 1995). Some of these secondary herbal metabolites are briefly discussed as follows.

Carbohydrates and related compounds

Plant-derived carbohydrates and related compounds with medicinal and therapeutic potential

include fiber, cellulose and its derivatives, starch and its derivatives, dextrans, fructans, mucillages (uronic acid containing polymers), pectins (polysaccharide complexes formed from partially methoxylated polygalactouronic acid) and gums (Bruneton, 1999). In addition to their use as bulking agents in pharmaceuticals, carbohydrates and related compounds have been shown to have immunomodulatory, anti-tumor, anticoagulant (e.g. heparin), hypoglycemic or antiviral activities (Gurib-Fakim, 2005).

Alkaloids

Alkaloids often contain one or more rings of carbon atoms, usually with a nitrogen atom in the ring. Many have declared pharmacological activity (Harborne, 1998). Most alkaloids have a strong bitter taste and are very toxic, for these reasons they are used by plant to protect themselves against herbivory, and attacks by microbial pathogens and invertebrate pests (Harborne, 1998). Several alkaloid containing medicinal herbs are reported to have been used by the early man as pain relievers, as recreational stimulants or in religious ceremonies to enter a psychological state to achieve communication with ancestors or God (Henrich et al., 2004; Gurib-Fakin, 2005). Alkaloids are classified into several groups either on the basis of their basic ring system (e.g. atropine, indole, quinoline, isoquinoline, imidazole, piperidine alkaloids), plant sources (e.g. opium, belladonna, vinca, cinchona and ergot alkaloids) or their pharmacological properties (e.g. analgesic, stimulant or anti-malarial alkaloids) (Kinghorn and Balandrin, 1993; Bruneton, 1999, Harborne, 1998; Henrich et al., 2004). Their botanical source(s), as well as their pharmacological properties are summarized in Table (1). Alkaloids normally occur in the herb as salts or free bases. Hence, their extraction from herbal materials is generally based on their differential solubility in aqueous acids and organic solvents (Starmans and Nijhuis, 1996; Jones and Kinghorn, 2005). A regular procedure is to initially extract alkaloids and their salts with 2% sulphuric acid. The resultant acid extract is then made alkaline with an ammonia solution and shaken gently with an organic solvent in a separating funnel. The alkaloids, as the free bases partition in favor of the organic layer leaving behind unwanted free non-basic substances (Jones and Kinghorn, 2005). A general chemical test for alkaloids involves addition of a drop of either Mayer's reagent or Dragendorff's reagent to the acid extract. A pale precipitate or an orange-red precipitate indicates the presence of alkaloids. (Gupta et al. 2005).

Table 1: Botanical source(s), and pharmacological properties of some well known alkaloids.

Alkaloids	Examples	Botanical Sources	Medical Properties	REFERENCES
Opium alkaloids	Morphine , heroin	Papaver Somniferum (Opium poppy)	Analgesics (pain relievers and narcotics)	Kinghorn & Balandrin (1993), Gurib –Fakim (2005) Heinrich et al (2004)
Belladonna alkaloids	Cocaine, atropine, scopolamine, hyoscyamine	Datura species, Atropa belladonna, Hyoscyamus niger (henbane)	Anti cholinergic (local anesthetics) and stimulants	Kinghorn & Balandrin (1993), Newman et al (2003) Heinrich et al (2004)
Cinchona alkaloids	Quinine , quinidine	Cinchona species	Antimalarial, antiarrhythmic activities	Kinghorn & Balandrin (1993), Heinrich et al (2004)
Catharanthus alkaloids (Vinca alkaloids)	Vincristine , vinblastine	Catharanthus roseus (Madagascar rosy periwinkle)	Anti cancer (antileukemic) activity	Kinghorn & Balandrin (1993) Kong et al (2003), Lesney (2004)
Rauwolfia alkaloids	Reserpine	Rauwolfia Species	Anti hypertensive activity	Kinghorn & Balandrin (1993); Brueton (1999); Gupta et al (2005)

Phenolics

Phenolics are a class of herbal secondary metabolites that are characterized by the presence of one or more hydroxyl (-OH) groups attached to a benzene ring or to other complex aromatic ring structures (Bruneton, 1999, Harborne, 1998 and Heinrich et al., 2004). Phenolic herb secondary metabolites are widely distributed in herbs and are responsible for color development, pollination and protection against UV radiation and pathogens (Bruneton, 1999; Heinrich et al., 2004). They also contribute to the color and astringency of some foods. On the basis of their structure phenolics compounds can be classified into two broad classes: the non-flavonoids and the flavonoid phenolic compounds (Bruneton, 1999; Heinrich et al., 2004).

Non-flavonoid phenolic compounds

Non-flavonoid phenolic compounds include simple phenols (eugenol, catechol, hydroquinone, phloroglucinol hydroquinone, and p-anisaldehyde) (Jadhav et al., 2004), the C6-C1 benzoic acids (vanillic acid, gallic acid and protocatechuic acid), the C6-C3 phenyl propanoids and their derivatives (cinnamic acid, caffeic acid, ferulic acid myristicin and sinapyl alcohol), coumarins (scopoletin; warfarin and dicoumarol), hydrozable tannins (gallotannins and ellagitannins) and lignans and related compounds (Kumar R, et al. 2010). Examples of non-flavonoids of pharmacological interest together with their botanical sources and their pharmacological properties are given in Table (2).

Table 2: Botanical source(s) and pharmacological properties of some non-flavonoid phenolic of pharmacological interest Flavonoid phenolic compounds.

References	Medical Properties	Botanical Sources	Examples	Non-Flavonoid Phenolic
Bruneton,(1999) jadhav et al.,(2004)	Anti-bacterial , anti-inflammatory , local anesthetic activities	Berries,red and green tea, coffee beans	Ellagic acid, tannic acid; vanillin, hydroquinone, eugenol	Simple phenols
Bruneton,(1999) Harborne,(1998)	Choleretic activity, hepatoprotective, anti-oxidant activity	Cynara scolymus (artichoke),rosmarinus officinalis rosemary)	Gallic acid, protocatechuic acid	Benzoic acids
Bruneton,(1999) Harborne,(1998) Gurib-Fakim,(2005)	Antiseptic, component of sunscreen lotions and for treatment of dyspepsia	Fruits and vegetables Cinnamon, Myroxylon, balsamum(Peruvian balsam)	Cinnamic acid,coumaric acid, caffeic acid ,ferulic acid	Phenyl propanoids
Harborne(1998) Heinrich et al. ;(2004)	Antifungal; blood anticoagulants; treatment of capillary fragility	Potato plant(Solanum tuberosum),Citrus	Scopoletin,warfarin, and dicoumarol	Coumarins (benzopyrone derivatives)
Bruneton (1999) Harborne(1998) Heinrich et al., (2004)	Anti-diarrhea; antidote in poisoning by heavy metals	Dicotyledonous herbs	Gallotannins, ellagitannins Stilbenoids	Hydrolyzable tannins
Harborne(1998) Heinrich et al.; (2004)	Antifungal	Heartwood of Pinus species	Resveratrol , Pinosylvin	Stilbenoids
Harborne(1998) Heinrich et al.; (2004)	Anti tumor, antiviral anti allergic, anti-rheumatic activity	Flax seed and other grains	Secoisolaricresinol, pinoresinol	Lignans and related compounds

Flavonoid phenolic compounds

Flavonoids are a large and complex group of compounds containing a three ring structure with two aromatic centers (rings A and B) and a central oxygenated heterocyclic ring (C) (Bohm, 1998; Hollman and Katan, 1999).

The six major classes of flavonoids are flavones, flavonols, flavonones, catechins (flavanols) anthocyanidins and isoflavones (Bohm, 1998; Bruneton, 1999; Pietta, 2000; Scalbert et al., 2005; Goutam and Dilip, 2006). Flavonoids have several

proven medicinal properties, such as anti-inflammatory, anti-oxidant, anti cancer, antibacterial and antiviral properties (Valsaraj R, 60, et al 1997, Valsaraj R, 58, et al. 1997, Hollman and Katan, 1999; Harborne and Williams, 2001; Chynier, 2005; Manach et al., 2004). Specific examples of each of the major subclasses of flavonoids, their botanical sources as well as some of their pharmacological properties are summarized in Table (3).

Table 3: Specific examples of each of the major subclasses of flavonoids, their botanical sources as well as some of their pharmacological properties.

References	Medical Properties	Botanical Sources	Examples	Flavonoids
Bruneton, (1999) Harborne et al,(1998) Pietta(2000)	Anti- inflammatory analgesic	Fruits of various citrus trees	Naringenin, hesperetin	Flavonones
Bohm(1998) Bruneton(1999)	Anti-tumor activity	Generally in herbaceous families, e.g. Labiatae, Umbelliferae	Apigenin, luteolin	Flavones
Bohm(1998) Bruneton(1999) Goutam&Delip(2006)	Antioxidant and microbial activities Enzyme inhibitors	Generally in woody angiosperms, anions and green tea leaves	Myricetin kaempferol, quercetin,	Flavonols
Manach et al(2004) Cheynier(2005)	Powerful antioxidants	Found in tea leaves	Catechins, galocatechins	Flavanols
Hollman & Katan, 1999),Pietta(2000), Scalbert et al(2005)	Anti-hepatotoxic, anti-lipolytic, vasodilatory effects	Fruit and vegetables	Pelargonidin, cyanidin,malvidin	Anthocyanidins
Manach et al(2004) Cheynier(2005)	Powerful anticancer and heart disease properties	Cereals and legumes	Daidzein, genistein, glycitein	Isoflavonoids
Hassan et al(2006) Gurib-Fakim(2005)	Antioxidant, anti-cancer , anti HIV activities	Abundant in grapes, wine and coffee pulp	Procyanidin, prodelphinidins	Condensed tannins (proanthocyanidin)

Less polar flavonoids (e.g. isoflavones, flavones, methylated flavones and flavonols) are generally extracted by solvents of medium polarity and polar solvents (chloroform, dichloromethane, diethyl ether or ethyl acetate). Polar flavonoids (anthocyanidins and flavanols) are generally extracted with alcohol or alcohol-water mixtures in the presence of a small amount (0.1-1%) hydrochloric acid, whereas tannins may be extracted with alcohols and acetone (Elangovan V, et al. 1994, Satyajit et al., 2006). All phenolic compounds (flavonoids and non-flavonoid phenolics) react with ferric chloride to give a characteristic color (Harborne, 1998, Das K. et al., 2010).

Terpenoids

Terpenoids, also known as isoprenoids constitute the largest group of herbal secondary metabolites (Bruneton, 1999). Terpenoids are involved in defense, wound scaling and thermotolerance of plants as well as in the pollination of seed crops (Heirich et al., 2004). They are also responsible for the flavor of fruits, the fragrance of the flowers and the quality of agricultural products. Terpenoids are classified as monoterpenes (C10),

sesquiterpenes (C15), diterpene (C20), triterpenes (C30) and tetraterpenes (C40) on the basis of the number of isoprene units. (Banthorpe, 1991; Bruneton, 1999; Heirich et al., 2004; Gurib-Fakim, 2005). Monoterpenes and sesquiterpenes are the main components of essential oils and are commonly found in plant families Labiatae, Myrtaceae, Pinaceae, and Rutaceae (Harborne, 1998, Heirich et al., 2004). Diterpenes include resin acids and plant hormones (gibberellins) (Harborne, 1998). Many of the diterpenes are toxic, but some, for example, forskolin (from gymnosperms), taxol (from the Pacific yew) and ginkgolides (from Ginkgo biloba) are used in modern medicine for the treatment of hypertension, cancer and memory loss respectively (Bruneton, 1999; Heirich et al., 2004; Gurib-Fakim, 2005). Triterpenoids are the most abundant plant terpenes, they include plant steroids and are components of saponins and steroidal glycosides (Harborne, 1998; Bruneton, 1999) The most common tetraterpanoids are the carotenoids which are responsible for most of the yellow and orange plant pigments (Heirich et al., 2004). Tetraterpenoids also include the xanthophylls found in many yellow fruits and flowers (Bruneton, 1999). Terpenoids are in general soluble in common organic

solvents. However, low molecular weight terpenoids such as essential oils, are thinly soluble in water. Hence terpenoids are generally extracted with non-polar solvents. However, the volatile essential oils can be steam distilled (Satyajit et al., 2006). Table (4)

provides a summary of examples of each class of terpenoids, together with their botanical sources and pharmacological properties.

Table 4: Specific examples of each of the major subclasses of terpenes, their botanical source (s) as well as some of their pharmacological properties.

References	Medical Properties	Botanical Sources	Examples	Terpenes
Harborne, 1998 Heinrich et al., (2004) Gurib Fakim,(2005)	Analgesic and anti-inflammatory activities	Essential oils of some Pinus Spp and coniferous woods	Camphor, limonene	Monoterpenes (C ₁₀)
Bruneton(1999) Heinrich et al., (2004)	Antibacterial, antifungal,antimalarial, mulluscicidal	Essential oils of many plant species	Bisabolol, Ngaione, Hymenoxin, Santonin	Sesquiterpenes(C ₁₅)
Bruneton(1999) Heinrich et al., (2004)	Anti hypertensive Anti cancer activities	Gymnosperm woods (Larix spp) Taxus(brevifolia)	Forskolin, Phorbol esters, Taxol (Paclitaxel)	Diterpenes (C ₂₀)
Bruneton(1999) Heinrich et al., (2004) Gurib-Fakim(2005)	Anti inflammatory Hemolytic properties	Bark of the birch Betulaalba,Larix,Picea,Pinus,Fagus, Quercus spp	Betulin (Pentacyclic triterpene) Phytosterols β-Sitosterol and campesterol	Triterpenes (C ₃₀)
Heinrich et al., (2004) Gurib-Fakim(2005)	Antioxidant activity	Vegetables such as carrots and pumpkin	β-Carotene	Tetraterpenes (C ₄₀) Carotenoids

Glycosides

Glycosides are herbal secondary metabolites made up of two components, a carbohydrate component known as the glycone and a non carbohydrate component known as the aglycone. The glycone component usually consists of one or more glucose units whereas the aglycone may be any one of the secondary herb metabolites discussed above (Bruneton, 1999; Heirich et al., 2004; Gurib-Fakim, 2005). The solubility of glycosides depends on the nature of the aglycone and the number and type of sugar molecules linked to the aglycone (Starmans and Nijhuis, 1996). Aglycones tend to be soluble in organic solvents and sugar part in aqueous solvents. In general, glycosides can be extracted with acetone, ethanol or an aqueous/ethanol mixture (Jones and Kinghorn, 2005). Medicinally important glycosides consist of anthraquinone glycosides, coumarin glycosides and steroidal (cardiac) glycosides.

Anthraquinone glycoside

Herbs such as Cassia senna, rhubarb (*Rheum palmentum*), cascara (*Rhamnus purshiana*) and Aloe vera have long been known for their laxative property (Bruneton, 1999; Heinrich et al., 2004; Gurib-Fakim, 2005). This property has been attributed to the presence of anthraquinone and entrones glycosides present in these plants (Heinrich et al., 2004). When ingested anthraquinone glycosides hydrolyze in the large intestine (colon) to liberate the aglycones which stimulate peristalsis and increase water retention in the colon (Bruneton, 1999).

Coumarin glycosides

Coumarins glycosides are phytoalexins, and are synthesized by the plant in response to bacterial or fungal infection, physical damage, chemical injury, or a pathogenic process (Gurib-Fakim, 2005). For example, scopoletin is synthesized by the potato (*Solanum tuberosum*) following fungal infection. Coumarin glycosides are very fragrant. They are the source, for instance, of freshly-mown hay scents (Heinrich et al., 2004). Medicinally, coumarin glycosides have been shown to have hemorrhagic, anti fungicidal, and antitumor activities (Bruneton, 1999). The aglycones of a coumarin glycoside dicumarol and its synthetic structural analog, warfarin are used in modern medicine as anticoagulants.

Steroidal glycosides

Steroidal (cardiac) glycosides are naturally occurring drugs whose actions include both beneficial and toxic effects (at higher doses) on the heart (Bruneton, 1999; Gurib-Fakim, 2005; Heinrich et al., 2004). Herbs containing cardiac glycosides contain *Digitalis purpurea* (foxglove) and *Strophanthus*. Foxglove is the source of two potent glycosides used as a heart stimulants, digoxin and digitoxin. Both digoxin and digitoxin are widely used in the modern treatment of congestive heart failure, atrial fibrillation and flutter (Heinrich et al., 2004). These glycosides prolong the relaxation phase of the heart (ventricular diastole), thus allowing the left ventricle to fill with more blood. In accordance with Starling's Law of Contraction, the increased blood volume in the left ventricle results in a more forceful contraction (ventricular systole), thereby pumping more blood out into the aorta (Sherwood et al., 2004). *Strophanthus*, a

genus of a South African shrub produces the cardiac glycoside, ouabain (G-strophanthin). Like digitalis glycosides, ouabain is also used in modern medicine to treat congestive heart failure (Heinrich et al., 2004). The chemical structure of ouabain is similar to that of digitoxin except that it has the sugar rhamnose instead of digitoxose.

Pharmacological investigation of herbal materials

Reviews of literature involving research of medicinal plants suggest that scientists follow more or less the same general strategy to investigate herbal materials for their pharmacological properties (Kinghorn and Balandrin, 1993; Heinrich et al., 2004).

Selection of herbal species

Any plant species and herb parts collected randomly can be investigated using available phytochemical methods. However, a more targeted approach is often preferred to a random selection (Kinghorn and Balandrin, 1993; Harborne, 1998; Heinrich et al., 2004). The herbal material to be investigated can be selected on the basis of some specific traditional uses (ethnobotanical bioprospecting approach). Extract prepared from herb used as traditional remedies to treat certain diseases are more likely to contain biologically active compounds of medicinal interest (Heinrich et al., 2004). Alternatively, the plant can be selected based on chemotaxonomical data. In the chemotaxonomic approach, knowledge that a particular group of plants contain a certain class of natural products may be used to predict that taxonomically related plant may contain structurally similar compounds (Heinrich et al., 2004). Some herbal materials can be selected following a combination of the above mentioned approaches. The use of literature data base early in the selection process can provide some preliminary information on the type of natural products already isolated from the plant and the extraction methods employed to isolate them (Heinrich et al., 2004). Another approach known as the information driven approach, utilizes a combination of ethnobotanical, chemotaxonomic and random approaches together with a data base that contains all relevant information concerning a particular plant species (Kinghorn and Balandrin, 1993; Harborne, 1998; Heinrich et al., 2004). The database is used to prioritize which herbs should be extracted and screened for biological activity. This approach is favored by large organizations (particularly pharmacological companies) interested in screening thousand of samples for bioactivity as it may reduce costs by a process known as dereplication; the process of avoiding the repeated discovery of common or known drugs (Heinrich et al., 2004).

Collection and identification of plant material

The whole plant or a particular part can be collected depending on where the metabolites of

interest (if they are known) accumulate. Hence aerial (e.g. leaves stems, flowering tops, fruit, seed, and bark) and underground (e.g. tubers, bulbs, roots) parts can be collected separately. Collection of herb materials can be influenced by factors such as the age of the plant and environmental conditions (e.g. temperature, rainfall, amount of daylight, soil characteristics and altitude) (Williams et al., 1996; Harborne, 1998). Thus, it is important to take this into consideration for the re-collection purpose, in order to ensure reproducible profile (nature and amount) of metabolites (Satyajit et al., 2006). The plant from which the material is collected must also be identified correctly. A plant taxonomist or a botanist should be involved in the detailed authentication of the plant (i.e. classification into its class, order, family, genus and species) (Satyajit et al., 2006). Any feature related to the collection, such as the name of the plant, the identity of the parts collected, the place and date of collection, should be recorded as part of the voucher (a dried specimen pressed between sheets of paper) deposited in a herbarium for future reference (Harborne, 1998; Satyajit et al., 2006).

Extraction of plant materials

Herbal materials are commonly extracted by means of liquid solvents in what is known as the "solid-liquid solvent extraction". Typical solid-liquid solvent extraction processes for herbal materials involve drying and grinding of the herbal material, choosing a suitable extraction solvent and extraction procedure (Starmans and Nijhuis, 1996; Cheng et al., 2001; Jones and Kinghorn, 2005).

Drying and grinding the plant material

Once the herbal material has been collected, it needs to be dried as soon as possible. A common practice is to leave the sample to dry on trays at ambient temperature and in a room with adequate ventilation (Heinrich et al., 2004; Satyajit et al., 2006). Dry conditions are essential to prevent microbial fermentation and subsequent degradation of metabolites. Herbal materials should be sliced into small pieces and distributed evenly to facilitate homogeneous drying. Protection from direct sunlight is advised to minimize chemical reactions (and formation of artifacts) induced by ultraviolet rays (Satyajit et al., 2006). To facilitate the drying process, the material can be dried in an oven. This can also minimize reactions (e.g. hydrolysis of glycosides) that can occur as long as there is some residual moisture present in the herbal material. The dried material should be stored in sealed containers in a dry and cool place. Storage for prolonged periods should be avoided as some constituents may be decomposed (Heinrich et al., 2004; Jones and Kinghorn, 2005). After drying, herbal materials are commonly grounded into a fine powder. Grinding of plant

materials into smaller particles facilitates subsequent extraction procedures by rendering the sample more homogeneous, increasing the surface area, and facilitating the penetration of solvents into cells (Harborne, 1998; Satyajit et al., 2006). Mechanical grinders (e.g. hammer and cutting mills) are employed to shred the herbal material into various particle sizes. Potential problems of grinding include the fact that some material (e.g. seeds and fruits rich in fats and volatile oils) may clog up the sieves and that heat generated may degrade thermolabile metabolites (Harborne, 1998).

Choice of a suitable extraction solvent

The choice of the extraction solvent depends mainly on the polarity and hence the solubility of the bioactive compounds of interest. Although water is usually applied as a solvent in many traditional protocols, organic solvents of varying polarities are often used (either alone or in different combinations) in modern methods of extraction to exploit the various solubilities of herbal ingredients (Lapornik B et al., 2005, Handa SS et al., 2008). The polarity and chemical profiles of most of the common extraction solvents have been determined (Ayaffor et al., 1994; Eloff, 2001; Cowan, 1995) and are summarized in Table (5).

Table 5: Polarity and chemical profiles of most of the common extraction solvents.

References	Extracted chemical profile	Solvent	Polarity
Ayaffor et al(1994), Cowan(1999)	Fatty acids, waxes , terpenoids	n- Hexane	Low
Perett et al (1995),Cowan, (1999),Bruneton(1999)	Fatty acids, waxes , terpenoids	Chloroform	
Bruneton(1999), Scalbert et al(2005)	Less polar and polar flavonoids , tannins, terpenoids	Dichloromethane	Medium
Bruneton(1999), Scalbert et al(2005)	Less polar and polar flavonoids , tannins, terpenoids	Ethyl- acetate	
Eloff, (1998), Bruneton(1999), Scalbert et al(2005)	Less polar and polar flavonoids , tannins, terpenoids, glycosides	Acetone	
Cowan, (1999),Bruneton(1999)	Polar flavonoids, tannins , glycosides (saponins)	Ethanol	High
Bruneton(1999),Scalbert et al(2005)	Carbohydrates, lecithin, amino acids, polypeptides, phenolic acids, phenylpropanoids, polar flavonoids, glycosides and alkaloids	Methanol	
Kaul et al(1985), Jones & Kinghorn (2005)	Carbohydrates, lecithin, amino acids, polypeptides, phenolic acids, phenylpropanoids, polar flavonoids, glycosides and alkaloids	Water	
Bruneton(1999)	Alkaloids	Aqueous acid or base	

Thus, if the polarity or the solubility of the compounds of interest is known, information such as the one in the above table can be used to select a proper extractor solvent or a mixture of two or more solvents of different polarity (Kaul et al., 1985). Alternatively, a solvent such as acetone, which has the capacity to extract both polar and non-polar substances, and has been recommended by Eloff (2001) for the extraction of most polar and nonpolar compound. If the polarity of the compounds of interest is not known, the powdered herbal material can be extracted simultaneously with a mixture of different proportions of two or more solvents of different polarity (Bruneton, 1999, Cowan, 1995). Alternatively, the powdered herbal material can be extracted sequentially with solvent of different polarity in what is known as a sequential extraction procedure (Bruneton, 1999).

Choice of the extraction procedure

The choice of the extraction procedure depends on the nature of the source material and the compound to be isolated. Solvent extraction

procedures applied to herbal products include but not limited to maceration, percolation, soxhlet extraction, steam distillation and sequential solvent extraction (Starmans and Nijhuis, 1996; Harborne, 1998; Jones and Kinghorn, 2005).

Maceration

This simple, but still widely used procedure involves leaving the pulverized plant to soak in a suitable solvent in a closed container at room temperature (Harborne, 1998). Occasional or constant stirring of the preparation (using mechanical shakers or mixers) can increase the speed of the extraction. Maceration involves soaking the herbal material in a suitable solvent, filtering and concentrating the extract (Harborne, 1998; Jones and Kinghorn, 2005). The use of a cold solvent reduces decomposition, but the process takes longer and uses larger amounts of solvent.

Percolation

This is similar to the maceration process, but hot solvent is refluxed through the herbal material. It is quicker and uses less solvent, but decomposition

due heat may occur (Jones and Kinghorn, 2005; Satyajit et al., 2006).

Soxhlet extraction

Soxhlet extraction is a form of continuous percolation with fresh solvent, which uses special glass ware. In this procedure, the herbal material is separated from the extract by encasing it in a paper thimble beneath the dropping condensed solvent. When full, the solvent in the thimble siphons off into the main vessel containing the extractant, and the process continues (Jones and Kinghorn, 2005). The advantage of this procedure is that fresh solvent continually extract the herbal material more effectively with minimum solvent, however, heating and hence decomposition of compounds is again a disadvantage (Nikhal SB et al., 2010).

Steam distillation

There is a special apparatus for distilling volatile oils which are immiscible with water. If compounds being extracted are water soluble, the method is less useful because a large volume of aqueous extract is produced. However, in some cases a partition system may be used to concentrate the extract (Jones and Kinghorn, 2005; Satyajit et al., 2006).

Sequential solvent extraction

If the polarity and solubility of compounds that are isolated is not known, a convenient and frequently used procedure is sequential solvent extraction. In sequential solvent extraction, the herbal material is extracted with a series of solvents of different polarity (Starmans and Nijhuis, 1996). The usual way is to start with a non-polar solvent and exhaustively extract the herbal material followed by a series of more polar solvents until several extracts are obtained of increasing solute polarity. For example, a first step, with dichloromethane, will extract terpenoids, less polar flavonoids (flavones, flavonols, flavonones) and other less polar materials (Jones and Kinghorn, 2005, Okwu DE 2001). A subsequent step with acetone or ethyl acetate will extract flavonoid glycosides and other medium polar constituents. A subsequent extraction with an alcohol or water will extract highly polar constituents (Jones and Kinghorn, 2005). Once the extraction is complete, the extractant is usually concentrated under vacuum, for large volumes or solvents and blown down under nitrogen for small volumes, ensuring at the same time that volatiles are not lost. Aqueous extracts are generally freeze-dried and stored at 20°C as this low temperature reduces the degradation of the bioactive natural product (Starmans and Nijhuis, 1996). Extraction protocols may sometimes be modified depending on the type of molecules being extracted, for example, acids may be added to extract alkaloids as their salts (Jones and Kinghorn, 2005).

Screening the extract for biological activity

Once the extract has been obtained, the biological activity within is usually verified by means of an in vitro bioassay method. In vitro screening methods for biological activity are generally divided into two formats; the low-throughput screening and high-throughput screening methods, depending on the number of extracts to be screened. (Valsaraj R, 58, et al 1997, Okwu DE 2001). In low-throughput screening (LTS), small numbers of extracts (a single extract up to hundred of extracts) are dispensed into a format that is compatible with the bioassay (e.g. microtiter plate or sample tube) (Kinghorn and Balandrin, 1993,). This approach is used widely in academic laboratories where only a relatively low number of extracts are assessed. In high-throughput screening (HTS), thousand of extracts are dispensed into a format (usually a microtiter plate with many wells) and screened in the bioassay (Heinrich et al., 2004). This approach is favored by the pharmaceutical industry. This may have hundreds of thousands of samples (both natural and synthetic) for biological evaluation. (Rawat AK. Et al., 1997). This large scale approach means that decisions can be made rapidly about the status of an extract, which has an effect on the cost of the drug discovery process (Kinghorn and Balandrin, 1993).

Bioassay guided fractionation and isolation of active compounds

Active fractions are fractionated using a bioassay guided fractionation. In bioassay-guided fractionation, a crude mixture is fractionated into its fraction components using chromatographic procedures, followed by biological evaluation (bioassay) of each fraction. (Vaidya and Antarkar 1994). Only fractions which display biological activity in the bioassay are selected for further fractionation. The cycle of fractionation and testing and further fractionation is repeated until a pure compound with the desired activity is isolated (Rimando et al., 2001).

Characterization and structure elucidation of isolated compounds

Once the biological evaluation has been performed and the separation of the natural product has been achieved, the chemist will try to attempt the elucidation of the compound. Structure elucidation depends on classical spectroscopic techniques such as: Nuclear Magnetic Resonance (NMR) 1-D and 2-D Proton NMR as well as C-13 NMR, Infra Red (IR), Mass Spectrometry (MS) and X-Ray analysis (Harborne, 1998).

Preclinical and clinical studies

Once innovation and structure of the bioactive compound has been established, large amounts of the bioactive compound are isolated and the decision is made as to whether the compound can

be synthesized de novo or whether chemical modification needs to be made to enhance the biological activity (Vaidya and Antarkar, 1994). The bioactive compound will undergo extensive in vivo studies to establish activity, toxicity and efficacy. These studies are sometimes known as preclinical studies (Ebadi M, .2002). Only once all these steps have been completed will a drug lead enter clinical studies, which is the most extensive evaluation stage of a drug candidate during which many drug may fail through toxicity or lack of efficacy in humans. (Gupta SS. 1994). Successful achievement of these trials usually results in a product license, which means that the compound is now a drug. (Vaz J, et al. 1998, Dalvi SS. et al. 1994). Given the complexity of the process described above, it is not surprising that many natural product drug leads fail to make their way onto the market. Some estimates state that only 1 in 10,000 of plant-derived drug leads may actually make their way to the market (Kinghorn and Balandrin, 1993). The process is also lengthy and it may take 12-15 years from the collection of the original herbal material to the granting of a license for the new drug.

Conclusion:

Phytochemical monitoring of medicinal herbs is essential to discover new sources of therapeutical and pharmacological compounds. There is an increasing interest in correlating phytochemical ingredients of herbs with its pharmacological activity. Since it is necessary to initiate vital steps for monitoring secondary metabolites of medicinal herbs, scientists also have even started associating the botanical properties of herbs with their pharmacological activity. The phytochemical screening and quantitative and qualitative analysis of chemical constituents of the medicinal herbs showed presence of various secondary metabolites like alkaloids, terpenoids, flavonoids, steroids, coumarins, tannins and saponins. They were known to show medicinal and pharmacological activity. However majority of the drugs are at the experimental stage and have to still undergo clinical trials. There is still a rarity of medical studies which are carried out in randomized, controlled, double blind manner. Today, parallel consumption of medicines from different disciplines is a common finding. Very few studies, however, attend to the problem of drug interactions. Also nonstandardized methods of extraction may lead to the degradation of the secondary metabolites present in the herbs and may fall to the variations of bioactive compounds. So selection of best extraction method to achieve high quality of herbal ingredients is important for producing of herbal medicines.

The herbal studied in this review are potential sources for useful medicines. In future, more co-

ordinated multicentral research in order to isolate, identify, characterize and elucidate the structure of the bioactive compounds to detect secondary metabolites properties of medicinal herbs for specific pharmacological activities is expected.

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References:

1. Acharya, D., and Shrivastava, K., (2008). *Indigenous Herbal Medicines: Tribal Formulations and Traditional Herbal Practices*, Aavishkar Publishers Distributor, Jaipur- India. ISBN 9788179102527. pp 440.
2. Ayaffor, J.F., Tehuendem, M.H.K., Nyasse, B., (1994). Novel active diterpenoids from *Fromomum aulacocapos*. *Journal of natural products* 57:917-923.
3. Banthorpe, D. V., (1991). Classification of terpenoids and general procedures for their characterization, in *Terpenoids* (Charlwood, B. V. and Banthorpe, D. V., eds), *Methods in Plant Biochemistry* (Dey, P. M. and Harborne, J. B., eds.), vol. 7, Academic Press, San Diego, pp. 1–41.
4. Barter, C.D., and Daly, W.J., (2000). "Clinical pharmacology in the Middle Ages: Principles that presage the 21st century", *Clinical Pharmacology & Therapeutics* 67(5): 447-450.
5. Bohm, B., (1998). *Introduction to flavonoids*. Amsterdam: Harwood Academic Publishers.
6. Bruneton, J., (1999). *Pharmacognosy, Phytochemistry and Medicinal Plants*. Intercept. Ltd. England, U.K.
7. Cheynier, V., (2005). Polyphenols in foods are more complex than often thought. *Am. J. Clin. Nutr.* 81(suppl.): 223S-229S.
8. Cooper EL.,(2004) Drug discovery, CAM and natural products. *Evid Based Complement Alternat Med.*; 1:215-217.
9. Cowan, M.M., (1999). Plant products as antimicrobial agents. *Clinical Microbiology Review* 12:564–582.
10. Dalvi SS, Nayak VK, Pohujani SM, (1994). Effect of guggulipid on bioavailability of diltiazem and propranolol. *J Assoc Phys India*;42:454-5.
11. Das K, Tiwari RKS, Shrivastava DK. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of Medicinal Plants Research*; 4(2): 104-111.

12. Ebadi M. , (2002). *Alternative Therapies, Pharmacodynamic Basis of Herbal Medicines*. CRC Press Boca Ratan, London, 10-11.
13. Elangovan V, Ramamoorthy N, Balasubramanian S, (1994). Studies on the antiproliferative effect of some naturally occurring bioflavonoidal compounds against human carcinoma of larynx and sarcoma-180 cell lines. *Indian J Pharmacol*;26:266-9.
14. Eloff, J.N., (2001). Antibacterial activity of *Murula* (*Sclerocarya birrea* (A. rich) Hochst. Subsp. *Caffra* (Sond) Kokwaro) (*Anacardiaceae*) bark and leaves. *Journal of Ethnopharmacology* 76: 305–308.
15. Fransworth, N.R., (1994). *Ethnopharmacology and drug development*. In Chadwick, D.J., and Marsh, J., (eds). *Ethnobotany and the Search for New Drugs*, CIBA Foundation Symposium 185, John Wiley and Sons, Chichester, New York, pp 42-51.
16. Goldman, P (2001). "Herbal medicines today and the roots of modern pharmacology". *Ann. Intern. Med.* 135 (8 Pt 1): 594–600.
17. Goutam, B., and Dilip, G., (2006). Progress in the research on naturally occurring flavones and flavonols: An overview. *Current Organic Chemistry* 10(8):873-898.
18. Griggs, B., (1981). *Green Pharmacy, a history of herbal medicine*; J.Norman & Hobhouse Ltd.; London.
19. Grover, J.K., Yadav, S.; and Vats, V.,(2002). Medicinal plants of India with antidiabetic potential. *J. Ethnopharmacology* 81:81–10.
20. Gupta. R., Gabrielsen, B., and Ferguson1, F.M., (2005). *Nature's Medicines: Traditional Knowledge and Intellectual Property Management. Case Studies from the National Institutes of Health (NIH), USA* *Current Drug Discovery Technologies* 2: 203-219.
21. Gupta SS. (1994), Prospects and perspectives of natural plant products in medicine. *Indian J Pharmacol*;26:1-12.
22. Gurib-Fakim, A., (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine* 27;1–93.
23. Handa SS, Khanuja SPS, Longo G, Rakesh DD, (2008). *Extraction Technologies for Medicinal and Aromatic Plants*. International centre for science and high technology, Trieste, 21-25.
24. Harbone, J. (1998). *Phytochemical methods: a guide to modern techniques of plant analysis*. Springer, Nature, 302 pages
25. Harborne, J.B., and Williams, C.A., (2001). Anthocyanins and other flavonoids. *Nat. Prod. Rep* 18:310-333.
26. Hassan, S.W., F.L. Bilbis, M.J. Ladan, R.A. Umar and S.M. Dangoggo et al., (2006). Evaluation of antifungal activity and phytochemical analysis of leaves, roots and stem barks extracts of *Calotropis procera* (*Asclepiadaceae*). *Pak. J. Biol. Sci.*, 9: 2624-2629.
27. Hassan, S.W., R.A. Umar, M. Lawal, L.S. Bilbis and B.Y. Muhammad, (2006). Evaluation of antifungal activity of *Ficus sycomorus* L. (*Moraceae*). *Biol. Environ. Sci. J. Tropics*, 3: 18-25.
28. Heinrich, M., Barnes, J., Gibbons, S., and Williamson, E.M., (2004). *Fundamentals of Pharmacognosy and Phytotherapy*. Churchill Livingstone, Elsevier Science Ltd., UK.
29. Hollman, P.C., and Katan, M.B., (1999). Health effects and bioavailability of dietary flavonols. *Free Radic Res* 31(Suppl):S75–S80.
30. Jadhav, B.K., Khandelwal, K.R., Ketkar, A.R., and Pisal, S.S., (2004). "Formulation and evaluation of mucoadhesive tablets containing eugenol for the treatment of periodontal diseases". *Drug Dev Ind Pharm.* 30 (2):195–203.
31. Jones, W.P., Kinghorn, A.D., (2005). Natural product isolation: Extraction of Plant Secondary Metabolites. *Methods in Biotechnology* 20:323-351.
32. Kalthor R. (1997). Contribution of Persian physician to development of Islamic medicine. *Med Arh*;51:9-12.
33. Kaul, T.N., Middletown, Jr. E., Ogra, P.L., (1985). Antiviral effects of flavonoids on human viruses. *Journal of Medical Virology* 15: 71-75.
34. Kinghorn, A.D., and Balandrin, M.F., (1993). *Human medical agents from plants*, American Chemical Society, San Francisco, USA.
35. Kong, J.M., Goh, N.K., Chia, L.S., and Chia, T.F., (2003). Recent advances in traditional plants drugs and orchids. *Acta Pharmacologia Scinc* 24: 7-21.
36. Kumar R, Sharma RJ, Bairwa K, Roy RK, Kumar A., (2010). Pharmacological review on natural antidiarrhoeal agents. *Der Pharma Chemica*; 2(2): 66-93.
37. Lapornik B, Prosek M, Wondra, A. G., (2005). Comparison of extracts prepared from plant by-products using different solvents and extraction time. *Journal of Food Engineering*; 71: 214–222.
38. Lesney, M.S., (2004). *Nature's Pharmaceuticals: Natural Products from Plants Remain at the Core of Modern Medicinal Chemistry*. *TCAW* 13(7):26-31.
39. Manach, C., Scalbert, A., Morand, C., Rémésy, C., and Jimenez, L., (2004). Polyphenols: food

- sources and bioavailability. *Am. J. Clin. Nutr.* 79:727-747.
40. Newman, D.J., Cragg, G.M., and Snader, K.M., (2003). Natural products as sources of newdrugs over the period 1981-2002. *Journal of Natural Products* 66: 1022-1032.
 41. Nikhal SB, Dambe PA, Ghongade DB, Goupale DC. (2010). Hydroalcoholic extraction of *Mangifera indica* (leaves) by Soxhletion. *International Journal of Pharmaceutical Sciences*; 2 (1): 30-32.
 42. Okigbo, R.N., and Mmeka, E.C., (2006) An appraisal of phytomedicine in Africa *KMITL Sci. Tech. J.* 6(2).
 43. Okwu DE (2001). Evaluation of the chemical composition of indigenous spices and flavouring Agents. *Global J. Pure Appl. Sci.* 7(3): 455- 459.
 44. Philipeon, J.D., (2001). Phytochemistry and medicinal plants. *Phytotherapy* 56:237-243.
 45. Pieta, P.G., (2000). Flavonoids as antioxidants. *Journal of Natural Products* 63:1035-1042.
 46. Rawat AK, Mehrotra S, Tripathi SC, Shome U., (1997). Hepatoprotective activity of *Boerhavia diffusa* L. roots a popular Indian ethnomedicine. *J Ethnopharmacol*; 56:61-6.
 47. Rimando, A. M., M. Olofsdotter, S.O.D., and Dayan, F.E., (2001). Searching for rice allelochemicals: an example of bioassay-guided isolation. *Agron. J* 93:16–20.
 48. Saad B, Azaizeh H, Said O., (2005). Tradition and perspectives of arab herbal medicine: A Review. *Evid Based Complementary Alternat Med*; 2:475-479.
 49. Satyajit, D., Sarker, Z., Latif, A., and Gray, I., (2006). Natural product isolation. Second edition, Humana Press Inc ISBN 1-59259-955-9.
 50. Scalbert, A., Johnson, I.T., and Saltmarsh, M., (2005). Polyphenols: antioxidants and beyond. *Am. J. Clin. Nutr* 81(suppl):215S-217S.
 51. Sherwood, L., (2007). *Human physiology: From cells to systems*, 6th edition, Thomson Brooks/Cole.UK. p 323.
 52. Starmans, D. A. J., and Nijhuis, H. H., (1996). Extraction of secondary metabolites from plant material: a review. *Trends Food Sci. Technol.* 7:191–197.
 53. Tapsell, L.C., (2006). "Health benefits of herbs and spices: the past, the present, the future". *Med J Aust* 1. PMID 17022438.
 54. Thomson, W.A.R., (1978). *Medicines from the Earth*. Maidenhead, United Kingdom: McGraw-Hill Book Co.
 55. Vaidya AB, Antarkar VDS., (1994). New drugs from medicinal plants: opportunities and approaches. *J Assoc Phys India*; 42:221-8.
 56. Valsaraj R, Pushpangadan P, Smitt UW. (1997). Antimicrobial screening of selected medicinal plants from India. *J Ethnopharmacol*;58:75-83.
 57. Valsaraj R, Pushpangandan P, Smitt UW. (1997). New anti- HIV-1, antimalarial, and antifungal compounds from *Terminalia bellerica*. *J Nat Prod*;60:739-42.
 58. Vaz J, Kulkarni C, David J, Joseph T., (1998). Influence of caffeine on pharmacokinetic profile of sodium valproate and carbamazepine in normal human volunteers. *Indian J Exp Biol*;36:112-4.
 59. WHO, (2001). *Legal status of Traditional Medicines and complementary/Alternative Medicine: A worldwide review*. WHO publishing 1.
 60. Williams, E.M., Okpato, D.T., Evans, F.J., (1996). Selection, preparation, and pharmacological evaluation of plant material.

Assessing the relation of selected mix marketing And brand equity of mobile phones among engineers society of consulting engineering company Mahab Ghods

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Abstract:As for the power that the brand equity gives to its brand, in this research we tried to assess how it develops. A questionnaire including 8 variables of brand building activities to create brand equity for mobile phones has been proposed. These variables are: Perceived quality, price, advertisement, awareness and association of brand, mental image of store, brand loyalty, spread of distribution and promotion cost. Based on the results, it is better to more concentrate on the elements which affect on brand equity development such as Perceived quality, brand loyalty, awareness and association of brand that are influenced by factors such as mental image of supplier stores, spread of distribution, advertisement and promotion costs. Based on the regression model and obtained Coefficients, we can conclude that among all the factors, perceived quality, advertisement respectively have the most effect on brand equity. Organizations by investing on these factors can achieve to more Competitive advantages.

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Keywords: market; mobile phone; engineer; society

1. Introduction

Brand building is one of the best ways of doing business. A successful brand building gives a powerful competitive position to the producers and increases the power of retailers. It can also act as a defensive factor to protect market share in front of competitors.

Brand equity and customer value communicate value to company through increasing effectiveness and efficiency of marketing programs, brand loyalty, price and profit margins, brand development, Commercial pyramids, profit advantage. Brand building activities (such as distribution, widespread advertisement, using stores with desirable image, high prices and not Using of price promotions) has been suggested for developing brand equity for mobile phones.

Purpose of this study is definition of empirical relations about interactions among Marketing mix elements and brand equity's components and its effects on brand equity. In view of the methodology of collecting information, the study is descriptive statistical. In this study, a questionnaire concluding 9 variables has been filled out by 176 persons of consulting engineering company of Mahab Ghods in spring of 2011. Among these variables according to the analytical model of the investigation, perceived quality, brand loyalty, awareness and association of brand are Intermediate variables, and brand equity is dependent variable and the other variables are independent variables.

1.1.Literature review

Brand equity is: "a set of capabilities and finance appendixes of a brand, a name or sign which increase or decrease the presented value of the product by its company or its customer." (acro viva kims taller, 2000, page 17)

Regularly brand equity in marketing texts is two type: 1) those which include customer perceptions (such as awareness and association of brand or perceived quality) 2) those which include customer behavior. (such as brand loyalty and concentration on differences of price.).

2.Brand equity

5 brand equity's assets are sources of value creation. As it has been showed in the picture 1, they are:

1. brand loyalty
2. brand Awareness
3. Perceived quality
4. consistent of brand plus perceived quality
5. The other assets such as: Patents, brands, distribution channel member relationships

Uoeand cohorts (2000) uses Confirm atory factor analysis methods to assay brand equity. Brand equity is considered as a 3 dimensional structure in which awareness and association of brand have been combined and merged in one dimension.

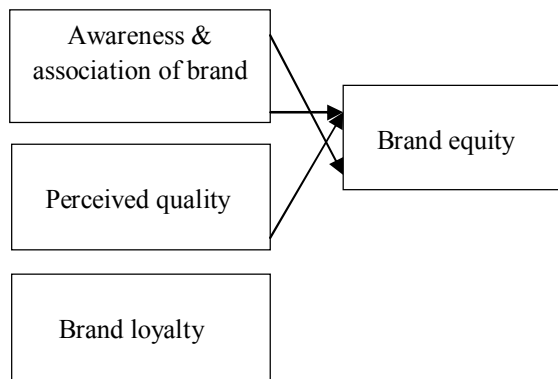


Image (1): brand equity's components

2.1. Brand Awareness

It refers to the power of a brand in consumer's mind and it is one of the primary and important part of the brand equity (Aaker, 1991, 140). Aaker has considered several levels for awareness of brand, from recognition of brand to dominance of brand, that the only brand which consumer remember is the desired one. Rossiter & Percy (1987, 56) have defined the awareness as a consumer's ability in recognition of brand while Aaker has defined it as a concept concluding both recognition and reminding of brand.

Brand association

Although a brand can have multiple sources of association Brand personality and organizational associations are 2 types of the most important brand associations which affect on brand equity (Aaker, 1991, p130; 1996b, p112). Brand personality is a fundamental factor of brand equity and is defined based on the characteristics which the brand has in the consumers' minds. In this study brand personality is considered as human characteristics related to brand (Aaker, 1997, p347). The concept that is well established in the marketing literature (Batra et al, 1993, p15; Biel, 1993, p70, Phau & Lau, 2000, p55).

2.2. Perceived quality

Is the other important dimension of brand equity (Aaker, 1991, p139) that is not actual quality of product and is just consumer's subjective evaluation (Zitn, 1988, p3). Right like brand associations, perceived quality is worthy for consumer because it gives a reason to consumer for shopping through differentiating the brand from the others. Zitn (1988, p3) define the perceived quality, "consumer's judgment about their preference and advantage of a product". specific experience in product, unique needs, consumption situations may be effective in consumer judging of the quality. a high perceived quality can be achieved during a

long-term experience of that brand hence the consumer can decide on distinctions and priorities of each brand.

2.3. Brand loyalty

Is the dependency of a consumer to a brand (Aaker, 1991, p39). consumer's loyalty to a brand causes that consumers buy a brand continually and resist the tendency to the other brands. Brand equity mainly occur through Brand loyalty.

3. Second-level factors

Second-level factors that affect the Brand equity are in the below:

3.1. Price

An amount that the customer is willing to pay to obtain a valuable product.

Usually considered to be based on the concepts of money, but it can be exchanged with any other valuable things.

3.2. Place (Also referred to as Distribution)

Where your business sells its products or services and how it gets those products or services to your customers. Due to the increasing intensity of competition, presenting Services and products in appropriate locations and the favorite locations for customers is a determining factor in attracting and keeping customers.

3.3. Advertisement

Means communicating message. It is introducing something into others or faking something is good or bad. it includes visual and verbal messages which is promoting an opinion with the product from a source through advertising channels to all the society and will spend money for it.

3.4. Price promotions

Whatever has value and is offered to customer in addition to the discount.

3.5. Image of store

Includes features such as physical environment, service levels and quality of goods. (Baker et al, 1994; Zimmer and Golden, 1988). Grewal and colleagues discovered that the image of the store has a huge amount of information in connection with the store environment, customer service, quality of product that gives to consumers And so it seems that the perceived quality of brand has a positive relationship with store image (Grewal et al., 1991).

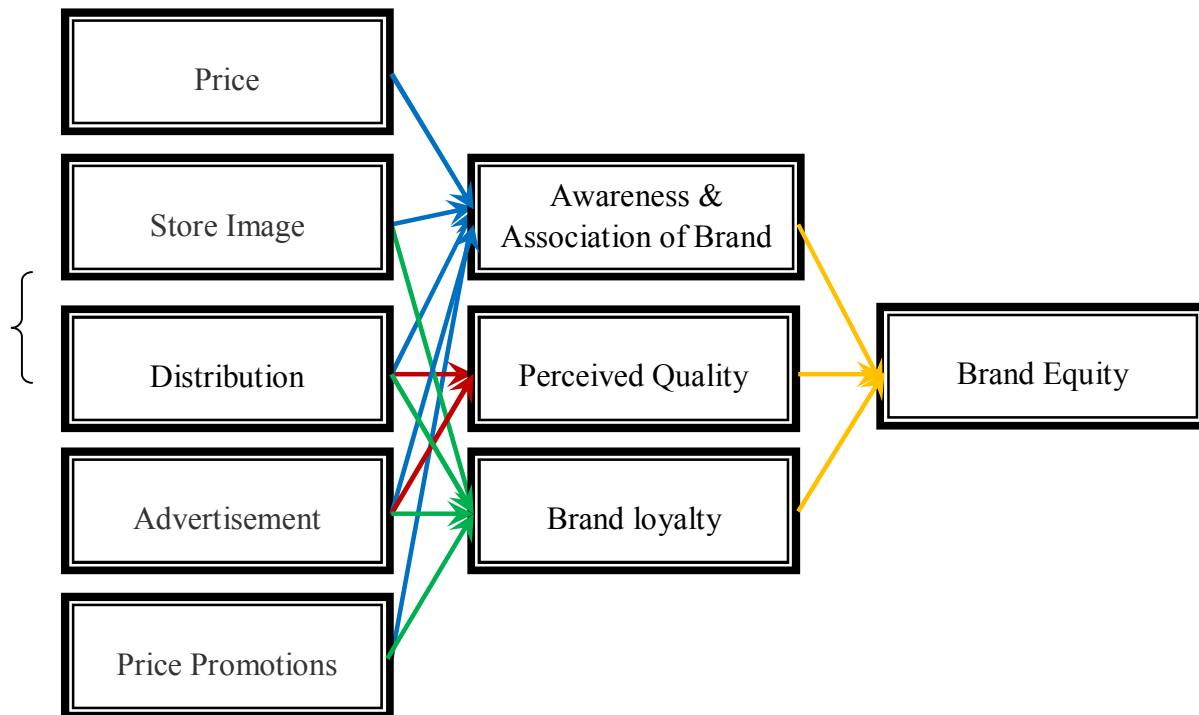


Image (2): Conceptual model for the study hypotheses

4. Research hypotheses

The model presented in the following research hypotheses are presented in two levels, like fig (2):

4.1. Methodology

For this study for Data collection about variables is used "field research" and "questionnaire". There are two types of survey questions in questionnaire:

- 1) general questions: including questions about demographic information and is generally
- 2) Technical questions: This section includes 42 questions that are designed on "5 point likert scale" .

4.2. Reliability and validity of the questionnaire

The reliability of this questionnaire is measured by "Cronbach's alpha" that is equivalent to 93%. On this basis we can say good reliability and is acceptable.

4.3. sampling

The study population consist of persons who have at least one trait that is distinctive others (Azar & ,Momeni:2005). to equality of opportunity for all people was used a "random sampling method". the statistical properties of the sample are as follows:

Location of study: Engineering Consultant Mahab

Time of study: Spring 1390

Sample size: 88 people

4.4. Statistics Analysis:

We use the method of analysis "Pearson correlation" to determine the relationships between variables and assumptions are presented and evaluated to determine the quantitative relationships between variables in the model. For this analysis:

$$H_0 : Sig > 0.05$$

$$H_1 : Sig < 0.05$$

H₀: There is no significant relationship between 2 variable

H₁: There is significant relationship between 2 variable

1. The relationship between quality and high price:

There is no significant relationship between quality and high price.

Table(1): The analysis of Pearson correlation between quality and Price

	Pearson Correlation	Sig. (2-tailed)
Price & Quality	0.038	0.614

2. The relationship between quality and store image:

There is a significant relationship between quality and store image.

Table(2): The analysis of Pearson correlation between quality and Store image

	Pearson Correlation	Sig. (2-tailed)
Quality & Store image	0.526	0.000

3. The relationship between quality and extent of distribution:

There is a significant relationship between quality and extension of distribution.

Table(3): The analysis of Pearson correlation between quality and distribution

	Pearson Correlation	Sig. (2-tailed)
Quality & distribution	0.35	0.000

4. The relationship between quality and advertising:

There is a significant relationship between quality and advertisement.

Table(4): The analysis of Pearson correlation between quality and Advertisement

	Pearson Correlation	Sig. (2-tailed)
Quality & Advertisement	0.167	0.027

5. The relationship between quality and Price Promotions :

There is a significant relationship between quality and Price Promotions.

Table(5): The analysis of Pearson correlation between quality and Price Promotions

	Pearson Correlation	Sig. (2-tailed)
Quality & Price Promotions	0.171	0.023

6. The relationship between loyalty and Price Promotions:

There is a significant relationship between brand loyalty and brand extension of its distribution.

Table(6): The analysis of Pearson correlation between Price Promotions and Distribution

	Pearson Correlation	Sig. (2-tailed)
Distribution & Price Promotions	0.291	0.000

7. The relationship between loyalty and advertisement

There is a significant relationship between loyalty and advertisement.

Table(7): The analysis of Pearson correlation between Loyalty and Advertisement

	Pearson Correlation	Sig. (2-tailed)
Loyalty & Advertisement	0.242	0.001

8. The relationship between awareness and store image:

There is a significant relationship between awareness and image of store:

Table(8): The analysis of Pearson correlation between Store image And Awareness

	Pearson Correlation	Sig. (2-tailed)
Store image & Awareness	0.445	0.000

9. The relationship between awareness and distribution:

There is a significant relationship between awareness and distribution

Table(9): The analysis of Pearson correlation between Distribution And Awareness

	Pearson Correlation	Sig. (2-tailed)
Distribution & Awareness	0.346	0.000

10. The relationship between awareness and Advertisement:

There is a significant relationship between awareness and Advertising.

Table(10): The analysis of Pearson correlation between Advertisement And Awareness

	Pearson Correlation	Sig. (2-tailed)
Advertisement & Awareness	0.28	0.000

11. The relationship between awareness and price promotions :

There is a significant relationship between awareness and Price Promotions.

Table(11): The analysis of Pearson correlation between Awareness and Price Promotions

	Pearson Correlation	Sig. (2-tailed)
Awareness & Price Promotions	0.251	0.011

12. The relationship between brand equity and Price Promotions :

There is a significant relationship between perceived quality of brand equity

Table(12): The analysis of Pearson correlation between Price Promotions And Brand Equity

	Pearson Correlation	Sig. (2-tailed)
Price Promotions & Brand Equity	0.402	0.000

13. The relationship between brand equity and loyalty:

There is a significant relationship between brand equity and loyalty.

Table(13): The analysis of Pearson correlation between Brand Equity And Loyalty

	Pearson Correlation	Sig. (2-tailed)
Brand Equity & Loyalty	0.356	0.000

14. The relationship between brand equity and awareness:

There is a significant relationship between brand equity and awareness.

Table(14): The analysis of Pearson correlation between Brand Equity And Awareness

	Pearson Correlation	Sig. (2-tailed)
Brand Equity Awareness	0.429	0.000

15. Relationship between brand equity and prices:

There is no significant relationship between brand equity and prices.

Table(15): The analysis of Pearson correlation between Brand Equity And Price

	Pearson Correlation	Sig. (2-tailed)
Brand Equity & Price	0.024	0.000

16. The relationship between brand equity and store image:

There is a significant relationship between brand equity and store image.

Table(16): The analysis of Pearson correlation between Brand Equity And Store image

	Pearson Correlation	Sig. (2-tailed)
Brand Equity & Store image		0.000

17. The relationship between brand equity and distribution :

There is a significant relationship between brand equity and Distribution.

Table(17): The analysis of Pearson correlation between Distribution and Brand Equity

	Pearson Correlation	Sig. (2-tailed)
Distribution & Brand Equity	0.37	0.000

18. The relationship between brand equity with price promotions:

There is a significant relationship between brand equity and price promotions.

Table(18): The analysis of Pearson correlation between Price Promotions and Brand Equity

	Pearson Correlation	Sig. (2-tailed)
Price Promotions & Brand Equity	0.402	0.000

Table(19): Regression Coefficients for Dependent Variable: Brand Equity

Model	Unstandardized Coefficients		Standardized Coefficients	T	Sig.
	B	Std. Error			
(Constant)	-.344	.450		-.764	.046
Quality	.246	.118	.184	2.087	.038
Loyalty	.106	.110	.083	.967	.035
Awareness	.138	.106	.119	1.296	.047
Price	.070	.073	.068	.964	.037
Storeimage	.154	.081	.056	.673	.042
Distribution	.052	.073	.053	.704	.042
Advertisement	.227	.092	.194	2.465	.015
Price Promotions	.222	.075	.213	2.976	.003

$Brand\ Equity = (.246) * Quality + (.106) * Loyalty + (.138) * Awareness + (.070) * Price + (.154) * Storeimage + (.052) * Distribution + (.227) * Advertisement + (.222) * Price\ Promotions - .344$

Regression

In this regression, the dependent variable "brand equity" and the independent variables are: "Price, Photo Shop, Perceived quality, brand image, widespread distribution, advertising, promotion and awareness of the brand price." Based on the last column, Sig, are smaller than 0.05. As can be seen in the table of regression coefficients, can be concluded that the regression equation between the dependent variable and independent variables into a few shows, is expressed as follows The coefficients obtained, we can conclude that the obtained dimensions, components, quality, advertising, and by definition, have the greatest impact on brand equity and organizations can invest more on these components and to try to gain competitive advantage for its brand equity.

Conclusions

The present study found no significant relationship between high prices and customer

perceived quality. Vbalks direct correlation was found between the image catalog and wide distribution, and 3 Vtrfyat price factors of perceived quality, brand loyalty and brand awareness and brand equity associations. . The most effective agent "of the brand awareness and brand association" with the correlation coefficient is 0.429. Confirmed that the store image, perceived positive impact on quality is widely distributed and Price Promotions. And also the extent of advertising and distribution of three variables on perceived quality, brand loyalty and brand awareness and brand associations influence.

References:

1. Aaker,D.A.(2004), Brand Porfolio Strategy. Creating Relevance, Differentiation, Ennergy, Leverage and Clarity. NewYork, Free Press.
2. Aaker,D.A.(2004),"Leveraging the corporate brand" California Management Review,46(3),6-18
3. Aaker, D.A. and Joachimsthaler, E.(2000), Brand Leadership, London, Free Press
4. Azar,A, Momeni(2005)," Statistics and Its Application in Management", V.1 ,10 th printing, SAMT publication, p 5
5. Azar,A, Momeni(2006)," Statistics and Its Application in Management", V.2, 10th printing, SAMT publication
6. Baldauf,A.,Cravens,K.S.andBinder,G.(2003),"performanc e consequences of brand equity management: evidence from organizations in the value chain", Journal of Product & Brand management, Vol.12No.4, pp.220-236.
7. Balmer, John, M.T, and Gray,E.R.(2003) ,"Corporate Brands: What Are They?What of them?,"European Journal of Marketing,37(7/8),972-997
8. Davis,S.M. and Dunn, M. (2002), Building the Brand-Driven Buisness: Operationalize Your Brand to Drive Profitable Growth,SanFrancisco,josey Bass.
9. Davis,S.M.(202), Brand Asset Management:Driving Profitable Growth through Your Brands, SanFrancisco, josey Bass.
10. De chernatony, L.(1999), "Brand management Through Narrowing the Gap Between Brand Identity and Brand Reputation",Journal of Marketing Management, 15,157-179.
11. DelaVar,A, (2002), "Theoretical and scientific research in the social sciences", growth Publications of, Tehran
12. Doyle, P. (2001a), "Shareholder-value-based Brand Strategies", Brand Management,9(1),20-30.
13. Hatch, M.J, and Schultz, M. (2003), "Bringing the Corporation into corporate Branding ", European Journal of marketing, 37(7/8),1041-1064.
14. Kapferer, J.N (1997), "Strategic Brand Management "Great Britain, Kogan page
15. Kayaman, R and Arasi, H.(2007), "Customer based equity: evidence from the hotel industry",managing Service Quality, Vol.17No.1,2007pp.92-109
16. Kim,H.B. and Kim, W,G, (20050, "The relationship between brand equity and firms' performance in luxury hotels and chain restaurants", Tourism Management 26,549-560
17. King, S.(1991), "Brand Building in the 1990's".Journal of Marketing Management ,7(1),3-13.
18. Knox,S.andBickerton,D.(2003),"The Six Conventions of Corporate Branding", European Journal of Marketing, 37(7/8), 998-1016
19. Konecnik, M., and Gartner, W.C., (2007), "Customer-Based Brand Equity For A Destination ",Annals of Tourism Research,Vol.34,No. 2,pp.400-421
20. Logman,M.(2004)," The LOGMAN Model:A Logical Brand Management Model", Journal of Product and Brand Management, 13(2),94-104.
21. Mcwilliam,G and Dumas ,A.(1997),"Using Metaphors in New Brand Design", Journal of Marketing Management, 13,265-284.
22. Netemeyera,R.G.,Krishnanb,B.,Pulliga,C.,Wangc,G.,Yag cid,M., Deane,D., Ricksf,J. and Wirthg,F. (2004), "Developing and validating measures of facets of customer-based brand equity", Journal of Business Research,Vol.48 No.1,pp.209-224.
23. Pappu,R.,Qeester,P.G,Cooksey,R.W.,(2005),"consumer-based brand Equity: improving the measurement – empirical evidence",Journal of Product & Brand Management,14/3,143-154
24. Rahj,E.,Vranesevic,T.andTolic,D.,(2003),"creation food induestry-brand equity in selected product categories", British Food Journal, Vol.105 No.4/5, pp.263-73.
25. Sakaran,U, (2004), "Research in Management", translated by Mohammad Saebi & shirazy, first printing, Center for Governmental Management. Tehran.
26. Sarmad,Z, Bazargan,A, Hejazy,E., (2005), "Research methods in behavioral sciences", fifth printing, Agah Publication, Tehran.
27. Speak, K.D. (1998), "Brand Stewardship", Design management journal,Winter,32-37.
28. Urde,M.(1999),"Brand Orientation:A Mindset for Building Brands into Strategic Resources"„Journal of Marketing Management,15,117-133.
29. Urde,M.(2003),"Core Value-Based Corporate Brand Building",European Journal of Marketing, 37 (7/8), 1017-1040.
30. Yasin,N.M,Noor,M.N,mohamad,O.(2007)."Does image of country-of-origin matter to brand equity?", Journal of Product & Brand Management, 16/1 (2007) 38-48.
31. Yoo, B. and Donthu, N(2002), "Testing cross-cultural invariance of the brand equity creation process", Journal of Product & Brand Management, Vol.11 No.6, pp.380-98.

1/21/2012

An innovative algorithm for code Obfuscation

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Abstract: The code obfuscation is a process of transforming any program into an incomprehensible form for protecting it from malicious attempts. To achieve this objective, many algorithms are found in the literature. Some of them based on program instructions reordering and block reordering which are difficult to implement as well as resource requirements are very high. In addition, some associated constructs are needed to run such applications and hence demand more user expertise. In this research paper, we propose a new user friendly obfuscation algorithm based on insertion of zero impact instructions and additional code insertion. Obfuscation can be carried out of any code however we choose assembly language programs as reverse engineers always translate high level language codes to it for stealing the intellectual properties. The algorithm is implemented in Microsoft visual basic for Intel machines.

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Keywords: Reverse engineering; Source code; Structural complexity; Insertion; Compilation; Obfuscation.

1. Introduction

The need of the safety of intellectual property of software developers has become clear in current years of rapid development of multimedia technologies. Now a days it has become difficult from attacker's perspective to understand the source code due to its availability in binary formats, however the reverse engineering process has made the attackers to understand the correct behavior of the software and to take out the actual logic out of the program [14], so code obfuscation came into being and this is a technique which employed to reduce the risk of the theft of this intellectual property. This research paper focuses on the methodology proposed for obfuscation. Reverse engineering is a mechanism which prevents the implementation of piracy prevention methodology. This technique actually allows the user to by pass the code detecting key and starts from the process of disassembling the code written in any language. After extracting the logic, de-compilation procedure is applied and high level abstraction from this assembly code is found. Most of the research on code obfuscation has been fixed on perplexing this de-compilation phase. In contrast of focusing on this disassembly stage our goal is to perturb the disassembly procedure to make the program harder to disassembly. Results obtained by majorly reverse engineering tools being used portray the effectiveness of our method.

Compilation is the process of translating a source code written in any language to machine code. This process consists of series of steps; each step

produces some low level representation than upper level step. Reverse engineering is a dual process of recovering high level structure and semantics from a machine code program. The compilation and reverse engineering processes are shown in Fig 1.

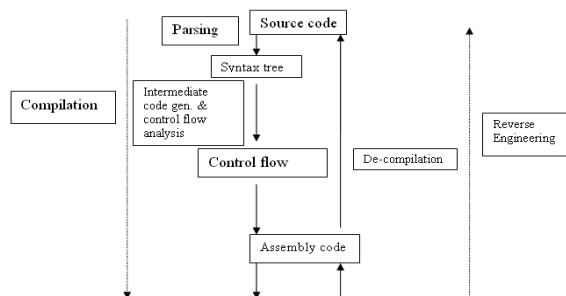


Figure 1. The Processes of Compilation and Reverse Engineering

The whole reverse engineering process can be divided into two parts [2].

- (i) Disassembly: which produces assembly code from machine code
- (ii) De-compilation: which reconstruct the high level semantic structure of the program from the assembly code?[5]

The most of prior work regarding code obfuscation was focused on different aspects of de-compilation; our goal in this paper is to increase the difficulty level of disassembling the program [2]

2. Techniques being used for software code protection

There are numerous publications on software obfuscation and their accomplishment. A complete nomenclature of obfuscating transformations was introduced in 1997 by Collberg et al. [8]. To gauge the consequence of an obfuscating transformation, Collberg defined three metrics: cost, potency, and resilience. Software complexity metrics ([1, 6, 11, 8, 21, 13, 1]), which were designed to decrease the complexity of the software, can be used to assess this in spite of subjective metric. In compare to potency that gauges the power of the obfuscating transformation in opposition to humans, flexibility defines how beautifully it withstands an assault of an automatic de-obfuscator. This metric technique evaluates both the programmer effort (that is much effort was required to develop a de-obfuscator) and the de-obfuscator attempt (the try of space and time necessary for the de-obfuscator to run). An ideal obfuscating transformation has high strength and resilience values, but small costs in terms of extra memory usage as well as greater than before execution time. In practice, a trade-off among potency, resilience and costs has to be compromised.

Preventing disassembling is almost impossible in situations where attackers have complete control over the host containing this software; the ordinary solution is to make the effect of disassembling valueless for additional static analysis by preventing the retransformation of control flow graph. To the end of this [6] and [5] use so-called branch functions to conceal the target of CALL instructions: The explained methods substitute CALL instructions with jumps (JMP) to a general function (branch function), which function is to call is decided on run time. Under the supposition of a static analyzer being the branching function is a black box, the call objective is not exposed until the real execution of the code. This successfully prevents the control flow graph being rebuilding using static analysis. Nevertheless, the idea of a branching function does not defend against dynamic examination. An attacker can run the software on a variety of inputs and watch its performance. Medou et al. [13] argues that newly anticipated software protection models would not survive attacks that unite static and dynamic examination techniques. Even now, Dynamic analysis can be made harder using code obfuscation.

One more approach to watch cryptographic keys embedded within software is the utilization of White-Box Cryptography (WBC), which attempts to build a decryption methods that becomes challenging

against white-box" attacker, who is smart enough to monitor each step of the decryption method. In WBC, the code is implemented as a arbitrary system which is dependent on any key for lookup tables. The implementation of white-box DES was firstly brought into the scene by Chow et al. [7]. On the Basis of this approach, further AES and DES white-box implementations have been suggested, but all have been broken. Billet et al. [4], Wyseur et al. [4], Jakob et al. [14], and Michiels Gorissen [4] introduced a method of white-box used cryptographic method which is able to make any software resistant against any attempt of tempering it. In this approach, the software code which is purely executable is used for the white-box lookup table for the purpose of cryptographic key. If the code has been changed it would message as an unacceptable key. On the other hand, owing to the lack of safe WBC implementations, the safety and security of the construction is ambiguous. Hardware-based methods will permit to shield the real execution of Program from the attacker completely. However, this merely moves hits to the hardware tamper resistance, while challenges like support difficulty for inheritance systems and elevated costs are rising. For that reason, hardware-based code protection techniques are obsolete

2.1 Research Challenges

Collberg's and Chenxi Wang's Algorithms are extensively used in the techniques of obfuscation. Due to the inclusion of some high level constructs, building of secondary structures, Inclusion of classic procedures as input and requirement of additional sources to make it user friendly, these algorithms are hard to implement and problems are solved theoretically. Implementation of the proposed algorithm using IZII and ACI techniques in the form of user friendly software which has not been implemented up till now is the objective of the paper as available literature presents theoretical solutions using IR and BR techniques which need some associated constructs to be implemented which makes the program very hard to compile. Designed software's simplicity is unique in a way it takes an assembly language and on a single click converts it into an obfuscated program completely different from original one and un-understandable as far as its logics are concerned. Furthermore obfuscated programs get compiled with the production of same out put as was before with out any problem. At the end the comparison of complexity measures of theoretical solutions discussed above and our implemented solution is given in tabular form.

3. Code Obfuscation Techniques

We have two types of methods to protect the software property (I) legal and (II) technical [12]. Legal methods include all possible laws which are being acting against illegal users and retailers making them face some legal actions in the form of fine and punishment, where as technical methods are Server-side execution, Code authentication, Encryption and Obfuscation. Here in this research paper we focuses only on the last method i.e. code obfuscation. Before going into the detail of the obfuscation method, we preceded our research on the basis of following hypothesis

1. In the complexity point of view machine code is more suitable than any high level language code.
2. In quality perspective, the algorithm is not bad than any so far proposed algorithms.

To prove these hypotheses we developed very effective obfuscation method for machine code. In our research work low level of programming was chosen because of the following reasons.

- The analysis of the machine code is harder than the code written in HLL.
- Decomposition of the machine code becomes impossible due to some inherited properties in compiled code from high level language [12].
- Obfuscation algorithm becomes very simple due to easier parsing methods in machine code.
- Investigation of machine code obfuscation was not found in reported literature.

Actualization of the main goal was decomposed into the following sub tasks.

- I. Specific analytical methods for measuring the complexity of the programs were adopted.
- II. Some empirical methods for measuring the obfuscating method's efficiency were worked out.
- III. A background for obfuscation algorithm for machine code was created.
- IV. An efficient method for obfuscation of machine level code was developed.
- V. This developed method was implemented for most renowned architecture.
- VI. After performing some measurements and experiment some appropriate and valuable conclusions were drawn.

Our approach is the combination of the obfuscation techniques working for both dynamical and static reverse engineering. Where static reverse engineering is the process of reverse engineering any

software automatically with out executing it actually. Machine code can be translated into assembly language by an attacker using a disassembler and the control flow graph could be redesigned with out execution of the code. We can make reconstruction of static flow more difficult by the insertion of indirect jumps that hides the details of jump target and the utilization of branching functions. A universal method for creating an algorithm of obfuscation working in machine level code can be designed by knowing two fundamental elements: Obfuscation transformation and the results of research in the structure of a specific program. Using this methodology an algorithm of obfuscation was designed and then implemented. Following four activities were under our discussion while proposing our new methodology.

- Program's instructions reordering (IR).
- Blocks reordering of the program (BR).
- Insertion of zero impact instructions (IZII).
- Additional code insertion (ACI).

All above activities would be independent of each other, which means a different result would be produced by changing the order of execution. On the basis of methods given above a sample algorithm is designed which works on Intel* 8086 machines. From above given methods only two IIZI and ACI methods were selected for implementation because these methods have great influence on the quality of the obfuscation [11].

3.1 Structure of the Algorithm

To initialize the code obfuscation algorithm following steps are followed:

- All global objects are assigned some starting values.
- Some additional local variables are added into the source code and base addresses are found using assembly language instructions.
- Memory is allocated to obfuscated program
- The process of data flow analysis is launched
- Main loop of the program as shown in Fig 3 is started.

As stated above that this research paper focuses only on the implementation of two techniques used for obfuscation i.e IZII and ACI. Detail of these is given below.

3.1.1 Insertion of zero impact instructions (IZII)

Some instructions whose over all result is zero are embedded in the program at particular place. Zero impact instructions are of following three types.

3.1.1.1 Jumps of over all zero effects

At particular location within the program is searched out and a set of jump instructions which ultimately brings the instruction pointer at the point where it was before the insertion are inserted. This makes the program lengthy and hard to understand. For example.

```

mov ecx, 5
    cmp eax, ebx
    jg greater
    jl less
    mov edx, 2
greater:
    mov edx, 2
less:
    mov edx, 2
    sub edx, 10
    
```

3.1.1.2 Insertion of free elements

Free elements means any register whose value does not effect the over all result of the program, For example **add. W d3,d2** as d3,d2 do not have any significance in the program. This free element is made independent of all dependencies between last inserted instructions and the current instruction and then instruction is added to the program.

3.1.1.3 Insertion of opaque constructs

Opaque constructs are the set of instructions which are not clear to understand. For example

Funcion	Ranges of parameters
Func1(a)	a=1,2,...,255 b = 0,1,...,5
Func2(a,b,c)	a = 1,2 b = 32, 33,...,127
Func3(a,b,c)	a = 0,2, 3, 6

Adding few global variables aa, bb, cc, we can make new opaque constructs. Into the function Func2 (a,b,c) we can insert for an example the expression bb = (a + b + c) AND bb, which value will be always less than 100000. A not used element and a place of jump from opaque construct are chosen and after drawing the opaque construct type instructions are inserted.

3.1.2 Additional code insertion (ACI)

3.1.2.1 Insertion of reversible operations

Reversible operation is one that gives the value as was before for example following two instructions are reversible:

AX = BX-5

AX= BX+5

A used element and an operation to be inserted are picked up. All dependencies on used elements get cleared and the set of instructions performing some reversible operation is inserted.

3.1.2.2 Insertion of meaningless code

Meaningless means the insertion of those registers and flags which are not used in the program. A block of code that has no meaning is added at particular location these meaningless codes may be a string, array or declaration of some not used variables. This meaningless code reduces the readability of the original code. Locations where all above insertions are made are found by our software. Original assembly program is read from top to bottom and places are found where IZII and ACI are implemented. These places are fixed on the following parameters.

- Where the loop is being started
- Where loop is being terminated
- Where mathematical operation appears
- Look for not used registers
- Where move operation is being performed
- Where an array is declared

The architecture of main loop of the algorithm is given below in Fig 3.

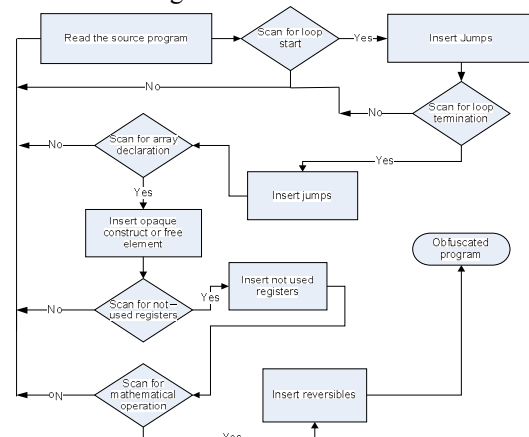


Figure 3: The main architecture of the algorithm for code obfuscation

4. Implementation of the Algorithm

The implementation phase of our research is the most important phase which we have contributed in present research. To get good performance and efficiency we designed proper data structure to store all information needed to run the program and by use of .NET technology we implemented above mentioned algorithm. Screen short of our obfuscated machine is given in Fig 4. Left hand side is the given source file and on the right hand side the program has

been obfuscated which is larger in size, difficult to understand but same in functionality.

The complete structure of program of code obfuscation consists on following six modules:

Step1. Loading of the source program- Program is loaded into the obfuscator

Step2. Basic analysis of the program - Investigation of the number of local variables and all parameters along with the jump addresses.

Step3. Data flow analysis - calculation of physical address of every instruction using pointers in the program and assembly language is very rich to provide physical address of each instruction..

Step4. Optimization of data flow - Calculation of loop holes in the context.

Step5. Obfuscation of the program – By scanning (reading each instruction and deciding the type of insertion) whole program from top to bottom an obfuscated program is obtained.

Step6. Saving of the obfuscated program – All transformations of the program is saved.

The basic thing which has been processed in this algorithm is an instruction. As obfuscated and source program are stored in the form of array structures so it is also possible to obfuscate any already obfuscated program and this is called iterative method of obfuscation[11].

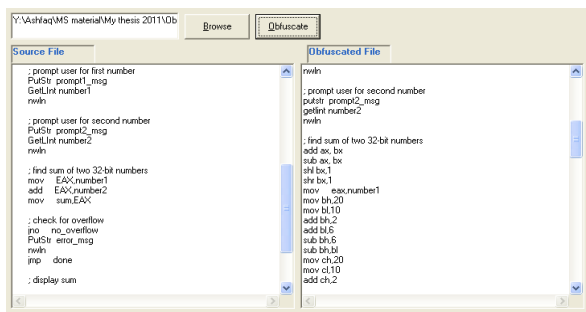


Figure 4 Screen shot of obfuscating machine

5. Results discussion

The most important parameter of obfuscating algorithm is its efficiency which reflects the power of the obfuscation process on the capacity to read the exact meaning of obfuscated program. Efficiency of obfuscation is directly proportional to the average complexity of the program Complexity as defined by Basili, is a measure of the resources expended by a system while interacting with a piece of software to perform a given task [15]. If the interacting system is a computer, then complexity is defined by the execution time and storage required to perform the computation. If the interacting system is a programmer, then complexity is defined by the difficulty of performing tasks such as coding, debugging, testing, or modifying the software. The

term software complexity is often applied to the interaction between a program and a programmer working on some programming task. For practical tests and research we have taken 5 sample programs of various tasks and obfuscated process was applied on them then the efficiency along with different parameters is measured. Detailed result discussion of all these programs is given below.

All given test programs were obfuscated with the methodology proposed by us and an analysis of obfuscated and un-obfuscated programs are made on the basis of following three parameters.

- (i) Resources used by the computer for an un-obfuscated and an obfuscated program.
- (ii) Efforts put by human to extract the logic from an un-obfuscated and an obfuscated program.
- (iii) Structure of an obfuscated and an un-obfuscated program.

Comparative study of the resources used by the computer to run and store an obfuscated and an un-obfuscated program is carried out and the results are accumulated in table 5.1. Execution time- Calculated by C++ function **ibmts_calcTimeStamp ()**. Storage is the space taken by the program on hard disk.

Program	Un-obfuscated program		Obfuscated program	
	Execution time (microseconds)	Storage Kb	Execution time (microseconds)	Storage Kb
Bubble sort	8889	4	9015	5.1
Sum Array	538	6.2	635	6.9
Sum of col and rows	745	5.4	810	6.1
Transpose of matrix	3548	3.9	3612	4.9
Array search	7345	4.7	7502	6.2

Table 5.1 Comparison of resources used by computer

Space taken by obfuscated program is enhanced due to the fact of addition of some extra code. Execution time remains almost same with slight difference which proves that our method of obfuscation effect no more on execution time and the performance of the obfuscated program remain unaffected. A tentative comparison of different people who try to extract the logic of obfuscated and un-obfuscated programs is given in table 5.2.

Table 5.2: An average time taken by different people to extract the logic of the program

Program	Un-obfuscated program			Obfuscated program		
	Student (h)	Engineer (h)	Cracker (h)	Student (h)	Engineer (h)	Cracker (h)
Bubble sort	0.21	0.18	0.11	3.13	2.30	2.01
Sum Array	0.29	0.19	0.12	3.43	3.12	2.10
Sum of col and rows	0.39	0.25	0.17	4.11	2.37	1.46
Transpose of matrix	0.40	0.23	0.10	4.27	3.31	2.51
Array search	0.32	0.17	0.06	2.46	1.19	0.47

It is obvious from this table that obfuscated program becomes harder to the people of different walks of life for extracting the logic hidden in the program and obfuscated program becomes less readable. Third parameter on the basis of which our obfuscator was tested is structural complexity. It is the measure of length E_L and flow E_F .

Measure of length E_L (See figure 1) describes specific length of program P containing N instructions, considers also number of arguments in instructions, according to the formula given below.

$$E_L(\tau, P) = \sum_{i=1}^N C_i d_{lac} = \begin{cases} 0 & \text{When instruction has no arguments} \\ 0.5 & \text{When instruction has one argument} \\ 1 & \text{When instruction has two arguments} \end{cases} \quad (1)$$

Values of C_i were selected empirically, starting from the rule that value 1 corresponds to instructions which have most often occurring number of arguments. Remaining values were selected in a way creating diversified values of measure E_L for selected test programs.

Measure of flow E_F (see Equation 2) is a rational number, describing the average number of references to local memory in basic block of program by the formula given below. Basic block is defined as continuous sequence of instructions lying between two nodes of control flow graph.

$$E_F(\tau, P) = \frac{1}{M} \sum_{i=1}^M a_i \quad (2)$$

Where M is number of basic block in program, a_i is number of references to local memory in block i . A comparison of structural complexity of obfuscated and un-obfuscated programs is given in table 5.3.

Table 5.3: Values of Structural complexity measures

Program	Un-obfuscated program		Obfuscated program	
	E_L	E_F	E_L	E_F
Bubble sort	0.67	7	0.88	3
Sum Array	0.70	3	0.79	1
Sum of col and rows	0.58	10	0.87	6
Transpose of matrix	0.66	17	0.78	11
Array search	0.75	6	0.91	4

After obfuscation, value of E_L is increased which shows that the program's length is increased after obfuscation making it difficult to understand and decreased value of logic flow E_F indicates that logic of the program becomes harder for reader to understand

6. Conclusion and future work

Implementation of our proposed algorithm for Intel architecture. The proposed approach looks very promising in the following areas of comparison.

- scalability - Describe the controllability of an

obfuscation process by user

- flexibility - How easy is to use an implemented algorithm in different development environment or programming language
- portability - It describes easiness to transfer an implemented algorithm from one machine to another
- The low complexity of our algorithm is due to easiness of semantic analysis of machine languages and simplicity of implementation of data flow analysis on the low level of programming: Other available algorithms are not very portable, use very specific opaque constructs

Our proposed algorithm allows obfuscating already obfuscated programs. Programs obfuscated in such a way will have significantly different control flow graph (in comparison to programs obfuscated one time only), in the way dependent on the kind of inserted opaque constructs. The main drawback of all developed algorithms of obfuscation so far is the fact, that they remove all effects of code optimization, done by compilers. In modern processor it causes very often breaking of data processing stream, which slows down execution. That's way critical loops and highly optimized fragments should not be obfuscated. Our method of program code obfuscation is very general - it does not depend on specific properties of any computer architecture, but to general idea of context and instruction only. To convert an implemented algorithm for a new machine, it is only required to handle the specifications of its architecture (like special instructions set).

It can be seen that efficient obfuscation is also possible with low-level approach. Using the results from empirical research, we estimated parameters of obfuscation required to obtain well protected software. The main aim of the work, production of efficient algorithm of code obfuscation on the assembler level, simpler than algorithms making full analysis of structures of programs written in high-level languages, was made with satisfying conditions. Our obfuscator was tested on three parameters resources used by computer, human efforts, and structural complexity and found our algorithm efficient and very complex for reverse engineering.

Our main contribution is the implementation of the proposed algorithm using IZII and ACI in the form of user friendly software which has not been implemented up till now. Whole literature available provides only theoretical solutions. Software designed by us is so simple in use that it takes an assembly language file and on a single click converts it into an obfuscated program completely different from original one and un-understandable as far as its logics are concerned. Screen shot given in Figur4 is

the demonstration of whole software. Left hand side is the source file and on the right hand side is the program that has been obfuscated.

Obfuscation of high level language may be carried out by finding more cut off points where insertions could be made. Furthermore obfuscation process could be made intelligent by merging obfuscation of all languages in single software and allowing it to select obfuscating technique automatically.

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References

1. Basili, V.R. Qualitative software complexity models: A summary. In Tutorial on Models and Methods for Software Management and Engineering. IEEE Computer Society Press, Los Alamitos, Calif, 1980.
2. Alfred V. Aho, Ravi Sethi, Jeffrey D. Ullman, Compilers: Principles, Techniques, and Tools, Addison-Wesley Publishing Company, 2001.
3. F... Allen, Control flow analysis, SIGPLAN Notices 5(7):1-19, July 1970.
4. B.S. Baker, n algorithm for structuring flowgraphs, Journal of the ACM, 24(1):98-120, January 1997.
5. Ooaz Barak, Oded Goldreich, Russell Impagliazzo, Steven Rudich, Amit Sahai, Salil Vad-han, Ke Yang, On the (Impossibility of Obfuscating Programs, Advances in Cryptology — CRYPTO'01, Springer Lecture Notes in Computer Science vol 2139, pp 1-18, Santa Barbara, CA, November 2001.
6. V. Basili, D. Hutchens, An Empirical Study of a Complexity Family, IEEE Transactions on Software Engineering, Volume 9, No 6, November 1983, pp 664-672
7. Rex Jaeschke, Encrypting C source for distribution, Journal of C Language Translation, 2(1), 2005.
8. Christian Collberg, Clark Thomborson, Watermarking, Tamper-Proofing, and Obfuscation -Tools for Software Protection, Technical Report #170, Department of Computer Science, The University of Auckland; also: Technical Report 2000-03, Department of Computer Science, University of Arizona (2000)
9. Christian Collberg, Clark Thomborson, Software Watermarking: Models and Dynamic Embeddings, Technical Report, Department of Computer Science, The University of Auckland (1998)
10. Christian Collberg, Clark Thomborson, On the Limits of Software Watermarking, Technical Report #164, Department of Computer Science, The University of Auckland (2000)
11. Christian Collberg, Clark Thomborson, Douglas Low, Breaking Abstractions and Unstructuring Data Structures, IEEE International Conference on Computer Languages, ICCL'98, Chicago, IL, May 1998
12. Christian Collberg, Clark Thomborson, Douglas Low, Manufacturing Cheap, Resilient, and Stealthy Opaque Constructs, SIGPLAN-SIGACT POPL'98, ACM Press, San Diego, CA, January 2003
13. Christian Collberg, Clark Thomborson, Douglas Low, Taxonomy of Obfuscating Transformations, Technical Report #148, Department of Computer Science, The University of Auckland, 2006
14. Frederick B. Cohen, Operating System Protection through Program Evolution, 1992
15. Cristina Cifuentes, Doug Simon, Antoine Fraboulet, ssembly to High Level Language Translation, Technical Report 439, Department of Computer Science and Electrical Engineering, The University of Queensland, August 1998.

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E-Hafiz: Intelligent System to Help Muslims in Recitation and Memorization of QuranAslam Muhammad¹, Zia ul Qayyum², Waqar Mirza M.³ Saad Tanveer⁴, Martinez-Enriquez A.M.⁵, Afraz Z. Syed⁶^{1,3,4,6} Dept. of CS & E, University of Engineering & Technology, Lahore⁴Department of Computing and Technology, IQRA University, Islamabad, Pakistan⁵Dept. of CS, CINVESTAV-IPN, D.F. Mexico(Corresponding author email: maslam@uet.edu.pk)

Abstract: Recitation of the Holy book of Muslims, The Holy Quran, is a regions duty and hence is done with utmost care such that no mistakes are made while reading it. These mistakes may include the wrong utterance of words, misreading words, and punctuation and pronunciation mistakes. Believers of Islam are spread all over the world and hence there also can be a difference in accent. To avoid this, tajweed rules are implemented to ensure that the utterance is done according to some rules. These rules ensure that there is no variance in the recitation of the Holy book for different reciters. For further improvements, the people are encouraged to memorize the whole book and the person who does that is called a Hafiz. Having known the whole book by heart down to every word with Tajweed rules he/she can a guide to correct other learners who intend to learn by listening to learners and correcting their recitation. But the availability of a Hafiz can be a problem where Islam is not a dominant religion. Furthermore the competency and level of expertise are of epic importance. To get around this problem we have designed and developed a system E-Hafiz. It is based on an idea that Tajweed rules are used to train learners how to recite Quran. To achieve this we used on Mel-Frequency Cepstral Coefficient (MFCC) technique. We extract the features of recorded voices using MFCC and compared with experts' voices stored in database. Any mismatch on word level is pointed out and ask the user to correct it.

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Keywords: The Holy Quran, Islam, Muslims, Tajweed, Hafiz, Voice recognition, feature extraction, MFCC.

1. Introduction

As Islam is the second largest religion of world's population, and their Holy book being in Arabic with only 3.12% of the world's population speaking Arabic (<https://www.cia.gov>). The adherents of Islam are 1.57 billion in 200 countries all over the world in the year 2009 (<http://pewforum.org>). Such diverse geographical distribution leads to the fact that most of the Muslim population does not have Arabic as their primary language. Regarding to problems in understanding and reading Arabic, the religion binds Muslims strongly to Arabic as their Holy book is in Arabic. The Holy Quran also gives an insight in science, engineering, social sciences, law and management (Abdul Rashid Sheikh, 2000). This fact encourages people to read and understand the Quran for both religious and scientific purposes. The Holy Quran was compiled as book nearly 1400 years ago. During this time Arabic as all other languages evolved and underwent changes. So the text reading is not same as Arabic we know today. To solve this barrier, some rules are implemented to ensure the correct reading of the Holy book. These rules have come to be known as Tajweed rules (H. Tabbal et al., 2006) (M.S. Bashir et al.).

The recitation of Holy Quran by use of Tajweed rules is an art and reciters follow the

Tajweed rules to build their recitation attractive (H. Tabbal et al., 2006). If someone wants to learn the Tajweed then he/she has to consult an expert Hafiz at some Learning institute. Learning is done manually as the student and Hafiz have to sit face to face and the learner recites and the Experts points out and corrects the mistakes if any occur. Presently, there exist institutes and websites that teach the recitation in manual method. The main problem consists here is the accessibility and feasibility of a Hafiz expert. Availability of Hafiz in countries where Islam is not a dominant religion can be quite less to come across. In addition, Hafiz's level of expertise is a big issue. As a human being a Hafiz can also make mistakes while listening, so not all Hafiz can be tutors.

The goal or objective for the development of E-Hafiz system is to facilitate the learning/memorizing of the holy Quran, minimizing errors or mistakes of all kinds, and the systematization of the recitation process. Using this system any reciter can learn the recitation skills at any place and any time. The presence of an expert hafiz would not be needed. Consequently, this system helps the Hafizes in preparation of recitation for 5 times prayers and Traveeh prayer in the month of Ramdhan.

Many audio enabled applications are available in the markets which offer the Holy Quran as audio streams. One of the most popular and commonly used is the Quran Auto Reciter (QAR) (<http://www.searchtruth.com/download.php>). QAR provides a user interface where user can select the verse they intend to listen and the verse can be played stopped and paused. And as the words are played the text also gets highlighted. QAR can help learn the Quran and recitation improvements can be made as well as some basics can be learned. But it cannot judge the user's accuracy and performance as there is no way QAR can indicate or detect the mistakes made by user. If software has the utility to detect and correct the mistake the learning and expertise can be improved substantially. So, the learning process through QAR is one only sided. A user can only listen his desired surah many times to improve recitation ability however he cannot know about the mistakes if any he made during his own recitation. Therefore, to solve this problem, he must consult to a hafiz/expert of Quran to know either he recite the holy Quran correct or make any mistake. So, reliability on other person is still exists in this system. Hence this system fulfill required objective in limited area not completely.

(Hassan Tabbal et al., 2006) introduce an automated delimiter that can extract the verses from the audio file using the open source Sphinx framework and speech recognition techniques. In whole processing, two models acoustic and language are discussed. These models takes parts with feature vectors to generate search space for HMM nodes. These two models are:

Acoustic Model: A set of phonemes symbols was used to train the state of HMM that corresponds to the acoustic model. For generating the Acoustic model for the application, the audio recitation of surah Al-Ikhlass (The Holy Quran) (recited by different reciters near about 1 hour) alongside with corresponding dictionary mapping is feed to the sphinxTrain application that generate acoustic representation of each word.

Language Model: It was not easy to choose a language model for the holy Quran, as the high precision and accuracy required for recognition. So, for this system, a language model based on the Java Speech Grammar Format (JSGF) specification was chosen that is compatible with sphinx framework and fulfills all the requirements of the system. The JSGF rules used for this system are similar to those used for conversational system and generated them as such to reflect the structure of the Surah.

The sphinx frame work automatically provides the core recognition process by using appropriate language and acoustic models. For this

purpose, the sphinx frame work is configured by an xml based configuration file that includes the feature extraction algorithms and all other aspects that needed by any speech recognition systems. The system design phase is divided into two sub phases.

Data Preparation: The frame of 10ms and a threshold of 10db were selected for speech segment extractor. To make the recognition ratio more accurate, a 2-stage pre-emphasis filter with factor values (0.92 and 0.97) is used. A raised cosine widower with the 512 points FFT analysis is used for the system and a Mel Filter Bank followed by a Discrete Cosine transformation is used for extracting MFCC features. MFCC have the ability to transform the frequency from a linear scale to a non linear one which can be achieved by this system by using set of 30 triangular Mel filters. Finally, to reduce the distortion effects produced by the microphone, the Cepstral Mean Normalization (CMN) operation is performed. The CMN is achieved by subtracting the mean vector from each vector.

System Settings: The frontend phase output is feed to sphinx core recognizer which uses HMM as a recognizer tool. To translate the result of recognizer into common Arabic language, a hash map was used. The breath first search combined with beam search algorithm is used by the decoder to search to the same words obtained from the recognizer. When the words matched with the stored words, the audio verse correspond to the obtained combination is extracted.

As we have seen, the above discussed application can help users to search a required verse form audio files but unfortunately it is not useful for the users who want to learn the recitation. This application is only used by those persons who know the recitation skill well but not for those who not know how to read the holy Quran. Fortunately, this application helped us very much in our research as it also uses MFCC feature extraction techniques for its implementation. So, the brief study of this application helps us a lot in establishing our ideas and solves the problems we faced.

(Zaidi Razzak et al., 2008) wrote a review paper presents techniques used in Quran Arabic verse recitation recognition and also mention there advantages and disadvantages by comparing them. The objective of this research is to found more effective and efficient technique of Quran Arabic verse recitation recognition for their system that will used to support in j-QAF learning process. According to the paper, process of recitation recognition is commonly divided into: pre-processing, feature extraction, training and testing, feature classification, as well as pattern recognition.

Preprocessing: In Pre-processing the information is organized to simplify the task of recognition. Three steps are performed: -End Point Detection specifies the start and end points of recorded words. -Smoothing reduces noise from the speech. -Channel normalization used to train a recognizer with recorded speeches. The recognition process is depending on speeches recorded from different microphones.

Feature extraction: To differentiate words, the unique, discriminative, and computation efficient features are extracted from the speech signal. Four techniques are treated: (a) Linear Predictive Coding (LPC) that is not considered as a good method, since LPC reduces high and low order Cepstral coefficient into noise when coefficient are transferred into Cepstral coefficient, (b) Perceptual Linear Prediction (PLP) that is better than LPC, since the spectral features remains smooth within the frequency band in PLP and the spectral scale is non-linear Bark scale, (c) Mel-Frequency Cepstral Coefficient (MFCC), based on the frequency domain of Mel scale for human ear scale. MFCC is considered the best technique because behavior of acoustic system remains unchanged during transferring the frequency from linear to non-linear scale. (d) Spectrographic Analysis is used for Arabic language phoneme identification. Arabic phonemes are identified by spectrograms that are represented by distinct bands.

Training and Testing: Speech sample is enrolled in the system database after constructing a model based on features extracted from the speech. Testing process determines similarity between score of newly speech word with the speech stored in DB. Three training and testing methods discussed are: a) Hidden Markov Model (HMM) in which each word is trained independently to get the best likelihood parameters and for this several utterances is performed to train each set of model. b) Artificial Neural Network (ANN) is a based mathematical model, that recognizes speech in such a way that a person applies to visualizing, analyzing, and characterizing the speech to measure its acoustic features. Here, we show that ANN is not well equipped with respect to HMM that solve problems. c) Vector Quantization (VQ) that uses a set of fixed prototype vectors called codebook and each vector in codebook is known as codeword. Quantization is performed by matching input vector against each codeword using distortion measure. d) Features Classification and Pattern Recognition that classify the object of interest into classes. The goal is to know patterns and classes referred to individual words. There are three methods for this purpose: HMM, VQ, and ANN. This process is also referred as feature matching. The author recommends HMM the

best approach for feature extraction and HMM or VQ is for training and testing. HMM is used when Arabic language recognition has to perform and VQ for English language.

There are many different methods suggested by (Zaidi Razzak et al., 2008) in to extract the features from the speech, the system suggests rules and regulations that should be followed during recitation. Again the basic learner is ignored. The system is useful for people who already know the correct pronunciation and Holy Quran rules. But, it is not suitable for non Arabic speakers.

So, a system to help naïve learners to recite the Holy book but also is effective for expert users to know Tajweed rules, pointing out mistakes made during recitation is suitable, tasks achieved by our E-Hafiz system.

We designed, implemented, and tested E-Hafiz application that helps learning like a Hafiz expert. Speeches signals will be gained by a sound speak by a person in microphone. By means of Mel-Frequency Cepstral Coefficient (MFCC) (Noor Jamaliah Ibrahim et al., 2008) transformation, voice features are extracted from the signal emphasized for further processing. MFCC transformation technique produces remarkable results, because the emulation of an auditory system behavior. The linear scale frequency is transformed to non linear one. MFCC is implemented by use of MATLAB framework. The extracted features are used to form a model of speech by use of Vector Quantization (VQ), and are stored in the Database which also contains a large number of speech vectors, obtained from different Quranic verses passed through the above process. Basically, the speech vector is an array of MFCC features. When user utters any verse, it is compared with the stored verse. Verses that are not matched with any registered one are considered as mistakes and pointed to user.

2. Material and Methods

Voice content matching is the process of comparing the voice content of a speaker with the relevant voices contents stored in database of system and make decision on the bases of this compression. At abstract level the content matching system has two phases: *Training phase* and *Testing phase*. During the Training phase, the system is trained with the experts' voices and during testing phase a user records his voice and this voice is matched with the experts' voices to generate results.

If we analyses the current method of teaching in existing organizations/institutes, we can find that most of the Islamic institutes follow the manual method of teaching recitations. In manual process, the teacher and student sit in front of each

other and student starts recitation. Whenever student makes mistake, teacher point it out and correct it like a real time system. The developed system does not work in this real time mode, but it can help the user to know his mistakes after he complete his recitation. The reason behind the development of this non-real time/offline mode system is that at initial level we have some kind of issue which we cannot resolve during user recitation like removing silence from the voice and filter out signals etc.

The figure 1 shows the core architecture of E-Hafiz system. In this system, the MFCC feature extraction techniques is used to get the feature vectors of some specific verses read by some experts and store in the system's database. In order to test the performance of the system, the voice sample of same verses read by ordinary persons are taken and the feature of these voices are taken too by using MFCC techniques. Then, these vectors are compare with the vectors stored in system's database to detect the mistakes if exists. The current system has the ability to detect mistakes on word level as the back end database is made at word level. So, any mistake made at word level can be identified. This is done by a word extraction module which extracts the words out the audio stream recorded. Whenever, a mistake is found the system gives option to the user to listen the verse again as in real life a Hafiz does. The user listens to the verse again to comprehend the right pronunciation and after that he again reads it until he passes the minimal criteria .This criteria however, provides more flexibility especially in Tajweed rules for beginners. But, they are stricter for experts. The dataset of E-Hafiz consists of 10 experts with database of first 5 Sorahs of the Holy Quran.

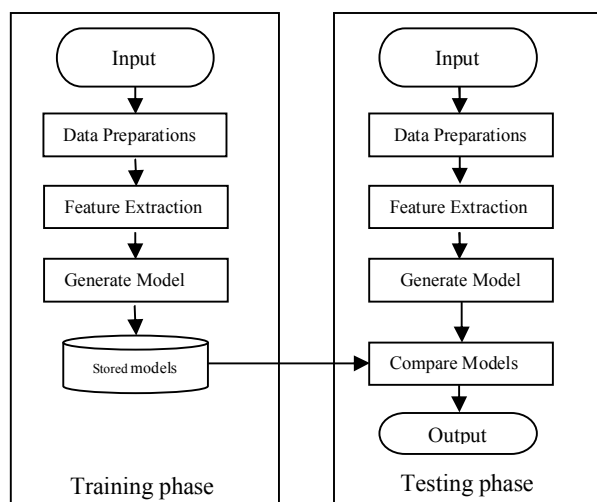


Figure 1. Architectural model of E-Hafiz.

The principal phases in E-Hafiz architecture are: data preparation, feature extraction, and

modeling, storing and comparison.

Data Preparation: In data preparation phase, the raw data (input speech signal) contains silences and noises, are filtered out in order to avoid the errors which may occur during processing and disturb the accuracy of results. The data preparation is subdivide into three steps:

Silence Trimming: The first verse of Surah Al- Fatiha (sarah-1, verse-1) (The Holy Quran) of holy Quran is uttered as an input audio. The recorder audio signal of this verse is shown in figure 2. As it is widely possible that at start time and end times in which the user may have recorded his/her recitation may contain long or short silence gaps. A module trims these silence gaps. This gives a content rich stream for processing. In order to remove silences trimming, the short-term energy method (Mark Greenwood *et al.*, 1999) is used. In short-term energy method, the energy of a speech signal can be calculated at any instance of time. So, the energy of each frame is calculated and removed all those frames whose energy is near about to zero.

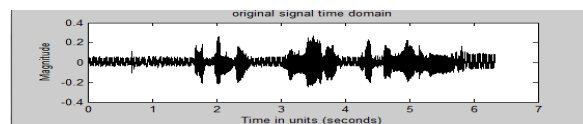


Figure 2. Audio signal of 1st verse of Surah "Al-Fatiha"

Word Extraction: The next module separates the words that are content of the audio stream on the basis of silence threshold that enables the system to store all words uttered separately into the database. This is done to ensure that we can match the error on word level as the comparison done is not on the whole audio stream but on the words that have been uttered.

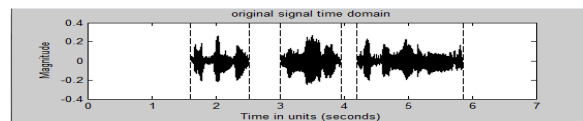


Figure 3. Audio signal of 1st verse of Surah "Al-Fatiha" after extracting words

The detection of words is also performed using short-term energy method (Mark Greenwood *et al.*, 1999). Here, we calculate the energy of each frame and whenever a set of 16 frames having energy approaches to zero is found it is consider an end of word and all the consequent zero energy frames after that are removed. On moving forward where the set of 16 frame having energy greater than threshold founds, it is a sign of starting next word and all the consequent frames are included until again found zero energy frames. This process continues till the

end of speech and finally we got all the available words in the speech (see Figure 3). Once words are extracted each of them undergoes these following steps.

Pre Emphasis: After extracting all available words, the next step of data preparation is the pre emphasis each sub-signal/word, giving raise to higher magnitude frequencies with respect to lower in order to improve the signal to noise ratio. The echoes lies in the signal are also eliminated and due to this quality this process is also known as noise canceling filter. To perform the pre-emphasis, the application of first order Finite Impulse Response (FIR) filter is imply on digitized signal. The equation used to apply FIR filter is:

$$H(z) = 1 - \alpha z^{-1} \quad (1)$$

Where α represents the pre-emphasis parameter, which may have value close to 1, in our case 0.935, it gives rise to the high frequency spectrum to more than 18dB amplification.

Feature Extraction: The voice features are extracted by means of Mel-Frequency Cepstral Coefficient (MFCC) (Noor Jamaliah Ibrahim *et al.*, 2008) transformation for further analysis purpose. MFCC is one of the best techniques used for feature extraction purpose that produces remarkable results in the field of voice content matching systems. It is because, it simulate the behavior of human ear and uses Mel Frequency scale. Here the Feature Extraction technique is performed on each extracted word of speech one after another to get their codebooks. The MFCC feature extraction technique consists of seven major components which are:

Pre-Processing: This is the process in which we prepare data for processing and make it ready to operate. In section 3.1, some necessary initial processing is performed. So, our date is already prepared and no further operation is needed.

Framing: Each word's signal is segmented into 23 mS frames to convert non-stationary signals into qui-stationary format. Also, theses frames are further blocked overlap i.e. every frame contains the 11.5 mS of its previous frame's data. The overlapping is performed to reduce the chances of losing information lies at the end of each frame, which may crash during segmenting of speech.

Windowing: Performing windowing means, multiplying each frame with Hamming Window. Windowing shrink the signal values toward zero level at the boundaries of each frame and hence, it reduces the discontinuity. The Hamming Window is obtained by:

$$w_n = 0.54 - 0.46 \cos\left(\frac{2\pi n}{N-1}\right) \quad (2)$$

In this equation N is the total number of

samples in each frame and n is any value from range 0 to $N-1$. Windowing is performed by multiplying each sample of each frame with each corresponding element of hamming window.

Discrete Fourier Transformation: In order to transfer each windowed frame from time domain to frequency domain, Discrete Fourier Transformation is applied by use of FFT algorithm. The windowed signal obtained from previous step, is given as an input to DFT and the output of this is a complex number, representing each frequency band (0 to $N-1$) having magnitude and phase of that frequency component in original signal. The DFT is obtained by the following equation:

$$Y_2[n] = \sum_{k=0}^{N-1} Y_1[k] e^{-2\pi jkn/N} \quad (3)$$

Where $k=0, 1, 2, 3, \dots, N-1$ and $Y_2[n]$ is the Fourier Transform of $Y_1[k]$.

Mel Filter Bank: Often low frequencies in speech signal contain more useful and important information as compare to higher ones. So, to emphasize these low frequency components, Mel scale is applied. The formula used to calculate Mels for a frequency f in Hz is:

$$\text{Frequency (Mel Scale)} = 2592 * \log_{10}\left(1 + \frac{f}{700}\right) \quad (4)$$

Logarithm: By use of logarithm, the multiplication effect of the magnitude of Fourier Transform is changed into addition. The Matlab command *log* is used to take the natural log of the Mel filtered speech segments. The effect of taking natural log is that it reduces the values of Mel filter bank.

Inverse Discrete Fourier Transformation: IDFT converts the speech signal back to time domain from frequency. As an output we will finally get the Mel Frequency Cepstrum Coefficients (MFCC) in the form of vector known as features vector. The equation used to get MFCCs is:

$$Y[n] = \sum_{k=0}^{N-1} x[k] \cos\left[n\left(k - \frac{1}{2}\right)\frac{\pi}{N}\right], \quad n = 1, 2, 3, \dots, L \quad (5)$$

Here $x[k]$ is the logged value of each Mel filtered speech segment gained from previous step. L is the required number of Mel Cepstral Coefficient taken from N filtertapes of each frame and in our case L is 12.

Modeling, Storing and Comparison of Codebooks: The output of second phase is used to generate model (a features codebook) of speech, and is stored in the Database. Basically, the feature vector is an array of MFCC features. As the speech have a large number of frames and each contains 12 feature vectors. So it is not easy to use all these feature vectors to form codebook. Hence, the numbers of features vectors are reduced by getting highly representative vectors

which is achieved by Vector Quantization (VQ) technique (R. M. Gray *et al.*, 1984).

Vector Quantization is the data compression technique in which probability density functions are modeled by the distribution of prototype vectors. In this technique a large set of vectors having similar number of points are grouped together and these group are represented by their centroid point gained through clustering algorithm. In this system LBG algorithm is used to implement VQ. LBG algorithm works by clustering the similar vectors and finding a single representative value (centroid) for each cluster. This centroid value is also called a code vector and collection of all code vectors correspond to a specific voice is called codebook.

For generating codebook, Mean is calculated for all features vector. So, to calculate the mean of a set of K vectors, the following formula is used:

$$M = \frac{\sum_{i=1}^k X_i}{k}, \quad (6)$$

Here we have a single Mean value representing the whole data. Now this Mean is split into two Means and for this, a very small positive number let say ε (read as epsilon) is used as follow:

$$M_1 = M + \varepsilon \quad (7)$$

$$M_2 = M - \varepsilon \quad (8)$$

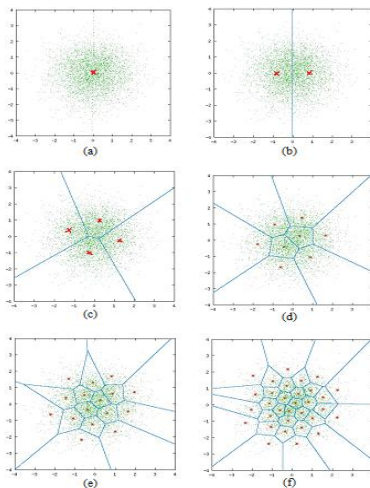


Figure 4. Process of VQ Codebook Generation

Now by use of these two Means two clusters of features i.e. Cluster1 contains all feature vectors having values close to M_1 and Cluster2 contains all feature vectors having values close to M_2 , are created. In order to generate clusters of vectors, the distance between each feature vector and Means values is calculated through Euclidean Distance Formula. Now these two clusters are further divided into four clusters according to method discussed above. This iterative process will continue till 32

clusters are created as shown in figure 4. In every cluster there is a mean value called the code vector of that cluster and all code vectors in all clusters is called a codebook.

Now for testing the system, an interface is given to user to select the Sorah and verse she/he intends to recite as shown in figure 5(a). The system also gives him the option to select his expertise level. On this expertise level, the system decides that with how many experts' voices, his voice is compare. For beginner, the voice of user is compare with all 10 experts' voices.

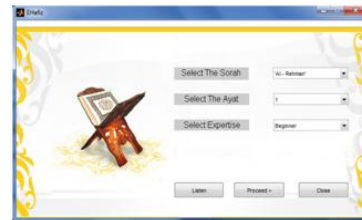


Figure 5(a). Main screen of E-Hafiz application

When user utters any verse, it is compared with the stored verses read by number of different experts; this number however depends on the difficulty level selected. The comparison is performed on word level and in case of any word not matched with any registered one is considered as mistakes and pointed to user. The whole process is as follow:



Figure 5(b). Result screen of E-Hafiz application

1. Gets the utterance of a selected verse recited by any user.
2. Extract the words from the voice sample taken.
3. Extract features of each word using MFCC technique discussed above.
4. Generate the codebook of these words and form an array represents whole verse.
5. From system's database, extract the codebooks array of the same verse recited by experts.
6. For each word of Expert's codebook array and user's codebook array, compute their averages and calculate distance between them.
7. The resultant distance value is compared with the Threshold value. If the distance is less than the threshold value (Dependant on the skill level of user value is set to 2.6 for beginners) it is considered matched, else mismatch word.
8. If all the words of one expert's array are

matched with the user's array, also the number of word in both arrays and their sequence is same then 1 match is considered.

9. In case of beginners at least 3 matches must be found and if not it is consider wrong utterance and pointed to user.
10. In case of wrong utterance result, the word which is found most mismatch is consider as wrong uttered word and that word is highlighted in result screen as shown in figure 7(b).

In result screen option given to user to listen verse in expert voice so that he can found where he misread the word and then he given another option to try it again. User does this process again and again till he recites the whole verse correctly.

3. Results

For experimentation, three groups of reciters men, women and children are chosen. Each reciter was asked to read some specific verse of the Holy Quran and his recitations were tested against the expert's recitation. All these experiments are performed in the presence of an expert Hafiz. It is because an expert hafiz knows better either a user recite correct or wrong. So, when any candidate recites any verse, the expert listen his utterance along with E-Hafiz. After evaluating the utterance of candidate, expert tells his decision which is actually the true result of user utterance. The result generated by E-Hafiz is compared with the expert's result. This comparison tells us the accuracy rates of E-Hafiz i.e. how much results generated by E-Hafiz are correct.

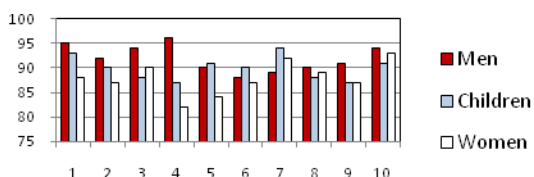


Figure 6. The accuracy rate of E-Hafiz against each candidate

The accuracy rate of E-Hafiz against each candidate is calculated by use of the following formula:

$$\text{Accuracy Rate} = \frac{\text{No. of correct identification by E-Hafiz}}{\text{Total No. of verses}} \times 100 \quad (9)$$

The accuracy rate of each candidate is shown in the figure 6 in which x-axis contains the candidate IDs and y-axis contains results. Also the global mean of all type of candidate is calculated by dividing the sum of all results of one type of candidate to the total number of candidate in that group as shown in table 1.

These results make us inspire and encourage us to make more improvements in this system and

enhance its performance much more than the current ones.

Table 1. Accuracy evaluation of E-Hafiz

Type of Reciters	Number of Reciters	Accuracy Rate
Men	10	92%
Children	10	90%
Women	10	86%

4. Discussions

The system solves a huge problem of arranging Hafiz for learning Quran or in cases where Hafiz could not be arranged learning in fear of mistakes. Users of the system have the possibility to finding mistakes and enhance recitation skills. After meticulous testing of system on many verses and test subjects the results obtained inspire us to further develop the system. With addition of word extraction feature E-Hafiz not only tackles the whole verse but also can identify the mistakes on word level. Our next endeavor is going to make this system capable of identifying the notes which are uttered, taking the recognition ability to letter level, where even a miss pronounced letter can be identified, further more a model assistance can be given on how to pronounce the word as per guided by rules in phonetics is underway.

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References

1. Abdul Rashid Sheikh, Teachings and reflections of Qur'an and Sunnah about Science, Engineering, Technology and Management. Quaid-e-awan University research journal of engineering, science and technology 2000; 1(1).
2. H. Tabbal, W. El-Falou and B. Monla, Analysis and Implementation of a "Quranic" verses delimitation system in audio files using speech recognition techniques. Proc. of the IEEE Conf. of 2nd Information and Communication Technologies 2006; 2(1): 2979 – 2984.
3. Institute for Research in Islamic education, The New Strait Times online – 26 Sep. 2007.
4. M.S. Bashir, S.F. Rasheed, M.M.Awais, S. Masud and S. Shamail, Simulation of Arabic Phoneme Identification through Spectrographic Analysis. CS, LUMS, Lahore, Pakistan.

5. Mark Greenwood and Andrew Kinghorn: SUVing, Automatic Silence/Unvoiced/Voiced Classification of Speech. Undergraduate Coursework, Department of Computer Science, The University of Sheffield, UK. (1999).
6. Noor Jamaliah Ibrahim, Zaidi Razak, Mohd Yakub, Zulkifli Mohd Yusoff, Mohd Yamani Idna Idris and Emran Mohd Tamil, Quranic verse Recitation feature extraction using Mel-Frequency Cepstral Coefficients (MFCC). Proc. the 4th IEEE Int. Colloquium on Signal Processing and its Application (CSPA) 2008, Kuala Lumpur, MALAYSIA. (2008).
7. Program j-QAF sentiasa dipantau, Berita Harian Online (2005).
8. R. M. Gray, Vector Quantization. IEEE ASSP Magazine 1984:4-29.
9. Satori, H. Harti, M. and Chenfour N. Introduction to Arabic Speech Recognition Using CMUSphinx System. ISCIII '07. International Symposium 2007:31-35.
10. The Holy Quran available in almost all languages.
11. Zaidi Razak, Noor Jamaliah Ibrahim, Mohd Yamani Idna Idris, Emran Mohd Tamil, Mohd Yakub, Zulkifli Mohd Yusoff and Noor Naemah Abdul Rahman, Quranic Verse Recitation Recognition Module for Support in j-QAF Learning: A Review. IJCSNS International Journal of Computer Science and Network Security 2008;8(8).

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Expert System to Diagnose Multiple Diseases using Association Factor

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Abstract: Many learning algorithms exist that are routinely used as commercial system. However, given knowledge in health domain, it is difficult to train computers for the decision making and learning. The problem becomes complex when some common symptoms of multiple diseases are present. Some knowledge based systems are available to find a particular disease but cases exist where patient may have more than one illness. We focus on this issue and develop an expert system which not only finds certain disease specifically, but also diagnoses the probability of other diseases to support in prescribing enhanced treatment. The proposed system learns based on a given knowledge, creating rules for making probable decisions and finds association among symptoms occurred mutually in previous assessments. The tested results are quite satisfied and it works accordingly. The system is flexible for new rule generation and association symptoms.

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Keywords: Rule based system, Disease diagnostic, Association factor

1. Introduction:

The expert systems are one of those programs that work on intelligence grounds and produce results on reasoning. Expert systems have two basic components: knowledge base, inference engine [1] and some have also the third component as working memory [2]. These systems are used in many applications of daily routine but are also used in health domain.

MYCIN [3] was designed to diagnose a "Blood Infection" disease and to recommend treatment. Quick Medical Reference QMR [4] diagnoses adult disease. These systems do not provide other patient diseases. Normally, disease itself does not exist; rather patients can be affected by a particular one. Thus, a real Knowledge Base System (KBS) should be exist to diagnose and to associate specific probability for possible diseases.

In addition, it is difficult to produce a differential diagnosis for diseases like Malaria, Dengue Fever and Bone Cancer having some common symptoms (e.g. vomiting, headache, nausea, fever and cough which can be found on <http://anytestkits.com>). Consequently, not discover on time a hidden disease, it may prolong and becomes severe, giving rise to loss of time, increase cost, and fatal result for patients.

In this paper we tackle this issue and come up with a system which diagnoses different diseases having common symptoms. Our Medical Diagnostic Expert System (MDES) uses associations among the

symptoms, trying to find it on probabilistic grounds. The system infers knowledge taking into account previous results since stores previous result for future decision. MDES uses inference rules and precedent decisions available. MDES increases all new knowledge. The system not only finds certain diseases specifically, but also diagnoses the probability of others which may help for enhancing treatment.

Many KBSs have the facility to diagnose by acquiring certain parameters, like MYCIN that diagnoses diseases, its inference rules are represented by IF THEN with a confidence factors. MYCIN uses basic backward chaining reasoning, i.e. is goal oriented.

Dxplain [5] online system assists doctors and paramedical staffs to process differential diagnoses on patients, given some signs, symptoms and laboratory test, the produced results are present and evaluated accordingly. Dxplain starts by acquiring different information about the patient or case in the form of answers binary questions and tries to close possible list of disease candidates.

QMR basically diagnoses adult diseases and also provides information about more than 700 diseases representing the enormous majority of disorder seen by internists in the routine practice along with compendium of less familiar disease.

Similarly GIDEON [6] uses symptoms, laboratory test results, signs and country region to generate a Bayesian ranked differential diagnosis

which is used for diagnostic support and simulation of all infectious diseases in all countries. The Knowledge repository of GIDEON was collected from famous resources from all over the world and the system also provides a monthly electronic literature.

HELP [7] is a hospital information system which provides function to the administration of the hospital in all aspects and also provides decision support system functionalities to the medical doctors to make their decision in efficient way while diagnosing or a performing a treatment. The decision support system part of the system provides important alerts and reminders, data interpretation, diagnosing a patient disease, patient management for suggestions and clinical protocols.

Decision making through association rules mining among large item sets available in different domains to fulfill the required result. There are number of algorithms available for decision making and support systems which works excellent in the relative environment. The algorithms are trying to find the frequency of association among items sets by applying different techniques like association [8], grid analysis [9], consensus theory and decision tables [10, 11]. For instance, Prutax [12] algorithm converts the problem into an acyclic directed graph to make decisions; Partition [13, 14] divides the large itemsets into small chunks for making a decision. Apriori [13] uses association among item sets to draw a final decision. Some extensions of apriori algorithms are AprioriTid [15], Apriori-C [8]

There is no such system to diagnose more than one disease at a time in a patient according to our knowledge but we done it using the Apriori [13, 16] type algorithm named Medical Diagnostic Expert System (MDES) described in detail in next section.

2. Materials And Methods

In order to ease the decision process even in complex situation, we designed, implemented, and tested the Apriori algorithm. Apriori mines a large database, providing relationships and discovering connections through different attributes that characterize diseases. Apriori algorithm is based on prior frequent item set properties for learning association rules [16].

The diagnosis of different possible maladies take into account different parameters associated to certain questions regarding internal and external patient structure, and examination systems. Each symptom consists of a set of characteristics like type, description. Similarly, while diagnosing, requires diagnosis to hit upon. All diagnosis like Kidney Failure, UTI, Blood Cancer or Heart Failure etc. can also have different parameters, serving into the diagnosis process.

Each query in MDES carries equal strength towards the diagnosing process. The criterion is pronounced as normal or abnormal. When the answer is under abnormal value category, this value is recorded and takes part into the process of diagnosing by providing association for the upcoming forecaster symptoms and diagnosis. The Apriori algorithm with the slightly change, takes into the probabilistic grounds. So the solution comes with more than one answer having different probabilities. For this we need the following information.

Categorization of Symptoms

A disease is characterized by different in particular one apparent to the patient and it can be classified by:

Boolean: symptoms with two values {yes, no} or {true, false} e.g. "Feet Swelling" and "Urine is irritable".

Enumerate: Symptoms whose value is a set of enumeration values at a time like and also one choice taking part in more than one symptom. Always, one was selected for future processing. For instance, "Urine Color" takes values into the set: {Normal, Dark, Yellowish, Brown}; "Amount of Urine" value can be {Normal, More Often, Less Often}.

Range of values: certain range values split into {min-max}, e.g. "Blood Pressure Diastolic" normal-value is into [50 to 100] and other than that value are consider to be abnormal.

Definition of the problem

The process of diagnosis can be defined by the followings tuple:

$$(P, s, d, p) \quad (1)$$

Where

- P: Patient to be treated
 s: Symptom available = $\{S_1, S_2, \dots, S_n\}$
 p= probability of proposed diagnose
 d: Diagnosis = (A, S') (2)

Where

- A: the association factor for the symptoms appearing for a given disease
 S': the previously inquired symptom

We consider the following two cases about the selection of the next symptom to be inquired based on the maximum value of associated factor (A).

The start process: When the previously inquired symptom does not exist, *i.e.* $S' = \text{Nil}$ and $A = 0$ (an inexistent previous symptom)

The start of process, when the first symptom begins to be asked, S' depends upon the choice of use. The selection starts the search by exploring the list of symptoms of different diseases.

Previous knowledge exists: Symptoms depend upon the previous knowledge

S' exists, then the choice of next symptom (S_i) takes place. S_i depends upon the highest value of association factor A_i w.r.t. S' & S_i

The association factor is the occurrence of symptoms that took place together in a particular disease as shown in Figure 1.

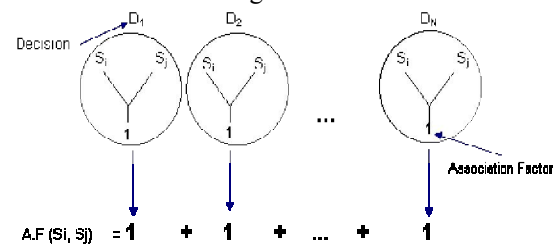


Figure 1 Calculating Association Factor

To calculate the total value of association factor of a certain symptom with respect to other, we count the occurrence of relative symptoms with each other in all decisions. So, to illustrate the functionality, we take some decisions with its symptoms as follow.

$$D_1 = S_1, S_5, S_7, S_{13}$$

$$D_2 = S_5, S_7, S_{10}, S_{12}$$

$$D_3 = S_1, S_5, S_{10}, S_{15}$$

Giving D_i decision, all mutual occurrence of S_i with respect to other S_j is added into the cell (S_i, S_j) (see Table 1). This process is repeated for all i from 1 to 15 symptoms in the present case. For instance, the association between symptoms S₅ and S₇, and then we follow the guidelines from Figure 1 and occurrences are counted: 2 in this case and represented in Table 1, cell (2, 3).

Table 1 Association of symptoms

Sympto	S ₁	S ₅	S ₇	S ₁₀	S ₁₂	S ₁₃	S ₁₅
S ₁	---	2	1	1	0	1	1
S ₅	2	---	2	2	1	1	1
S ₇	1	2	---	1	1	1	0
S ₁₀	1	2	1	---	1	0	1
S ₁₂	0	1	1	1	---	0	0
S ₁₃	1	1	1	0	0	---	0
S ₁₅	1	1	0	1	0	0	---

The values named association factor are used to rule the decision process. The support of the symptom towards a particular disease can be generated using simple percentage formula which can be derived as

$$\text{Presence of 'd' (\%)} = \frac{\text{Symptoms associated}}{\text{Total Symptoms Associated with 'd'}} \times 100$$

Considering the example, when there are seven symptoms associated with a disease “d” and some instance, only four symptoms qualify the

required criteria, and then by using the above formula, the disease “d” has occurred as under

$$d = 4 \times 100 / 7 = 57.14 \%$$

So, the algorithm predicts that 57.14 percent is the chance that of the disease may occur in the patient and keeping in view that all the

“Symptoms have same support value for diagnosing a disease”.

Implementation of MDES

The important objects of the algorithm are Symptom and Diagnose which have some attributes like its number of identification, name, and other related fields that may help in the processing of the algorithm. The process of diagnosing is described by the following pseudo code-

1. Procedure MDES ()
2. Symptom S₁, S₂ ... S_k
3. Diagnose D₁, D₂ ... D_k
4. Select Symptom s
5. While (Not Diagnose D)
6. Result R ← Result.Abnormal
7. Load Symptom Detail
8. // Check the type of the symptom s
9. if (value is not valid)
10. R ← Result.Abnormal
11. Confidence c ← Confidence [i]
12. if (c < s.Confidence)
13. R ← Result.Normal
14. Process.Add (s,value,R,patient)
15. if (R = Result.Abnormal)
16. Add(s)
17. //loading the next proposed symptoms
18. Symptom[]symptoms←ProposedSymptoms(s)
19. Diagnose[] diagnose ← ProposedDiagnosis(lstSymptoms)
20. s ← symptoms [i]//choice of selection
21. D ← diagnose [i]
22. End

The above algorithm is a complete solution for acquiring the disease when different types of symptoms were available in the process. Symptoms are available in a list: {S₁, S₂ ... S_k} which is a structure or class used to contain information about the symptoms used in the algorithm.

The recorded symptoms are added into a list for future for-casting further processes. The actual functionality of the algorithm starts from line no 4 where symptom s has been selected to run the process of learning and diagnosing certain disease. The choice of first symptom is most important as it will initiate the process and algorithm molds itself according to that symptom which has been selected so far. This process going on until some disease will

be identified with the “while” loop at line no 5 and ends at the line 21.

The type of symptom is checked at line no 8 and mode selection made by the user of which choice has been selected from the available symptom types and their relative results, the next option is to mark the confidence level, which starts from the min value of 10 and goes to max value of 100. The confidence level indicates the degree of authenticity over the selection made or answered the choices. The algorithm checks whether the value of confidence is greater than or equal to value of confidence associated with the symptom initially or not, if less than the defined percentage then the Result “R” value again set to “Normal” value because of its low degree of confidence otherwise the value will remain its value if it has selected as “Abnormal”.

At line 14, all process which has been carried out so far will be saved / added in the “process” list along with symptom asked, value to be answered, Result “R” and the patient to whom symptom is being asked. When the symptom result is abnormal, it is recorded into the list of Symptoms to note all the symptoms which were caused as abnormal values and used to perform the diagnosing process in the lines.

At line no 18, the proposed symptoms are loaded against the last symptom asked. The proposedSymptoms function provides sorted array of symptoms associated with the last symptoms in term of their occurrences (having maximum association first). For this, it uses a structure with three attributes {FirstSymptom, SecondSymptom and Association Factor}. The “Association Factor” holds the value of association as integer between the two symptoms.

Similarly, the ProposedDiagnosis function provides the array of proposed diagnoses which may be intimation to actual diagnose as all the provided diagnoses are based on the probability of occurrence. The list of asked symptoms is provided and it checks all symptoms one by one in the available diagnoses whether it contain the particular symptom or not and calculates its probability accordingly. At line 19, the provided diagnoses are stored in the array called “diagnose”.

After maintaining the proposed symptoms and diagnosis, one of the symptom is selected in the variable “s” on line no 20 for continuing the further process of diagnosing certain disease and a diagnose in variable “D” which is used in the while loop at line no 5 to terminate the process of diagnosing a disease on probability grounds. The process of determining carries on within the line of codes from 5 – 21 until unless some expected diagnose comes from the knowledge at line no 19.

The basic elements of the MDSE are described in Figure 2. ‘Patient’ element represents to which certain disease is to be diagnosed and it has some signs in the form of ‘symptoms’.

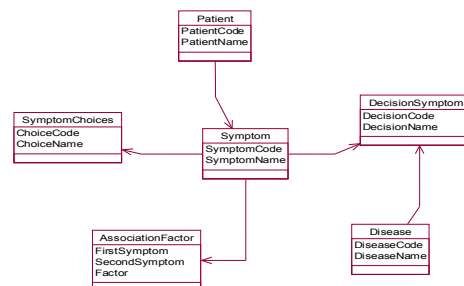


Figure. 2 MDSE Structure

The person has some symptoms which show some disorder of internal and external examination systems about it. The symptom has different choices associated with it and Disease object contains all the diseases in its repository and it is associated with “DecisionSymptoms” to show their relation with the list of symptoms. The set of symptoms which are associated with a particular disease is stored in this object. The object “AssociationFactor” used to store the association of one symptom with respect to all symptoms available in Knowledge base (KB) as a list. In the process of diagnosing a probable disease in a patient, the system gone through a starting symptom, the system shows all the possible choices attached with it like Boolean, enumeration as described in previous section. The user selects one of his / her best choice with confidence level, the system proceed to next step by verifying the defined criteria and then, proposing the best symptoms associated with the symptom asked. It also creates the probability of diseases found and display accordingly.

Here we present two major panels for the description and discusses functionality in detail with the help of figures as shown under.

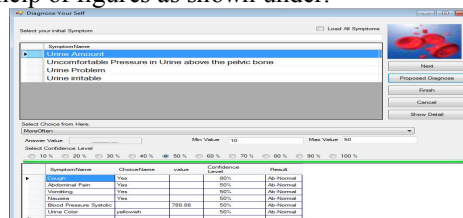


Figure. 3 MDSE System

When a symptom has been selected, the system loads its complete detail regarding its type, range value or a choice selection along with the confidence level. The confidence level ranges from 10 % to 100% with the provision of the answer, if this level is below than 50%, the system will not

marked it as “abnormal” value while the status shows it as an abnormal value. All the symptoms having confidence level more than 50% and choice of answer value not in normal range, the system marked these symptoms as abnormal and includes them in the process of decision making. The first text field is used to write the different symptom. The ‘Next’ button performs all the necessary process of selecting the next symptoms by calculating the association factor among the symptoms available in the KB. When range value symptom has been selected and its value lied in the certain range. So the field allowed the user to enter the value within the range specified by the min and max values besides field. The ‘Proposed Diagnose’ button allows checking the formed diagnosis so far during the process of decision making while answering the different symptoms towards the actual. The list in the bottom part of screen contains the resulted value entered by the user cum operator.

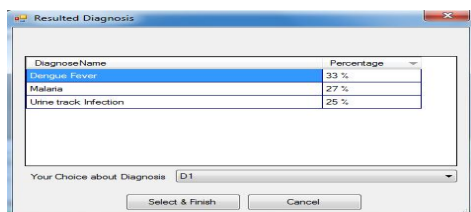


Figure. 4 Proposed Diagnoses

The Proposed Diagnose screen displays the list of diagnoses which are produced in the decision making process. The list shows two columns, one is ‘Diagnose Name’ to indicate the actual name of finding disease and the other column is ‘Percentage’ which indicates the probability in numeric form. E.g. “Urine Track Infection” has the value of 25 % in the list at number 3 (row wise).

3. Discussion

MDES system identifies diseases in the presence of symptoms. In order to illustrate its functionality, consider the following diseases with their relative symptoms.

Disease D_1 refers to Malaria and its symptoms are
 $S_1 \rightarrow$ Vomiting, $S_2 \rightarrow$ Fever, $S_3 \rightarrow$ Cough,
 $S_4 \rightarrow$ Abdominal Pain, $S_5 \rightarrow$ Headache

Disease D_2 refers to Dengue Fever
 $S_1 \rightarrow$ Vomiting, $S_2 \rightarrow$ Fever, $S_3 \rightarrow$ Cough,
 $S_6 \rightarrow$ Nausea, $S_7 \rightarrow$ Muscle Pain

Disease D_3 refers to Bone Cancer
 $S_1 \rightarrow$ Vomiting, $S_3 \rightarrow$ Cough, $S_6 \rightarrow$ Nausea,
 $S_8 \rightarrow$ Fatigue, $S_9 \rightarrow$ Constipation

The above symptoms are registered in Base of Fact and they have different degree in all foresaid patient.

Scenario:
 $D_1 \rightarrow S_1, S_2, S_3, S_4, S_5$
 $D_2 \rightarrow S_1, S_2, S_3, S_6, S_7$
 $D_3 \rightarrow S_1, S_3, S_6, S_8, S_9$

The association factor among the symptoms with respect to each other for diagnosing a particular disease is described in the following table.

Table 3 Association among scenario symptoms

	S_1	S_2	S_3	S_4	S_5	S_6	S_7	S_8	S_9
S_1	-	2	3	1	1	2	1	1	1
S_2	2	-	2	1	1	1	1	0	0
S_3	3	2	-	1	1	2	1	1	1
S_4	1	1	1	-	1	0	0	0	0
S_5	1	1	1	1	-	0	0	0	0
S_6	2	1	2	0	0	-	1	1	1
S_7	1	1	1	0	0	1	-	0	0
S_8	1	0	1	0	0	1	0	-	1
S_9	1	0	1	0	0	1	0	1	-

The association for each symptom is defined with all other symptoms available in KB and calculated by using simply the occurrence together in the past in any order during the process.

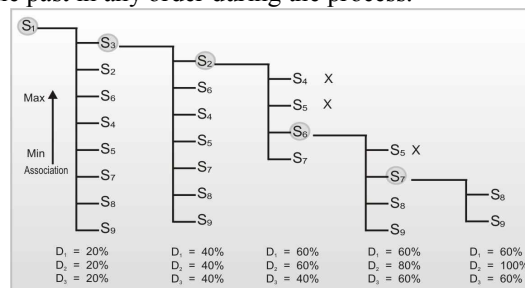


Figure. 5 Decision Process using association facto

Starting from S_1 symptom and after satisfaction of fulfilling the required criteria, the proposed algorithm loads all the symptoms which are associated with it in ascending order (having higher value first). One by one, the user selects its required option and gone through the process as listed in Figure 5. When the user progresses from one step to another, the systems records all the successful symptoms in its repository and tries to propose a disease list based on these symptoms so far. The support of each symptom in a disease is same and its value can be calculated as shown in lower part of the each list of the symptoms (see Figure .5). Similar case is repeated until required disease is found. At every step, it produces the associated symptom list in ascending order and calculates the probability for each disease based on asked symptoms. So, the successful sequence of symptoms asked so far is S_1, S_3, S_2, S_6 and S_7 and at this stage Disease D_1 has its max value of 100 and we can stop our procedure. The other minor diseases are also available with 60% value each. So proposed algorithm MDES is able to find the required disease by locating the symptoms

which had been asked through the process and other diseases as well.

4. Conclusion

MDES is a KBS which has been developed to ease the work of medical domain to make decision efficiently and reduce workload as well. The system allows them to start from a scratch but as long as its usage made, it became more efficient and learns accordingly.

In future, the system will be able to produce treatments based on diagnosed diseases accordingly, to facilitate the paramedical staff in more efficient approach.

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REFERENCES

1. Karl Anders Ericsson, Neil Charness, Paul J. Feltovich, Robert R. Hoffman, "The Cambridge handbook of expertise and expert performance", 2006, ISBN 052184097X, 9780521840972
2. Robert S. Englemore, Edward Feigenbaum, "Expert Systems and Artificial Intelligence", Chap #1, Published: May 1993; WTEC Hyper-Librarian
3. Buchanan, B.G.; Shortliffe, E.H. (1984). "Rule Based Expert Systems: The MYCIN Experiments of the Stanford Heuristic Programming Project", Addison-Wesley. [ISBN 9780201101720](https://doi.org/10.1002/9780470201101).
4. Randolph A. Miller, "Quick Medical Reference QMR", University of Pittsburgh, CA, 1980
5. G. Octo Barnett, MD, Kathleen T. Famiglietti, Richard J. Kim, Edward P. Hoffer, MD, Mitchell J. Feldman, "DXplain on the Internet", MD Harvard Medical School, Laboratory of Computer Science, Massachusetts General Hospital, Boston, Massachusetts, 1998
6. GIDEON online, Medical Expert System, Retrieved From <http://www.gideononline.com> on Dated 21-05-2011, ISSN: 1938-6508
7. Pryor R.A, Gardner RM, Clayton PD, Warner H.R, "The HELP System", Department Of

Medical Informatics, University of Utah, Salt Lake City. 1983, 7: 87-102

8. J Viktor and L Nada, "Classification Rule Learning with APRioRI-C", J. Stefan institute, jamova 39, 1000 ljubijana, Slovenia {Viktor. Jovanoski, Nada, Lavrac} ijw.wi, 2001
9. Ruth Mcguire, "Decision Making", Continuing Professional Development, The Pharmaceutical Journal (Vol. 269), 2007
10. Kamila Aftarczuk, Adrianna Kozierekiewicz and Ngoc Thanh Nguyen, "Using Representation Choice Methods for Medical Diagnosis Problem", Institute of Information Science and Engineering, Wroclaw University of Technology, Poland, 2006
11. János Fülöp, "Introduction to Decision Making Methods", Laboratory of Operations Research and Decision Systems, Hungarian Academy of Sciences, 2005
12. H Jochen, M Andreas, W Rudiger and G Ulrich, "A New Algorithm for Faster Mining of Generalized Association rules", Wilhelm Schickard institute, University of Tübingen, 72076 Tübingen, Germany, {hippj, myka, guentzer} @ informatik.uni-tuebingen.de, 1998
13. Javeed M Zaki, P Srinivasan, O Mitsunori and Wei Li, "New Algorithms For Fast Discovery Of Association Rules", {Zaki, Srin, ogihara, li}@cs.rochester.edu, Computer Science Department, Rochester, New York 14627, Technical Report 651, July 1997
14. H Jochen, G Ulrich, and N Gholamreza, "Mining Association Rules; Deriving a Superior Algorithm by Analyzing Today's approaches", Wilhelm Schickard-Institute, University of Tübingen, Germany, {jochen. hippj, guentzer}@informatik.uni-tuebingen.de, C Daimler AG, Research & Technology FT3/AD, 89081 Ulm, Germany, 2000
15. A Rakesh, S Ramakrishnan, "Fast Algorithms for Mining Association Rules", IBM Almaden Research Center, 1994
16. Nevcihan Duru, "An Application of Apriori Algorithm on a Diabetic Database", Department of Computer Eng. University of Kocaeli, 41440, Izmit, Kocaeli, Turkey, nduru@kou.edu.tr, 2005

2/20/12

Identification for the Specific Depression-like Behavior of 60-Minute Maternal Deprivation Rats in Early LifeMu-Hsin Chen¹, Jen-Haur Lin¹, Chih-Hung Lin^{1,*}¹Department of Optometry, Chung-Hwa University of Medical Technology, Tainan City, Taiwan.Earlylife555@yahoo.com.tw

Abstract: In clinic, it is well known that maternal deprivation can lead to certain forms of depression. However, the evidence from basic researches on the relationship between maternal deprivation and depression is still inadequate, a major impediment in this research field is lack of validated animal models. In previous studies, most evidence showed that animals experienced maternal deprivation in early life would behave more anxiously, it means that maternal deprivation animals will form anxiety animal models. However, Just little evidence showed that maternal deprivation animals would express depression-like behavior. Therefore, it is so urgent to develop a steady and optimal maternal deprivation animal model for the research of depression. In this study, rats after parturition will submit to maternal deprivation process for 60 minutes a day from postnatal day 2 to 14. When the isolated rats wean, they will be separated from mother and raised independently. In addition to fundamental physiological condition measurements, 5-week-old maternal deprivation rats will proceed to forced swim test, spontaneous motor activity test, antidepressant treatments and active avoidance test. However, the results show that maternal deprivation rats experiencing all the tests will express depression-like behavior, but not anxiety-like behavior. Therefore, we find a novel depression animal model due to rats experiencing maternal deprivation for 60 minutes daily in early life. It is expected to offer more beneficial contributions to basic medical studies on the relations between maternal deprivation and depression by this animal model.

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Keywords: Maternal Deprivation, Depression, Forced Swim Test, Antidepressants, Active Avoidance Test

1. Introduction

It is not until the middle part of the 19th century that the brain became the focus of efforts to understand the pathophysiology of depression [1]. However, a major impediment in depression research is the lack of validated animal models. It is so urgent to develop validated depression animal models for the depression research. It is well known that animal models of depression are used both as screening tests to discover and develop novel antidepressant drug therapies and as simulations for investigating various aspects of the neurobiology of depressive illness including the neuropharmacological mechanisms mediating the effects of antidepressant treatments [2, 3, 4]. Although there are some pitfalls for depression animal models in depression research, nevertheless, depression animal models are still very required for the field to formulate several hypotheses by which depression may occur and antidepressant treatments may work.

Depression is often described as a stress-related disorder, and there is good evidence that episodes of depression often occur in the context of some form of stress [1].

Animal models of depression are typically generated by exposure of animals to stressors of various kinds, resulting in behavioural changes reminiscent of aspects of depression [5, 6, 7, 8]. Some early life experiences are also known to increase the

risk for depression, particularly parental deprivation. Clinically, maternal deprivation induced early life stress is a crucial source of stress-related depression [9]. However, little evidence is revealed in basic research of maternal deprivation induced predisposition to depression. Therefore, the development of optimal maternal deprivation induced depression animal model is a pivot in studying the relationship between maternal deprivation and depression.

In previous studies, maternal deprivation induced animal models have been applied commonly [10]. However, there is a discrepancy existed in the final expression of behavior in rats experiencing isolation of early life. Most evidence shows that rats experiencing maternal deprivation produce anxiety-like behavior [11, 12, 13, 14]. And little evidence shows depression-like behavior in maternal deprivation rats of early life [15]. The difference in animal behavior may be due to various conditions of isolation protocols, it means different isolation duration and intensity will lead to different behavioral expression. Therefore, proper isolation conditions will be tested for obtaining optimal maternal deprivation induced depression models.

Tests for the validations of new built animal models are necessary. All available animal models of depression rely on two principles: actions of known antidepressant or responses to stress [16, 17, 18,

19]. Therefore, when rats experiencing 60-min maternal deprivation protocol in early life are grown up to 5 weeks old, they will be tested in forced swim test, spontaneous motor activity test, antidepressant treatments and active avoidance test. These validation criteria are conducted to identify this new depression animal model, and the results reveal that rats experiencing maternal deprivation for 60 minutes once a day from postnatal day 2 to postnatal day 14 will express behavior of predisposition to depression.

2. Material and Methods

Animals

This study is conducted in conformity with the policies and procedures detailed in the "Guide for Animal Care and Use of Laboratory Animals". The animal experimental protocols are confirmed by the Institutional Animal Care and Use Committee (IACUC) of Chung-Hwa College of Medical Technology and IACUC. Young adult male Sprague-Dawley rats weighing 130-150g at the time of testing are housed at a constant room temperature ($22\pm 1^{\circ}\text{C}$) and humidity ($50\pm 10\%$ RH) with a 12-h light: dark cycle. Food and water are available ad lib. The source of feeding diet comes from Lab Diet^R company in U.S.A. and trade code is "rodent diet 5001".

Maternal deprivation

The experimental protocols are modified from Husum et al. [20]. Timed -pregnant Sprague-Dawley rats arrived at the animal facility on gestational day 12. The day of delivery is designated as postnatal day (PND) 0. On PND 2, the pups were sexed and culled into litters of 8-10 male pups and randomly assigned to maternal deprivation for 60 min per day (MDP60) or untreated control group. The separation procedures took place at 8:00~9:00 P.M. daily from PND 2 to PND 14. During the separation, the pups are placed in a neonatal incubator to prevent metabolic changes caused by change of body temperature. Non-handled pups are left undisturbed until PND 23 when all pups were weans. From this day onwards, the rats are maintained four per cage at a constant room temperature of $22\pm 1^{\circ}\text{C}$ in a 12-h light/dark cycle (light on at 6:00 A.M.) with free access to chow and water.

Basal physiological conditions monitor

In this study, we measure two items of basal physiological conditions including body weight and temperature of rats. Five-week-old rats are under measurements. We put rats which are limited in a small transparent cage (the weight of small cage has been zeroed first) on the electronic weight scale and record the digital number of body weight. After weight measurement, animal-specific rectal thermometer is applied to measure the body temperature of rat.

Vaseline treated thermometer is put into 2-3 cm rectal lumen of rat and kept for 5 min, then take the thermometer out and record the body temperature.

Forced swimming test

Rats are immersed in plexiglass cylinders (diameter 18 cm, height 38 cm) filled to a depth of 25 cm with water at 25°C . On the first experimental day, rats are gently placed in the water for a 15 min period of habituation. On removal from the water, they are placed in a plexiglass box under a 60W bulb for 30 min to dry. The next day, they are replaced in the cylinders and observed for 5min. During this period, the total time that spent immobile (i.e., making only the movements necessary to remain afloat) is measured. After 5-min test, the rats are removed from the water.

Spontaneous motor activity test

In view of the physiological nature of rats in spontaneous running. We assign 4 rats at a time on the treadmill (Columbus Instruments) which partitioned into 4 channels by plastic boards (90 cm x 8 cm x 27 cm for each channel). The test protocol needs two days. On the first day, rats were placed at the center of treadmill for 10 min for familiarization with the environment. The next day, the training load of 9 m/min for 10 minutes is applied to the animals and test its spontaneous running time. The treadmill machine was modified. The start point of every run path is equipped with a sensor, which is responsible for the recording of retention time. After 10 min test, the spontaneous running time will be available by abstracting the retention time from total test time. A rat of low spontaneous running time is considered as impairment of moving activity due to physiological reasons. Based on the vivacious instinct of rats, the measurement of the spontaneous running time by treadmill can be used to assess the spontaneous motor activity of rats.

Antidepressants treatment protocol

Rats are treated with desipramine HCl and fluoxetine HCl (from Sigma Chemical Company) intraperitoneally. The treatment doses of desipramine and fluoxetine are 10mg/kg and 5mg/kg respectively. The treatment duration for desipramine is 21 days and that for fluoxetine is 3 days. The timing for the treatments of antidepressants is earlier than the time that rats grow up to 5 weeks old.

Active avoidance test

The anxiety level of rats is evaluated by active avoidance test. The apparatus is consisted of two compartments: one light area (27 L x 27 W x 27 H cm) illuminated by 100-W desk lamp is transparent, and the other dark area (18 L x 27 W x 27 H cm) is painted

black. The dark box is enclosed completely and the light box is open on the ceiling cover. The experiments are performed between 09:00 and 14:00. The retention time of rats in light area will be recorded by a timer. According to the recording time, we will quantify the anxiety level of rats. The more retention happens in the dark area, the more anxious level produces in rats.

Statistics

Results are expressed as mean \pm SEM. Sample sizes are indicated by n. Comparisons between groups are carried out with a one- or two-way analysis of variance (ANOVA). Differences between two groups are compared by using unpaired or paired Student's t-test with $p < 0.05$ considered statistically significant.

3. Results

The basal physiological conditions measured in normal and MDP60 rats

Five-week-old normal and MDP60 rats are submitted to the measurement of body weight and temperature. In body weight, figure 1A shows that there is no obvious difference in these two groups (normal rats: MDP60 rats, 141.2 ± 1.1 : 140.0 ± 2.2 , $n=8$, respectively, $F(1,14)=0.44$, $p > 0.5$) and the average weight of rats is about 140g. Figure 1B also shows that there is no significant difference between these two groups in body temperature (normal rats : MDP60 rats, 37.9 ± 0.1 : 38 ± 0.03 , $n=8$, $F(1,14)=1.81$, $p > 0.5$) and the average body temperature of rats is about 38.0°C .

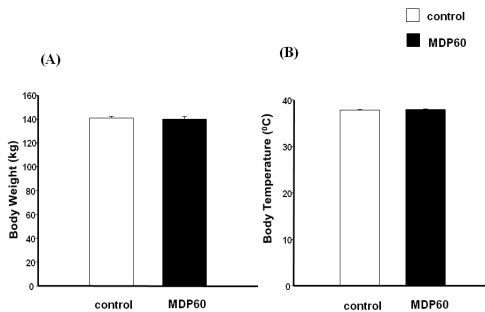


Figure 1

Figure 1. Measurement of body weight and temperature in normal and MDP60 rats 5-week-old normal and MDP60 rats are submitted to the measurement of body weight and temperature. (A) The data show that there is no difference between normal and MDP60 rats in body weight ($n=8$ in each group, $p > 0.5$). (B) The data also show that there is no difference between normal and MDP60 rats in body temperature ($n=8$ in each group, $p > 0.5$). It represents that rats experiencing maternal deprivation have no alteration in basal physiological conditions (vs. normal rats).

The immobility time measured in normal and MDP60 rats in FST

After the measurement of basal physiological conditions, rats are subjected to forced swim test. Figure 2 shows that rats experiencing maternal deprivation for 60 minutes once a day from PND2 to PND14 expressed more immobile than normal rats did in forced swim test (normal rats: MDP60 rats, $45.5 \pm 2.5\%$: $75 \pm 2.6\%$, $n=8$, respectively, $F(1,14)=76.5$, $***p < 0.001$). It means behavioral alterations happen in rats experiencing maternal deprivation in early life and the basal level of immobility in MDP60 rats is significantly higher than that in normal rats.

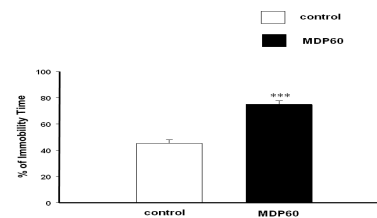


Figure 2

Figure 2. Measurement of immobility time in normal and MDP60 rats during FST. Forced swim test is conducted to normal and MDP60 rats. The data show that rats experiencing maternal deprivation in early life express significantly higher immobility time than normal rats do (normal rats: MDP60 rats, $45.5 \pm 2.5\%$: $75 \pm 2.6\%$, $n=8$, $***p < 0.001$). It demonstrates that behavioral alteration happens in MDP60 rats (vs. normal rats).

Spontaneous motor activity test in normal and MDP60 rats

Spontaneous motor activity test is conducted to evaluate the spontaneous motor activity of rats. Rats after two-day test protocol, figure 3 shows that rats experiencing maternal deprivation still express strong motor activity as well as normal rats (normal rats: MDP60 rats, $95.0 \pm 1.2\%$: $97 \pm 1.8\%$, $n=8$, respectively, $F(1,14)=4.54$, $p > 0.5$). And the percent of motor activity in MDP60 rats is almost 100%, it suggests that maternal deprivation seem not to influence the physiological motor function of rats.

The effects of antidepressants on forced swim MDP60 rats

MDP60 rats are treated with two antidepressants: one is desipramine, the other is fluoxetine. After the treatments of antidepressants, MDP60 rats are submitted to forced swim test and the effects of antidepressants on MDP60 rats are evaluated. Figure 3A shows that rats after 21-day desipramine treatment produce a significant decrease in immobility

time (MDP60 control : desipramine treated MDP60 , $75.7\pm 4.2\%$: $39.4\pm 3.4\%$, $n=6$, respectively, $F(1,10)=62.0$, $***p<0.001$). In figure 3B, the data also show the inhibitory effect of 3-day fluoxetine treatment on the immobility behavior of MDP60 rats (MDP60 control: fluoxetine treated MDP60, $72.0\pm 4.5\%$: $39.0\pm 7.0\%$, $n=6$, respectively, $F(1,10)=27.5$, $***p<0.001$). There is no change on immobility of MDP60 rats by treatment of saline solution ($69.5\pm 3.7\%$ in fig.3A, $69.0\pm 4.5\%$ in fig.3B, $n=6$ in each group, $p>0.5$).

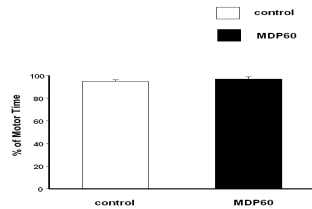


Figure 3

Figure 3. Measurement of spontaneous motor activity in normal and MDP60 rats. The monitoring on the spontaneous motor activity of rats is carried out. After two-day test protocol, the data show that no matter normal or MDP60 rats, there is no retardation took place in both rats (normal rats: MDP60 rats, $95.0\pm 1.2\%$: $97\pm 1.8\%$, $n=8$ in each group). It means rats experiencing 60-min maternal deprivation protocol has no alterations in motor activity.

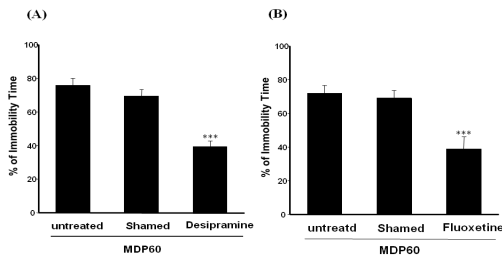


Figure 4

Figure 4. The inhibitory effects of antidepressants on the immobility time of MDP60 rats in FST. MDP60 rats are treated with two antidepressants: desipramine (10mg/kg, i.p.) and fluoxetine (5mg/kg, i.p.). (A) After 21-day desipramine treatment, MDP60 rats produce a significant reduction in the immobility time in FST ($n=6$ in each group, $***p<0.001$). (B) After 3-day fluoxetine treatment, MDP60 rats also show a obvious reduction in the immobility time in FST ($n=6$ in each group, $***p<0.001$). There is no effect on both rats with 0.9% normal saline treatment. It demonstrates that antidepressants can work on MDP60 rats very well.

The immobility time measured in normal and MDP60 rats in FST

After the measurement of basal physiological conditions, rats are subjected to forced swim test. Figure 2 shows that rats experiencing maternal deprivation for 60 minutes once a day from PND2 to PND14 expressed more immobile than normal rats did in forced swim test (normal rats: MDP60 rats, $45.5\pm 2.5\%$: $75\pm 2.6\%$, $n=8$, respectively, $F(1,14)=76.5$, $***p<0.001$). It means behavioral alterations happen in rats experiencing maternal deprivation in early life and the basal level of immobility in MDP60 rats is significantly higher than that in normal rats.

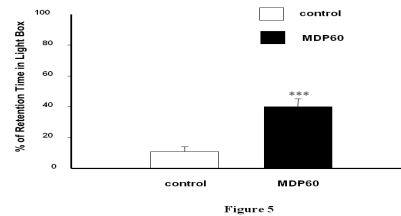


Figure 5

Figure 5. Measurement of anxious level in normal and MDP60 rats. Active avoidance test is carried out to estimate the anxious levels of normal and MDP60 rats. The data show that rats experiencing 60-min maternal deprivation protocol behave less anxious manner than normal rats (retention time in light box: normal rats: MDP60 rats, $11.0\pm 3.0\%$: 40.0 ± 5.05 , $n=8$ in each group, $***p<0.001$). It can demonstrate that MDP60 rats could not be an appropriate anxiety animal model.

4. Discussion

Early life stress involved in the etiology of depression has been revealed. However, the detailed mechanisms for the involvement of early life stress in depression are still inadequate, therefore, it is necessary to build a well early life stress animal models for the further research of depression. Here we find out MDP60 rats, a stable and solid maternal deprivation animal model. Rats experiencing 60-min maternal deprivation protocol in early life will express depression-like behavior, but not anxiety-like behavior. In previous studies, various maternal deprivation protocols show different animal behavior. 15-min isolation protocol makes no alterations in animal behavior, however, 180-min isolation protocol makes animals behave anxiously [20,21]. Here, we conduct 60-min isolation protocol and it makes the animals express a steady depression-like behavior. It may suggest that different isolation intensity or duration would make different final animal behavior, and 60-min isolation is an optimal protocol for the

formation of depression animal models.

In figure 1, MDP60 rats have no difference from normal rats in basal body weight and body temperature. The evidence elucidate that the basal physiological conditions or even the basal metabolism rate (BMR) in rats experiencing 60-min maternal deprivation protocol in early life have no alterations (versus normal rats). In brain regions, hypothalamus is the regulatory center of appetite and body temperature in mammals [22]. Since there is no changes in body weight and body temperature of MDP60 rats, it seems to suggest that rats submitted to this isolation protocol show no harm on the hypothalamus of rats. In other words, it seems to suggest that mild early life stress would not impact the normal function of hypothalamus, at least in the control of body weight and temperature. However, HPA axis dysfunction involved in depression has been well stated [1, 23]. Thus, it is worthy to investigate further whether 60-min isolation early life stress would elicit the dysfunction of HPA axis and contribute to the depression-like behavior formation in rats.

In figure2, MDP60 rats express an obvious increment in immobility time in FST (vs. normal rats). In view of the statement of Porsolt et al, the immobility time in forced swim test represents “despair” state [24, 25, 26]. Therefore, the interpretation for the behavior of MDP60 rats is depression-like behavior. On the other hand, the immobility time in normal rats will be interpreted as basal despair level. In human beings, it is accepted that men has certain basal level of emotional despair, and the persons that express more disappointed emotion will be understood as more depressive. However, there is still a pitfall for identifying the depression syndrome in animals. Therefore, other remedies will be applied to identify the depression state of animals. In this studies, we conduct spontaneous motor activity test、antidepressant treatments and active avoidance test to identify the behavior of MDP60 rats.

It is very common to question that whether the alteration on immobility time of rats in forced swim test is just a change of motor activity of rats, but not a mental change. For responding to the point, spontaneous motor activity test is used in the study. In figure 3, the data show that MDP60 rats behave a strong spontaneous motor activity as well as normal rats (normal rats : MDP60 rats, $95.0\pm 1.2\%$: $97\pm 1.8\%$). From this result, it suggests that rats experiencing 60-min isolation protocol have no damage or hurt on the motor activity of rats. Therefore, it can be deduced that the alterations on immobility time of MDP60 rats is due to mental changes but not physiological issues.

Through the tests of forced swim test and spontaneous motor activity test, it seems more convincing to identify the depression-like behavior of

MDP60 rats. However, antidepressant treatment is also a useful screening platform for depression-like behavior in animals. Here we treat MDP60 rats with desipramine and fluoxetine and observe how they are going on. In figure 4, desipramine, a tricyclic antidepressant, and fluoxetine, selective serotonin reuptake inhibitor, are treated in MDP60 rats. The data show that MDP60 rats treated with desipramine and fluoxetine show a significant reduction on immobility time in FST. It represents that antidepressants can also improve the immobility behavior of MDP60 rats as well as they work in clinic patients. Taking the data from figure2, figure3, figure 4 together, it get more convincing to the identification for the depression-like behavior of MDP60 rats.

Although MDP60 rats can express depression-like behavior, however, as we discussed in first paragraph of discussion section, various isolation protocols for rats may produce anxiety behavior. Therefore, active avoidance test is conducted to dissect the anxious level of MDP60 rats in the study. In figure 5, MDP60 rats spend more time in retaining in light area, it means that MDP60 rats behave less anxious than normal rats do (retention time in light box: normal rats : MDP60 rats, $11.0\pm 3.0\%$: $40.0\pm 5.0\%$). And the difference between normal rats and MDP60 rats in anxious level is significant. Based on this result, rats experiencing 60-min maternal deprivation protocol in early life show less anxiety behavior, reversely, we can state that the behavior of MDP60 rats is more specifically depression-like.

5. Conclusion

The establishment for depression animal models is a necessity in the research field of depression. We first demonstrate that rats experiencing 60-min maternal deprivation from PND2 to PND14 in early life will produce a stable and specific depression-like behavior, it means, an optimal depression animal model has been addressed, especially, a maternal deprivation induced depression animal model. Thus, it is expected that more fruitful and beneficial evidence will be elucidated by means of this animal model. At final in the research of maternal deprivation induced depression. In addition to the search of depression animal models, searching the screening platforms for identifying the depression syndromes or behavior in animals is still another crucial issue.

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References

1. Nestler EJ, Barrot M, DiLeone RJ, Eisch AJ, Gold SJ, Monteggia LM. (2002) Neurobiology of depression. *Neuron*, 34:13-25.
2. Duman RS, Heninger GR, Nestler EJ. (1997) A molecular and cellular theory of depression, *Arch Gen Psychiatry*, 54:597-606.
3. McKinney WT, Bunney WE. Animal model of depression. (1969) *Arch Gen Psychiatry*, 21: 240-248.
4. Willner P, Mitchell PJ. (2002) The validity of animal models of predisposition to depression, *Behav. Pharmacol*, 13:169-188.
5. Geyer MA, Markou A. Animal models of psychiatric disorders, In: *Psychopharmacology: fourth generation of progress*. Bloom FE, Kupfer D (editors), New York: Raven, pp. 787-798.
6. Weiss JM, Kilts CD. Animal models of depression and schizophrenia. In: *Textbook of Psychopharmacology*. Schatzberg AF, Nemeroff CB. (editors), Washington DC: American Psychiatric Press, Pp. 89-131.
7. Willner P. (1990) Animal models of depression: An overview, *Pharmacol Ther*, 45:425-455.
8. Willner P. (1984) The validity of animal models of depression, *Psychopharmacol*, 83:1-16
9. Robertson J. Bowlby J. (1952) Responses of young children to separation from their mothers, *Cour du Centre Internationale de L' Enfance*, 2:131-142.
10. Gutman DA, Nemeroff CB. (2002) Neurobiology of early life stress: rodent studies. *Semin Clin Neuropsychiatry*, 2:89-95.
11. Daniels WM, Pietersen CY, Carstens ME, Stein DJ. (2004b) Maternal separation in rats leads to anxiety-like behavior and a blunted ACTH response and altered neurotransmitter levels in response to a subsequent stressor, *Metab Brain Dis*, 19(1-2):3-14.
12. Huot RL, Thivikraan KV, Meaney MJ, Plotsky PM. (2001) Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment, *Psychopharmacol*, 158(4):366-73.
13. Kalinichev M, Easterling KW, Plotsky PM, Holtzman SG. (2002) Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats, *Pharmacol Biochem Behav*, 73(1):131-40.
14. Park HJ, Chae Y, Jang J, Shim I, Lee H, Lim S. (2005) The effect of acupuncture on anxiety and neuropeptide Y expression in the basolateral amygdala of maternally separated rats, *Neurosci. Lett.*, 377(3):179-84.
15. Lim S, Ryu YH, Kim ST, Hong MS, Park HJ. (2003) Acupuncture increases neuropeptide Y expression in hippocampus of maternally-separated rats, *Neurosci. Lett.*, 343(1):49-52.
16. Hitzemann R. (2000) Animal models of psychiatric disorders and their relevance to alcoholism. *Alcohol Res. Health*, 24: 149-158.
17. Lucki I. (2001) A prescription to resist proscriptio for murine models of depression, *Psychopharmacol (Berl.)*, 153:395-298.
18. Porsolt RD. (2000) Animal models of depression: utility for transgenic research, *Rev. Neurosci*, 11:53-58.
19. Willner P. (1995) Animal models of depression: validity and applications, *Advance in Biochem. Psychopharmacol*, 49:19-41.
20. Husum H, Mathe AA. (2002a) Early life stress concentrations of neuropeptide Y and corticotrophin-releasing hormone in adult rat brain. Lithium treatment modifies these changes, *Neuropsychopharmacol*, 27(5):756-64
21. Husum H, Termeer E, Mathe AA, Boludi GTG, Ellenbroek BA. (2002b) Early maternal deprivation alters hippocampal levels of neuropeptide Y, and calcitonin-gene related peptide in adult rats, *Neuropharmacol*, 39: 1463-1473.
22. Ganong WF. (1983) Review of medical physiology, 11th edition. LANGE Medical Publications, pp 183-192.
23. Kaplan HI, Sadock BJ. (1995) Comprehensive textbook of Psychiatry/VI, 1067-1079.
24. Arushanian EB, Makushkina EN. (1989) Behavioral "despair" in female rats and gonadal function, *Zh Vyssh Nerv Deiat Im I P Pavlova*, 39(6):1129-33.
25. Porsolt RD, Anton G, Blavet N, Jalfre M. (1978) Behavioral despair in rats: a new model sensitive to antidepressant treatments, *Eur. J. Pharmacol*, 47: 379-391.
26. Porsolt RD, Le Pichon M, Jalfre M. (1977) Depression: A new animal model sensitive to antidepressant treatments, *Nature*, 266:730-732.

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EFFECT OF USING POZZOLANIC MATERIALS ON THE PROPERTIES OF EGYPTIAN SOILS

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Abstract: The possibility to use a large amount of waste materials as a replacement for the imported virgin material in road construction has been recognized. RoadCem is a soil stabiliser provides cheap and more environmentally friendly source of materials for road construction to use with in-situ material. This results in reduction in the required thickness of the pavement consequently reducing costs and contributes to the solution of declining resource of imported materials. An extensive study was carried out on a sample of Egyptian soil. RoadCem as a primary stabiliser with ground granulated blast furnace slag (GGBS), lime and ordinary Portland cement (OPC) were employed. The results revealed that the unconfined compressive strength (UCS) and the modulus of elasticity (E_{40}) of the test soil increased while the free swelling percent (FSP) decreased with an increase in the total stabiliser and the curing period.

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Keywords: Clay, RoadCem, slag, lime and swelling soil.

1. Introduction and Problem Definition

The traditional section for road typically consists of different layers such as the surface coarse, base and sub-base courses. These layers are typically made of imported materials that require transport, environmental and other costs increase with the distance to the source of the materials. The reliance on imported material is the main problem, from a sustainability and efficiency point of view, of the traditional road and pavement design and construction. Due to the gradual depletion in the conventional resources, searching for a more rational road construction approach aimed at reducing the dependence on imported materials while improving the quality and durability of the roads is necessary [5].

Many chemical substances have been used to stabilise soils, e. g. lime, OPC, and GGBS. Lime and OPC are the two common additives which have been employed in stabilisation of Egyptian subgrade to produce a base or sub base layer instead of importing granular base course materials [7]. Modern societies produce large quantities of waste materials often disposed off in landfill and considered not to have any value. As the society develops, larger quantities of waste materials continue to be generated by people and these mountains of waste are also becoming a problem. The possibility to use a large amount of these waste materials as a replacement for the imported virgin material in road construction has been recognized as an option. Up to now only a limited portion of the waste stream could be brought to use in road construction due to pollution problems associated with reuse of waste materials in road construction [5].

RoadCem is a blend of special selected substances in various percentages with each individual, chemical specific characteristics. It is an additive and OPC improver, used in soil stabilisation typically for road construction [5]. The use of RoadCem can control of waste streams and pollution, on the other hand it can provide cheaper and more environmentally friendly source of materials for road construction. This results in the reduction of the required thickness of the pavement structures consequently reducing road costs and contributes to the solution of the problem of declining resource base for imported materials [5].

Although RoadCem may be expensive materials, using it in very small percentages together with OPC and/or other pozzolanic materials to produce major improvement in the soil properties may be economic. The main challenge is to secure sufficient funding both to maintain the existing network and to accommodate the extensions to the network that are deemed to be necessary for rural development and for the attainment of poverty reduction goals.

An extensive laboratory study was carried out on a sample of a test soil chosen from Ain Shams district, east Cairo, Egypt. Many additives were employed in this investigation such as RoadCem, OPC, GGBS and lime with different percentages according to the test programme listed below.

2. Materials and Methods

2. Materials

2.1. Test Soil

The main objective of this research was to evaluate the use of RoadCem, OPC, lime and GGBS, as stabilizers on a sample of natural soil. The particle

Table 3. Chemical composition of hydrated lime, RoadCem and GGBS

Composition	Lime [7]	RoadCem	GGBS [7]
SiO ₂	0.46 %	21.4%	34.8 %
Al ₂ O ₃	0.10	1.99	10.7
Fe ₂ O ₃	0.06	0.62	1.2
TiO ₃	0	0	0.6
Ca O	0	47.3	36.4
Mg O	0.83	4.1	1.9
Mn O	0	0	5.4
Ca (OH) ₂	96.79	0	0
Ca CO ₃	1.36	0	0
Ca SO ₄	0.06	0	0
Fe O	0	0	0.75
Fe	0.60	0	0
S	0	0	0.85
Ba O	0	0	6.0
K ₂ O	0	7.44	0
H ₂ O	0.34	16.45	0

3. TEST PROGRAM

Two different mixes in addition to a control mix and two different stabiliser contents for each mix were used in this investigation. The test program and composition of mixes are illustrated in tables 4 and 5 [4].

Table 4. Test programme

Mix 1	Mix 2
2% RoadCem	2% RoadCem
98% OPC	33% OPC
	50% GGBS
	15% Ca (OH) ₂

Table 5. Composition of mixes

ixes	Stabiliser %	Weight of components in grams				
		Natural soil	RoadCem	OPC	Lime	GGBS
1	6%	22500	27	1323	-	-
	12%	22500	54	2646	-	-
2	6%	22500	27	445	203	675
	12%	22500	54	890	406	1350

4. Experimental Study

4.1. Compaction Test

The compaction test was carried out for the determination of the optimum moisture content (OMC) and the maximum dry density (MDD). This test covers the determination of the mass of dry soil per cubic metre when the soil is compacted over a selected range of moisture contents, covering that giving the maximum. The first phase of this study involved a detailed investigation of the compaction characteristics of the test soil containing different percentages of stabiliser, in order to obtain the OMC and MDD. The moisture contents used in preparing specimens for all tests were OMC + 2% as the author believes that this moisture content gives better

conditions for the chemical reactions and the strength development to take place. This moisture content was kept constant for all mixes, to maintain consistency of the results.

4.2. Specimens Preparation

4.2.1. Mixing

Mixing of dry materials was performed using a Hobart variable speed mixer. A quantity of distilled water equal to the (OMC + 2%) as obtained from the standard compaction tests was used. The OMC varied from 9.54% for test soil to 11.28% for test soil + 12% of mix 2, as a percentage of dry weight of soil. Dry materials, enough to produce twelve compacted cylindrical test specimens 50 mm in diameter and 100 mm in length, were thoroughly mixed in a variable speed Hobart 1/4 hp mixer at the lowest speed for 3 minutes before slowly adding the calculated amount of water. The mixing paddle, the bottom and the inside of the mixing bowl were scraped free of the materials and then additional hand mixing with palette knives was carried out to ensure a uniform dispersion and to produce a homogenous mixture.

4.2.2. Compaction

A predetermined amount of materials, sufficient to produce one sample, was placed in a mould specially designed for this purpose. Prior to filling, the inside of the moulds were lightly covered with mould oil to facilitate extrusion after compaction. The amount of material placed in the mould was that required to achieve the previously determined MDD (from 2.0 T/m³ to 2.13 Mg/m³) for a height of 100 mm. The specimen was weighed, measured, and placed in sealed double polyethylene bags to ensure minimum loss of moisture during the curing period. The specimens were then labeled and stored under required curing condition.

4.2.3. Curing

Specimens were placed in the curing room which was maintained at (30 ± 2°C and 50% relative humidity). The curing periods varied from 3 days to 28 days. The curing conditions were selected to represent those that might be achieved in Egypt in most days of the year. The moisture content for all mixes after all curing periods was registered.

4.2.4. Unconfined compressive strength test procedure (UCS)

The second phase of this study involved a detailed investigation of the strength tests (UCS) and the volume stability test (FSP). To carry out these tests the specimens were taken out from the storage bags at the end of the curing periods, weighed to the nearest 0.01g and the dimensions were measured. All specimens were tested in a universal test machine with a loading rate of 1.2 mm/min, so that the specimen fails in about 5 minutes, until failure. A minimum of three specimens of each mixture were

tested. The results presented below are the average values.

4.2.5. Free swelling test procedures

After finishing the strength tests, about 50 g of materials was oven dried at 50° C, ground and passed through a 425 μ sieve. The soil powder was then placed loosely in a dry 25 ml cylinder up to the 10ml mark without any compaction. 50 ml of distilled water was placed in a 50 mm diameter measuring cylinder. The dry soil powder is then poured slowly into the water. The water and soil were then left for at least half an hour. The volume of settled solids was then measured (V_{ml}). Free swell was then calculated from the equation below [1].

$$\text{FreeSwell} = \frac{V - 10}{10} \times 100$$

5. Results

5.1. Unconfined Compressive Strength Of Mixes

The effects of adding stabilisers of different mixes on the UCS of the test soil for 6% and 12% total stabiliser are presented in figure 2. It can be observed that generally the UCS of the test soil increased with an increase in the curing period for the same combination. For example, the UCS of the test soil increased from 795 kN/m² to 2640 kN/m² with an increase in the curing period from 3 to 28 days of mix 1, at 6% stabiliser content. Replacement of 65% OPC by GGBS and lime (mix 2) increased the UCS to 2460 kN/m² and 2820 kN/m² after 28 days at 6% and 12% stabiliser respectively.

5.2. Free Swelling Testing Of Mixes

The initial (FSP) of the test soil was 30%. The effects of adding stabilisers of different mixes on the (FSP) of the test soil are presented in figure 3 for 6% and 12% stabiliser respectively. It can be seen that generally the FSP of the test soil decreased with an increase in the total stabiliser and with an increase in the curing period for the same combination at total stabiliser of 6%. Increasing the total stabiliser to 12% caused a further decrease in the FSP for the same combination. For example, the FSP of the test soil decreased from 30% to 7% with an increase in the curing period from 3 to 28 days for mix 1 at 6%, while the FSP decreased from 30% to 5% with an increase in the stabiliser content to 12%, keeping all other conditions constant. Replacement of 65% OPC of the total stabiliser by GGBS and hydrated lime (mix 2) reduced the FSP of the mix to 10% only at 6% total stabiliser after 28 days. However, no further decrease was observed in the FSP values at 12% total stabiliser.

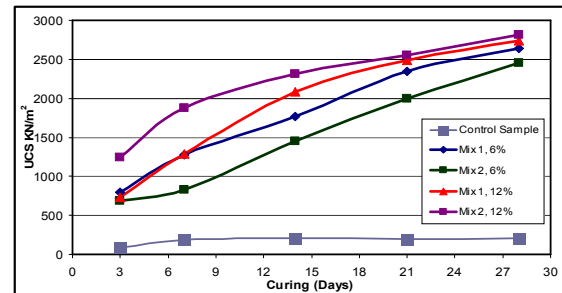


Figure 2. Effect of using stabilisers on UCS of the test soil

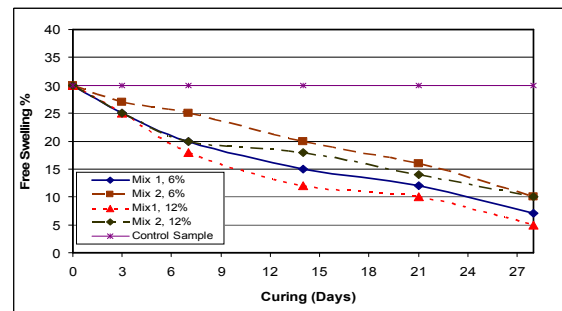


Figure 3. Effect of using stabilisers on the free swelling %

5.3. Modulus Of Elasticity Of Mixes

The effect of stabiliser on the stress / strain (σ/ϵ) behaviour as described by (E_{40}) of the test soil is given in figure 4. (E_{40}) is defined as the ratio of 40% of the maximum UCS to the corresponding strain. It was used in this investigation to study the effect of adding RoadCem, OPC, GGBS and lime on the elasticity behaviour of the test soil instead of using the initial modulus of elasticity to get more representative values as the initial modulus could be affected by the surface conditions of the specimens.

It can be seen that the modulus of elasticity (E_{40}) generally increased with an increase in the stabiliser content and with an increase in curing period, with only few exceptions. Also, (E_{40}), after 7 days, reached to (50% to 60%) of its maximum value after 28 days for the same mix. For example, (E_{40}) of the mix 2 with 12% stabiliser increased from 3680 kN/m² to 21550 kN/m² after 7 days and then further increased to 35255 kN/m² after 28 days. Replacement of 65% OPC with GGBS and lime caused a decrease in the (E_{40}) by about 20% at 6% stabiliser, while the (E_{40}) increased by 20% at 12% stabiliser for the same mix. The increase in (E_{40}) with an increase in the stabiliser content and curing period is probably due to the changes in the composition and the formation of the cementitious materials which have direct effects on the deformation properties of the test soil.

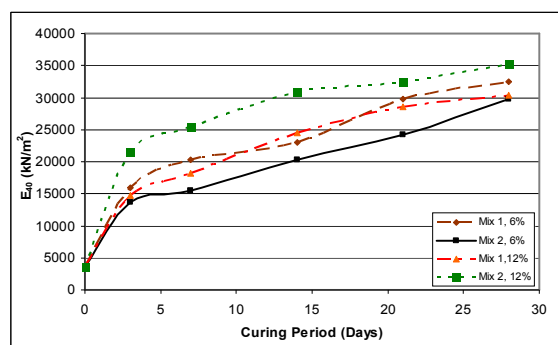


Figure 4. Effect of using stabilisers on the E₄₀

6. Discussion

The increase in strength represented by the UCS with an increase in the total stabiliser content probably results from the reaction of the stabiliser with the clay portion of the test soil and the formation of new cementitious materials. There is also a possible effect on the moisture content of the soil due to the water demand of the lime and/or changes of moisture content due to drying/wetting of the sample. Changes of water content could change the pore suction of the sample which could have a small effect on the strength [7]. The rate of the formation of cementitious materials, is the main reason for the strength increase with an increase in the curing period due to development in the crystallinity and percentage of the cementitious materials. The increase in elasticity expressed in (E₄₀) is associated with the increase in the strength which is primarily due to the formation of new cementitious materials.

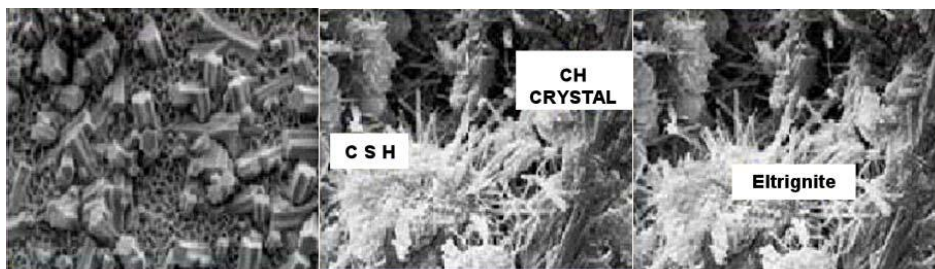
A previous work on similar mixes proved that the CBR values increased dramatically with the increase in the total stabiliser [8]. The great increase in the UCS means a greater increase in the CBR value of the mixes. In road design, it is well known that the thickness of asphalt layers inversely proportional to the CBR values. It may be economic to use soil stabilisation technique using these mixes to increase the CBR values of the subgrade soil and thus reduces the asphalt layer thickness and the base layer may not be used. Optimization study carried out by Ouf and El-Hakem, show that it is possible to reach a UCS of 1792 kN/m² with a 5% swelling by considering an 11% Lime/GGBS and a 7% stabiliser at 37°C curing temperature for 27 days. It can be observed from this investigation that approximately the same gains in strength and the same reduction in free swelling percentage can be obtained easily using 6% of stabiliser or low after 14 days [9].

Previous research [7, 12] on clay-lime system and pozzolanic reactions has showed that the type, form, amount and characteristics of the reaction products control the physical, chemical and

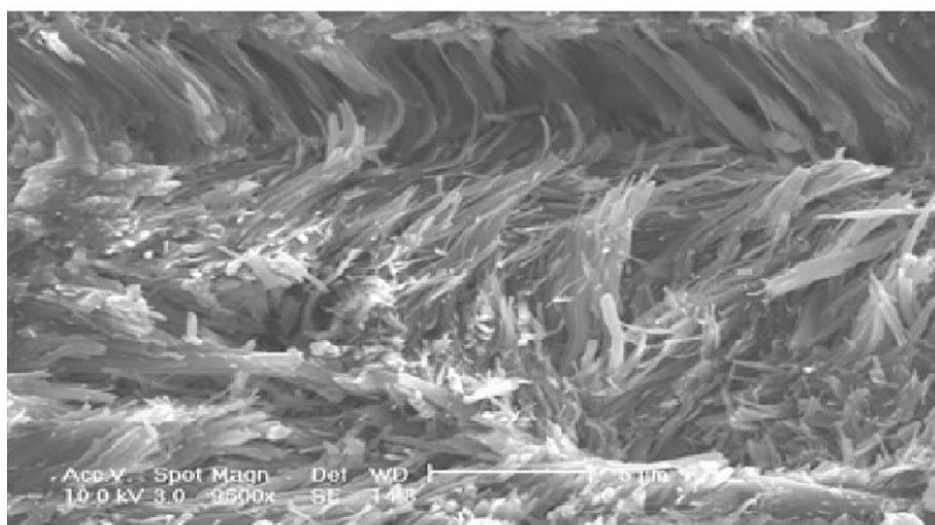
mechanical properties of the bulk material after stabilisation. Thus, the nature of the long-term cementation in clay stabilisation will, depending on the effect of the curing conditions and time. The primary cementing agent in all clay lime stabilisation systems is aluminium substitute calcium silicate hydrate (C-A-S-H) gel. The pore solution of these systems contains silicate and aluminate ions which are formed by the dissolution of clay particles in the highly alkaline environment provided by the dissolved lime [7]. In clay-GGBS-lime systems, the primary cementing agent is still C-A-S-H gel. Due to the high alumina content of GGBS, some alumina is expected to replace silica and C-A-S-H gel is probably also formed.

In clay-GGBS-lime-OPC systems (mix 2), two reactions were expected, hydration of GGBS activated by lime to produce C-A-S-H gel and hydrotalcite type phase containing magnesium, and clay-lime reactions producing C-A-S-H and calcium aluminate hydrates. The major reaction in the short-term is the hydration of GGBS activated by lime which normally starts immediately after mixing the dry materials with the required mixing water. GGBS hydration usually consumes a relatively large amount of water and a relatively small amount of lime [7]. Some free lime however, is still present after GGBS hydration in the mixes depending upon the percentage of lime added, as the required amount of lime to activate GGBS is very small [7]. As a result of GGBS hydration, the mixing water available for lubrication dramatically decreases and the air void content increases, and more water is needed to obtain the same level of lubrication at the same compaction effort. Therefore, the OMC increases with increasing total stabiliser.

OPC needs moisture for hydration and becomes an effective binder of other appropriate materials such as aggregate, sand etc. The reaction of water with the OPC is extremely important to its properties as a binder. When water is added to OPC, each of the compounds contributes to the final product. Tricalcium silicate and dicalcium silicate are responsible for the early and later strength respectively. Upon the addition of water, tricalcium silicate rapidly reacts to release calcium ions, hydroxide ions, and a large amount of heat. The reaction slowly continues producing calcium and hydroxide ions until the system becomes saturated. Once this occurs, the calcium hydroxide starts to crystallize. Simultaneously, calcium silicate hydrate (C-S-H) begins to form. The formation of the calcium hydroxide and (C-S-H) crystals provide "seeds" upon which more C-S-H can form.



A. End Product of Cement Hydration Showing Elements of Crystalline Structure Formed



B. End Product of Cement + RoadCem Hydration Showing Extended and More Pronounced Crystalline Structure Formation and “Wrapping” Effect

Figure 5. Electron micrographs for the typical structures of end products [5]

The C-S-H crystals grow thicker which makes it more difficult for water molecules to reach the anhydrate tricalcium silicate. The speed of the reaction is now controlled by the rate at which water molecules diffuse through the C-S-H coating. This coating thickens over time causing the production of C-S-H to become slower [5].

When RoadCem is used as an additive, moisture remains necessary for hydration and hardening. The five major compounds of the hydration process of OPC still remain the most important hydration products but the minor products of hydration probably change. Furthermore, the rate at which important hydration reactions occur and the relative distribution of hydration products changes as a result of the addition of RoadCem. In addition the crystallization of calcium hydroxide accordingly occurs at different rates and the reduction of heat generation from the hydration reactions occurs. There are more crystals formed, when RoadCem is added, during the reactions and the relevant crystalline matrix is much more extensive. Also when RoadCem

is mixed with OPC, it forms interlocking needles throughout the mixture, creating a strong 3D mineral structure. Also, the amount of water trapped as free water is reduced and the crystals grow into the empty void space. This makes the product less permeable to water and more resistant to all types of attack that are either water dependent or water influenced. Figure 7.A and 7.B show electron micrographs for the typical structures of end products with cement alone and with cement and RoadCem added respectively. It can be seen with these two photographs that the addition of RoadCem creates a different structure. Also, electrochemistry change is induced by the addition of RoadCem. It makes it possible to bind different materials even in acidic environments and when combined with the “wrapping effect” leads to a product which has superior characteristics and performance [5].

The durability, including physical and long-term leaching performance of the final product is a key consideration that should be determined. The approach selected to address durability questions will

depend on the design life, properties of the treated material and potential risk to receptors (e.g. groundwater). Durability can be assessed by considering the failure mechanisms that may affect the bonds between treated materials.

A simple definition of leaching is the transfer of a substance or compound from a solid to a liquid phase when the two are in contact. It is a complex phenomenon and occurs in nature as a result of physical and chemical weathering processes involving the interaction between a soil or rock and water. The amount, or rate, of leaching of a particular substance from a solid matrix, can be influenced by a large number of physical, chemical and biological factors [10].

Leaching behaviour is dictated predominantly by the mechanism of release (percolation or diffusion) [10]. A significant work has been carried out to harmonise leach tests for a wide variety of materials and comparison of numerous test methods has led to the development of standard tests to permit the characterisation of leaching performance for a range of disposal and re-use scenarios.

7. Conclusion

1. The UCS of the test soil generally increased with an increase in the total stabiliser and with an increase in the curing period for the same combination. Replacement of 65% OPC by GGBS and lime caused a further increase in the UCS at 12% stabiliser, while the UCS decreased at 6% stabiliser.
2. Generally the FSP of the test soil decreased with an increase in the total stabiliser and with an increase in the curing period for the same combination at total stabiliser of 6%. Increasing the total stabiliser to 12% caused a further decrease in the FSP for the same combination.
3. The (E_{40}) of the test soil increased with an increase in the total stabiliser and with an increase in the curing period for the same combination. Also, (E_{40}), after 7 days, reached to (50% to 60%) of the maximum value after 28 days for the same mix.
4. High temperature can accelerate hydration of stabilisers in road materials, resulting in raveling of the aggregate and brittle fracture of the layer. When RoadCem is used this mode of failure is largely eliminated.

References

1. Head K.H (1992): *Manual of Soil Laboratory Testing*, Volume 1: Soil Classification and Compaction Tests, London.
2. Higgins, D.D, Kinuthia, J.M, and Wild, S. (1998): Soil Stabilization Using Lime-Activated GGBS", *Proceedings of the 6th Int. Conference, Fly Ash, Silica fume, Slag, and Natural Pozzolans in Concrete*, Bangkok, Thailand, Vol. 2, pp.:1057-1074.
3. Kinuthia, J.M (1997):Property Changes and Mechanism in Lime-Stabilised Kaolinite in the Presence of Metal Sulphate", Ph. D thesis, School of the Built Environment, University of Glamorgan, U. K.
4. Laboratory Guide for RoadCem, powerCem technology, 2009.
5. Marjanovic, P., Egyed, C.E.G., De La Roij, P and de La Roij, R (2008): The Road to The Future. Manual for Working with RoadCem", PowerCem Technologies.
6. Marjanovic, P., Egyed, C.E.G., De La Roij, P and de La Roij, R (2008): The Road to The Future. The Economic Immobilizer with more security for mankind: Manual working with ImmoCem", PowerCem Technologies.
7. Ouf, M.S.A (2001), "Stabilisation of Clay Subgrade Soils Using Ground Granulated Blastfurnace Slag", Ph.D. Thesis, School of Civil Engineering, University of Leeds, U.K.
8. Ouf, M.S.A (2010), "Assesment of Using RoadCem As An Effective Soil Stabiliser For The Egyptian Soil", IC-NOCMAT, International Conference on Non-Conventional Materials and Technologies: Ecological Materials and Technologies for Sustainable Building National Center for Housing and Building, Cairo, Egypt.
9. Ouf, M.S and Al-Hakeem, A.A (2009): Optimum Soil Stabilization using A Mix of Artificial Intelligence Techniques", International Conference on Computational in Engineering, (Code 2009), Seoul, South Korea.
10. van der Sloot, H. A., Heasman, L. and Quevauviller, Ph. (1997): Harmonisation of Leaching Extraction Tests. *Studies in Environmental Science* 70. Elsevier, Amsterdam.
11. van der Sloot, H.A. (2002) Characterisation of Leaching Behaviour of Concrete Mortars and Cement-Stabilised Wastes with Different Waste Loading for Long Term Environmental Assessment. *Waste Management* 22, pp. 181-186.
12. Wild, S, Abdi, M. R. and Leng Ward, G (1993):Sulphate Expansion of Lime-Stabilized Kaolinite: II Reaction Products and Expansion", *Clay Minerals*, Vol. 28:569-583.

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Detection of CK19 mRNA in the blood of breast cancer Female Egyptian patients and its relation to established prognostic parameters

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Abstract: Purpose Breast cancer is a leading cause of cancer-related deaths in women Worldwide. The clinical course of this disease is highly variable and clinicians continuously search for prognostic parameters that can accurately predict prognosis. peripheral blood cytokeratin-19 (CK-19) mRNA-positive cells and its correlation with well established prognostic factors including pathologic parameters, hormonal status and biologic marker; HER 2/neu in breast female Egyptian cancer patients was studied. **Patients and Methods** A total of 60 peripheral blood specimens were collected for study. Patients were forty newly diagnosed breast cancer and 10 patients with benign breast lesions. The 10 apparently healthy donors and patients with benign lesions were used as the control group. They were analyzed for the presence of CK-19 mRNA-positive cells using nested reverse transcription polymerase chain reaction assay (RT-PCR). Immunohistochemical staining for HER 2/neu, estrogen and progesterone receptors were carried out for all cases. The association with known prognostic factors and the effect of CK-19 mRNA-positive cells on patients' prognosis was investigated. **Results** CK-19 mRNA-positive cells were detected in the blood of 14 patients (35%) of the 40 patients. There was statistically significant association between the presence of CK19 mRNA-positive cells and the patients' tumor size and histologic grade of the tumor, stage of disease and the involved lymph nodes $P = <0.001, 0.006, 0.007$ and 0.005 respectively. CK-19 mRNA-positive cell detection also showed high significance with HER2 expression receptor ($p=0.001$). None of the patients with CK 19 positive cells expressed dual ER & PR positivity being both negative and either negative. There was no statistically significant association between the detection of CK19 mRNA-positive cells and patient's age, menstrual status or pathologic type. **Conclusion:** Peripheral-blood CK-19 mRNA-positive cells might constitute a biologically active subset of breast cancer patients with high tumor burden and bad prognosis.

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Keywords: Cancer breast, Peripheral-blood, CK-19, RT-PCR, Prognostic factors.

1. Introduction

Breast cancer ranks first among cancers affecting woman throughout the world and its marked impact is not restricted to Western industrialized societies [1]. Carcinoma of the breast is the most prevalent type of cancer among Egyptian women and constitutes 29% of National cancer Institute cases. Median age at presentation is one decade younger than countries of Europe and North America and most patients are premenopausal. Tumors are relatively advanced at presentation [2].

Biological aggressiveness of breast cancer which is encountered in Egypt is more than in the West and is explained partly by the predominant premenopausal patients and partly by the late presentation of patients at an advanced stage [3, 4].

El-Bolkainy *et al.*, [5] reported clinical (T) categories in NCI series to be as follows: T1 (1.2%), T2 (30%) and T3 (26.4%), due to lack of screening programs and the lack of public awareness of the importance of early detection of breast cancer among Egyptian population, so most of Egyptian patients

lack investigations for early detection of breast carcinoma.

Breast cancer is considered a systemic disease because early dissemination may occur even in patients with small tumors [6]. Micro-metastases, which are undetectable by the classic images and laboratory studies, can contribute to disease relapse [7]. Therefore, their identification in patients with early breast cancer may have substantial effect on determining prognosis and individualizing treatment for those patients [8].

Since cancer cells are very heterogeneous, different cancers express different markers and even cells from the same tumor may not be identical so different assays have been developed for detection of tumor cells in the peripheral blood of patients with various malignancies [9]. Reverse transcription polymerase chain reaction (RT-PCR) amplification technique can identify cell-specific mRNA and detect up to one tumor cell in 10^7 normal peripheral blood or bone marrow mononuclear cells [10], which is at

least 10 times more sensitive than immunohistochemistry [11].

Cytokeratin 19(CK 19) is one of the main keratins expressed in simple or stratified epithelium and considered a general marker of epithelial cancers including breast cancer. It is cleaved by Caspase 3 and the soluble fragments are released and detected in cancer patients. It is one of the markers used for RT-PCR detection of circulating tumor cells (CTCs) in breast cancer patients and it is correlated to an unfavorable prognosis [12, 13] and seems to be the most sensitive and reliable tumor marker in both patients with operable and metastatic breast cancer [14, 15].

In the present study, we aimed at studying the prognostic significance of CK 19m RNA in peripheral blood of newly diagnosed, non metastasizing Egyptian females cases of breast carcinoma with relation to CK19 and the other well established prognostic factors including pathologic parameters, hormonal status and the biologic marker HER /2 neu.

2. Patients and Methods

A total of 60 peripheral blood specimens were collected for study. Patients were forty newly diagnosed breast cancer cases and 10 patients with benign breast lesions who presented to the outpatient clinic at the National Cancer Institute, Cairo University during the **time** period from December 2009 till June 2010. Ten apparently healthy donors were also included. The donors and patients with benign lesions were used as the control group. Our few cases can be justified by the time limit and by our conservative culture; our female patients are not willing to contribute with samples to research or acknowledge the presence of problems in their breast

Written informed consent was obtained before enrollment into the study.

Peripheral blood samples of (8ml in EDTA) were collected. All blood samples were obtained at the middle of vein puncture after the first 5 mL of blood were discarded. This precaution was undertaken to avoid contamination of the blood sample with epithelial cells from the skin during sample collection.

All cancer patients were subjected to chest x-ray, liver and bone scan to exclude metastasis.

RNA Extraction:

Peripheral blood mononuclear cells (PBMCs) were obtained by gradient density centrifugation using Ficoll-Hypaque 1077 (Sigma) at 1,200 g for 30 minutes at 4°C. The interface cells were removed, washed twice with 50 mL of sterile PBS (pH 7.3), pelleted, and resuspended in 1 mL of PBS. The cells were pelleted again at 1,200 g for 2

minutes. Cell pellets were kept at -80°C till RNA extraction.

Total RNA isolation was performed using RNA QIAamp RNA Blood Mini Kit Catalog no. 5230 according to the manufacturer's instructions. All preparations and handling steps were done under RNase-free conditions. RNA quantity and integrity were checked immediately prior to reverse transcription

RT-PCR Assay:

Reverse transcription of RNA was carried out using the GeneAmpGold RNA PCR Reagent Kit (P/N 4308206 Applied Biosystems) using 3-4 µg of RNA according to the manufacturer's instructions.

The CK-19 gene expression was evaluated by nested PCR as described by **Datta et al.** [16]. The sequences of primers used were (synthesized by Genet, Paris, France):

First round PCR:

(P1; forward): 5'AAGCTAACCATGCAGAACCTC AACGAC CGC 3'

(P2; reverse); 5'TTATTGGCAGGTCAGGAGAAGA GCC 3'

Second round PCR (nested):

(P3; forward); TCCCGCGACTACAGCCACTACTA CACGACC

(P4; reverse); CGCGACTTGATGTCCATGAGCCG CTGGTAC

These primers extend across at least an intron, so an eventual DNA contamination would not pose a significant problem.

Beta-actin: was used as a house keeping gene to indicate the presence of intact RNA and successful first-strand cDNA preparation. The primers' sequences used were: CATCCTGTCCGCAATGCCAGG (forward A1) and CTTCTTGGGCATGGAGTCCTG (reverse A2).

The corresponding sizes of PCR products were 745 base pairs for the CK-19 nested PCR and 540 base pairs for B-actin. The second round PCR was carried out using the nested primers and 0.5 µL of the first round product. The cycling conditions for the first round PCR of CK-19 were: one cycle at 95°C for 10 minute followed by 40 cycles at 94°C for 1 minute, 58°C for 3 minutes and a final extension at 72°C for 10 minutes. The conditions for the second round PCR were: one cycle at 95°C for 10 minutes, followed by 40 cycles at 94°C for 1 minute, 64°C for 3 minutes, and a final extension at 72°C for 10 minutes. The cycling conditions for beta-actin were: one cycle at 95°C for 10 minutes,, followed by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds and a final extension at 72°C for 4 minutes. Ten microliters of all PCR products were electrophoresed on 2% agarose gels and visualized with ethidium bromide

Cell lines:

The human mammary carcinoma cell line MCF-7, which expresses the CK-19 gene was obtained from the NCI, Tissue Culture Department. Cells grown in monolayer were harvested, counted and viability was assessed by trypan blue dye exclusion and stored at -70 °C.

To improve sensitivity and specificity of CK-19 mRNA Detection in Peripheral Blood, the following was done:

RNA extracted from MCF-7 was amplified using the set of primers described above. The MCF-7 cell line was consistently positive. Moreover, no amplification product could be detected by nested RT-PCR performed on RNA from the chosen cell line in the absence of the RT enzyme, which demonstrates that any contaminating DNA derived from the processed pseudo- gene would not amplify using the above-mentioned pair of primers subsequently; the sensitivity of CK-19 mRNA detection was evaluated by nested RT-PCR analysis. For this purpose, MCF-7 cells were mixed with PBMCs from healthy blood donors in a cell ratio ranging from 1:10 to 1:10⁶ cells, which mimics the clinical setting for detection of mammary cells in patients' peripheral blood. Representative results of a positive nested RT-PCR are shown in Fig 1, demonstrating 745-base pair whereas the 540-base pair corresponding to the beta-actin gene. This assay was capable of detecting one MCF-7 cell among 106 normal hematopoietic cells as described by 7-Stathopoulou *et al.* [13]. All of samples from control patients and healthy blood donors were positive for beta-actin which indicates the presence of intact RNA and successful first-strand cDNA preparation. These results indicate that the detection of CK-19 mRNA in the peripheral blood is highly associated with breast cancer, despite the fact that a small number of false-positive results (usually < 5%) may be obtained in healthy female blood.

Histopathologic evaluation

All cases undergone modified radical mastectomy or lumpectomy with lymphadenectomy. Tumor grading was evaluated according to Nottingham combined histologic grade (Elston-Ellis modification of the Scarff Bloom Richardson grading system) [17]. Tumor staging was evaluated according to American Joint Committee on Cancer (AJCC) [18].

Three Positively charged slides were prepared from representative tumor block of each case and stained with primary monoclonal antibodies against estrogen receptors (Dako, mouse monoclonal, clone 1D5, ready to use), progesterone receptors (Dako, mouse monoclonal, clone PgR 636, ready to use) and HER2/neu (Dako, rabbit polyclonal,

dilution 1:250). According to the manufacturer's instructions

ER and PR status were evaluated according to Allred scoring system considering only positive nuclear staining [19].

HER-2 immunostaining results was estimated according to HER2/neu scoring system used to evaluate Hercep Test [20]

3. Results:

The characteristics of 40 newly diagnosed breast cancer patients enrolled in the study are listed in **Table 1**. The patients' median age was 49 years (range, 29 to 80 years). Thirty cases with invasive duct carcinoma. Most of cancer patients were postmenopausal constituting 57.5%. None of the studied cases demonstrated any evidence of metastatic disease at time of diagnosis.

According to **American Joint Committee on Cancer (AJCC) [9]**, most of the studied patients were of; stage II constituting 60 %, T2 category constituting 47.5% of all cases. Most of cases of invasive duct carcinomas (**Figure 1**) were of grade 2 representing 80% of all case. Most of node positive cases were of N2 category constituting 52% of all studied cases. As regards hormonal status, 22/40 (55%) were positive for estrogen receptor (**Figure 2**) of which 10 cases were negative to progesterone receptors, while 19/40 (47.5%) were positive to progesterone receptor of which 7 cases were negative to estrogen receptor. Dual positivity for both estrogen and progesterone receptors were encountered in 12(30%) cases, while dual negativity was obtained in 11(27.5%) cases. HER-2/neu immunohistochemical results revealed 25% to be positive (score 3) (**Figure 3**).

None of the studied cases demonstrated any evidence of metastatic disease at time of diagnosis.

Results of RT-PCR amplification of CK19 mRNA:

CK19 was found positive in the peripheral blood of fourteen (35%), of the 40 breast cancer patients studied. Positive cases had a median age of 52.2 years (**Figure 4**), while only one healthy control case exhibited CK-19 positive. The majority of tumors (85.7%) were of invasive duct carcinoma. As regards tumor size, CK 19 positive cases included most of the tumors belonging to T2 & T3 categories (57.9% & 60%) respectively, and none of the T1 category. All patients with grade III tumors had also CK 19 positive cells in their peripheral blood. Only one case with node negative breast cancer was positive for CK 19, while 52% of node positive cases were positive. As regards tumor stage, none of patients with the stage I tumor expressed CK 19 positive cells in peripheral blood. None of the patients with CK 19 positive cells expressed dual ER

& PR positivity being both negative or either negative.

As regards HER-2/neu, all patients with positive reaction to HER-2/neu (score 3) also had CK 19 positive cells in their peripheral blood, while none of the HER-2/neu negative cases exhibited detectable CK 19 positive cells.

Relation between CK19 mRNA+ cases and clinicopathologic parameters, hormonal status and HER -2/neu expression [Table 2]:

Statistically significant association was recorded between CK19 mRNA-positive cells and tumor size ($p < 0.001$), histologic grade ($p = 0.006$), stage of disease ($p = 0.007$), lymph node status ($p = 0.005$) and hormonal status ($p = 0.003$).

There was no statistically significant association between the detection of CK19 mRNA-positive cells and patient's age, menstrual status or pathologic type.

There was a highly significant relation between HER-2 expression and CK-19 mRNA-Positivity recording $p < 0.001$. Interestingly, all the patients with no detectable CK19mRNA- cells were also negative for HER2 by immunohistochemistry and vice versa.

4. Discussion:

Breast cancer is a leading cause of cancer-related deaths in women Worldwide. The clinical course of this disease is highly variable and clinicians continuously search for prognostic parameters that can accurately predict prognosis, and indicate a suitable adjuvant therapy for each patient [1]. In developing countries as Egypt, poverty, illiteracy and limited resources for health care are extra challenges facing both patients and medical care providers.

The National Cancer Institute, Cairo University, Egypt is responsible for supplying medical care free of charge to patients who are mainly poor and with little education. We are aiming at choosing the best and most economic managements to serve as many patients as possible and achieve international cure rates.

The TNM system is incapable of identifying women who, although they have an early-stage breast cancer, may be at high risk of relapse and death. This is due to the early dissemination of malignant cells from the original tumor through hematogenous and/or lymphatic pathways and the failure of the adjuvant treatment to eliminate them [21]. Notably knowing that solid tumors usually contain multiple clones and it is possible that only a small subset of cancer cells of the primary tumor have the biologic characteristics to become disseminated tumor cells. Therefore, the likelihood of finding disseminated cancer cells may not necessarily

parallel the primary tumor load, nor can it be predicted by the well-known risk factors [13].

Especially for breast cancer, CK19 is stably and abundantly expressed on epithelial breast tumors but not on mesenchymal haemopoietic cells and has been successfully used for the detection of breast cancer cells in the bone marrow, lymph nodes and peripheral blood [22].

Up to our knowledge, limited studies demonstrated CK-19 expression by nested PCR and its behavior in Egyptian Female breast cancer patients and its relevance to prognosis.

CK-19 mRNA-positive cells were detected in the peripheral blood of 14 patients (35%). This finding is in agreement with the study by Stathopoulou, *et al.* [7] in which CK-19 mRNA-positive cells were detected in 30% of patients using the same technique.

Detection rates of CK-19 mRNA-positive cells in breast cancer have varied in published studies from 21% to 55 % [7- 9, 16, 23-25]. This variation could be attributed to different patient population, the different detection techniques used with different sensitivities and specificities, even in different investigators using the same primers may have different results [7, 8] depending on the amount of RNA used at the beginning of the reaction or the number of amplification cycles. Alternatively, the false-positive results may be due to the detection of CK-19 pseudo genes a and b [26] or even due to sample contamination with epithelial cells of the skin during vein puncture [27]. Especially for the nested PCR assay, it is feasible to reduce the sensitivity of detecting these low-level transcripts. [28]

In our patients, statistically significant association was found between CK19 mRNA-positivity in the peripheral blood and well established prognostic parameters; namely, tumor size, histologic grade of the tumor, lymph node status, the stage of the disease, and hormonal status. These results were in concordance with other studies that showed statistically significant with tumor size, clinical stage in their cases or association between ck-19 and tumor size [22, 29]. In our study the percentages of CK19+ cells in the peripheral blood samples of patients were increased as the illness grew worse. This result was similar with that of Ivy Wong and his group that positive expression level of CK19 correlates strongly with disease stage in colorectal cancer [30].

It has been mentioned that CK19 detection rate increased with tumor size [31]. Most of our CK19 positive patients had a tumour size of more than 2 cm. However, other reports found the presence of CK19 positive cells had nothing to do with clinicopathological prognostic factors [15, 25]. This could be explained by the fact that solid tumors

usually contain multiple clones; it is possible that only a small subset of cancer cells of the primary tumor have the biologic characteristics to become disseminated tumor cells. Therefore, the likelihood of finding disseminated cancer cells may not necessarily parallel the primary tumor load, nor can it be predicted by the well-known risk factors. Also the lack of correlation might be due to the fact that absence of CK19 transcript in blood samples does not exclude the presence of circulating tumor cells.

Also in this study, detection of CK-19 mRNA in peripheral blood showed direct statistically significant association with lymph node status. This was in contrast to a relative recent report [29]. This may be due to the different dissemination pathways that breast cancer cells utilize, ie, lymphatic spread of the tumor is independent of hematogenous dissemination. Moreover, it has been revealed that detection of disseminated carcinoma cells in the bone marrow using an antibody directed against a common cytokeratin epitope has an independent prognostic value, which is superior to that of the axillary lymph node status in women with early-stage breast cancer [32].

As regards expression of HER2 and in agreement with other studies [8, 25], detection of CK-19 mRNA-positive cells showed direct statistically significant association with immunohistochemical expression of HER-2. All our patients with no detectable CK19mRNA were HER-2 negative and vice versa. This supports that early hematogenous dissemination is associated a number of tumor associated characteristics, such as expression of urokinase plasminogen activator receptor, over expression of the erbB2 oncogene, and deficient expression of major histocompatibility complex class I molecules [21].

It is noteworthy to mention that at the time of diagnosis, all of our cases were subjected to bone scan as well as imaging studies to detect any metastatic deposits and all of them revealed negative results and showed significant relation with bad prognostic parameters. In this study CK-19 mRNA+ were detected in 14 of 40 (35%) patients, when these positive cases were re-evaluated for possibility of occurrence of metastasis after a period of 12 months, 5 out of 14 patients revealed evidence of metastasis by chest CT and bone scan. A longer period of follow up of the other positive cases might verify if they would metastasize or not. This highlights the paramount importance of application of standard routine techniques for detection of circulating tumor cells in the peripheral blood of newly diagnosed patients in order to identify a group of high risk patients who may need different therapeutic approach.

This finding is supported by other studies [21,32] which reported that the detection of occult tumor cells in the bone marrow or peripheral blood of patients with early-stage breast cancer has been shown to be an independent predictive and prognostic factor for early disease recurrence and decreased overall survival either with hormonal receptor positive or negative operable breast cancer. These findings support the role of CTC monitoring as an adjunct to standard clinical and radiographic methods in the evaluation of disease status during follow-up [24].

The detection of CTCs before adjuvant chemotherapy or during tamoxifen administration has been demonstrated to be an independent adverse prognostic factor in women with early-stage breast cancer. The prognostic value of CTC detection is of great significance in subgroups of patients with estrogen receptor-negative and human HER-2/ neu-positive tumors [24].

This opens the way to further investigation of important questions such as whether the detection of CTCs should be performed in all patients at the time of primary diagnosis to identify high-risk patients or whether CTCs detection at diagnosis should modify the adjuvant therapeutic strategy or, whether the detection of CTCs during the administration of adjuvant treatment would allow the development of secondary adjuvant therapeutic strategies.

Several groups of researches proposed a number of therapeutic strategies targeting CK19. In cancer cervix chemotherapeutic efficiency is restrained by the overexpression of Ck19 that will minimize the efficacy of chemotherapy in cervical cancers. Nevertheless, overexpression of Ck19 may provide an alternative approach to treat cervical cancers by radiolabelling mAb against Ck19 [33]. Several similar approaches are undergoing to cancer breast targeting CK19.

Our results strongly suggested that CK19 might be involved with a biologically active subset of breast cancer patients with high tumor burden and bad prognosis, a suggestion that is shared by others who stated that CK19-releasing cells might constitute a biologically active subset of breast cancer cells with high metastatic properties and that CK19⁺ cells in the human breast may have stem cell-like properties [34].

Since our findings were obtained using RNA extracted from peripheral blood mononuclear cells; therefore, blood could be a valuable, feasible and inexpensive method for early detection and monitoring CTCs as CK19 mRNA- positive cells. Also, could give a very early indication of possible prognosis of patients and which ones could be at more risk of metastasis than others. This is particularly useful in our category of patients who are

often irregular in their follow up and do not strictly follow treatment schedules.

It is noteworthy to report that none of the 15 patients with benign breast lesions (14 fibroadenomas and 1 granulomatous mastitis) were positive for CK-19 while only one of 10 apparently healthy volunteers had positive blood sample. Other investigators have also detected CK-19 transcripts in the PBMCs of healthy breast subjects [35, 3], but none reported if follow up of such women showed development of breast cancer later in life. The high specificity of the

method was made possible by avoiding contamination of skin epithelial cells during vein puncture, as well as by carefully designing the primers. Hence, amplification of the known CK-19 pseudo-genes and genomic DNA was avoided [24].

We strongly recommend that future studies should examine the use of RT-PCR CK-19 mRNA detection, preferably with a quantitative method, in evaluating CTC prior and after completion of therapy and monitoring minimal residual disease after the administration of novel adjuvant therapies.

Table 1: Patient characteristics

	Patients No (%)
Age(years)	
Median	49
Range	29-80
Menopausal status	
Premenopausal	17 (42.5)
Postmenopausal	23 (57.5)
Tumor size	
<=2cm	16 (40.0)
2-5cm	19 (47.5)
>5cm	5 (12.5)
Pathologic type	
Invasive duct carcinoma (IDC)	30 (75.0)
Invasive lobular carcinoma (ILC)	8 (20.0)
Mixed IDC & ILC	1 (2.5)
Mucinous adenocarcinoma	1 (2.5)
Tumor grade of IDC	
I	1 (3.3)
II	24 (80)
III	5 (16.7)
Axillary lymph nodes	
N0(-ve)	15 (37.5)
N1(1-3)	8 (20.0)
N2(4-9)	13 (32.5)
N3(=>10)	4 (10.0)
Stage	
I	11 (27.5)
II	24 (60.0)
III	5 (12.5)
Hormonal status ER	
Negative	18 (45.0)
Positive	22 (55.0)
PR	
Negative	21 (52.5)
Positive	19 (47.5)
Her2/neu	
Negative	22 (55.0)
Positive	10 (25.0)
equivocal	8 (20)

Table (2): Relation between clinicopathologic parameters, hormonal status, HER-2/neu and CK-19 mRNA

	Total	CK-19 mRNA(-ve) N (%)	CK-19 mRNA(+ve) N (%)	P value
Age(y)	40	26(65)	14(35)	0.3
Median	49	48	52.2	
Range	29-80	29-80	32-70	
Menopausal status	17	12(70.6)	5(29.4)	0.5
Premenopausal	23	14(60.9)	9(39.1)	
Postmenopausal				
Tumor size				<0.001
<2cm	16	16(100)	0(0)	
2-5cm	19	8(42.1)	11(57.9)	
>5cm	5	2(40.0)	3(60.0)	
grade				0.006
I&II	25	18(72)	7(28)	
III	5	0	5(100)	
Pathology type				0.4
IDC	30	18(60)	12(40)	
ILC	8	6(75)	2(25)	
Axillary lymph nodes				0.005
N0	15	14(93.3)	1(6.7)	
N1,2,3	25	12(48)	13(52)	
Stage				0.007
I	11	11(100)	0	
II,III	29	15(51.7)	14(48.3)	
Hormone receptor status				0.003
Both -ve, either -ve	28	14(50)	14(50)	
Both +ve	12	12(100)	0	
HER-2/neu				<0.001
Negative	22	22(100)	0	
Positive	10	0	10(100)	
equivocal	8	4(50)	4(50)	

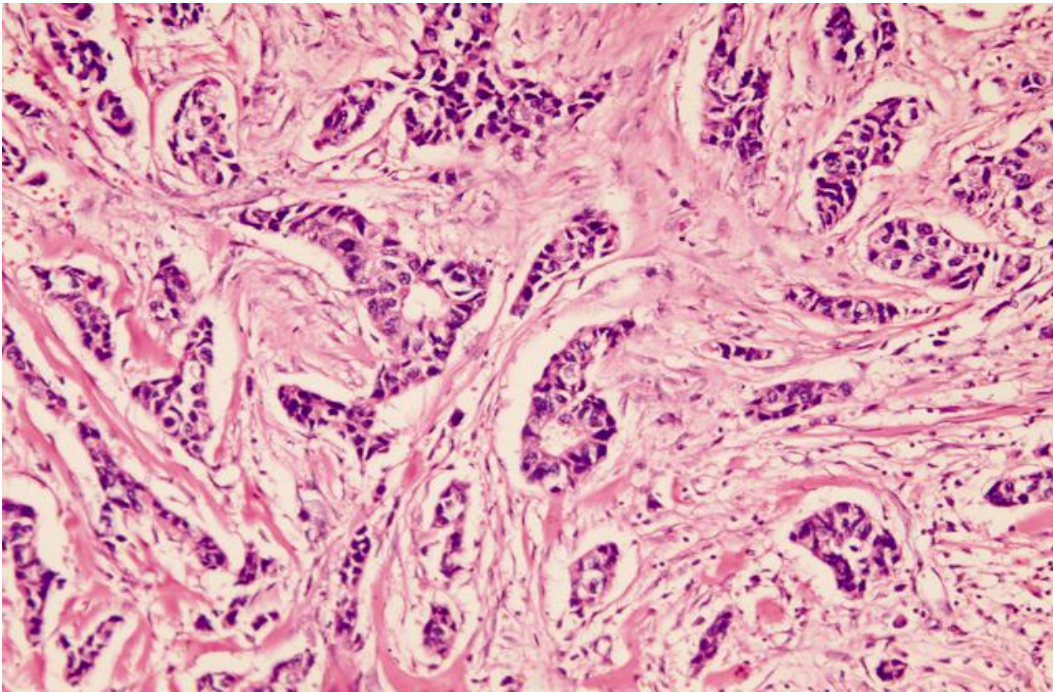


Figure 1: Case of invasive duct carcinoma grade II showing tubular and glandular formation. (H&E x200).

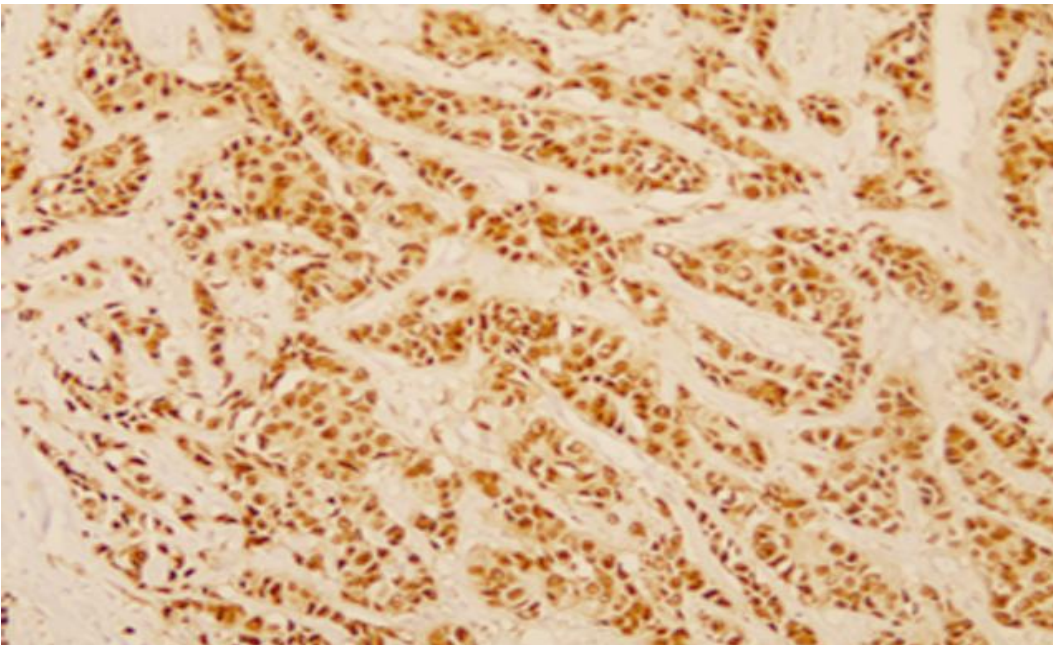


Figure 2: Case of invasive duct carcinoma strongly expressing ER. (x200).

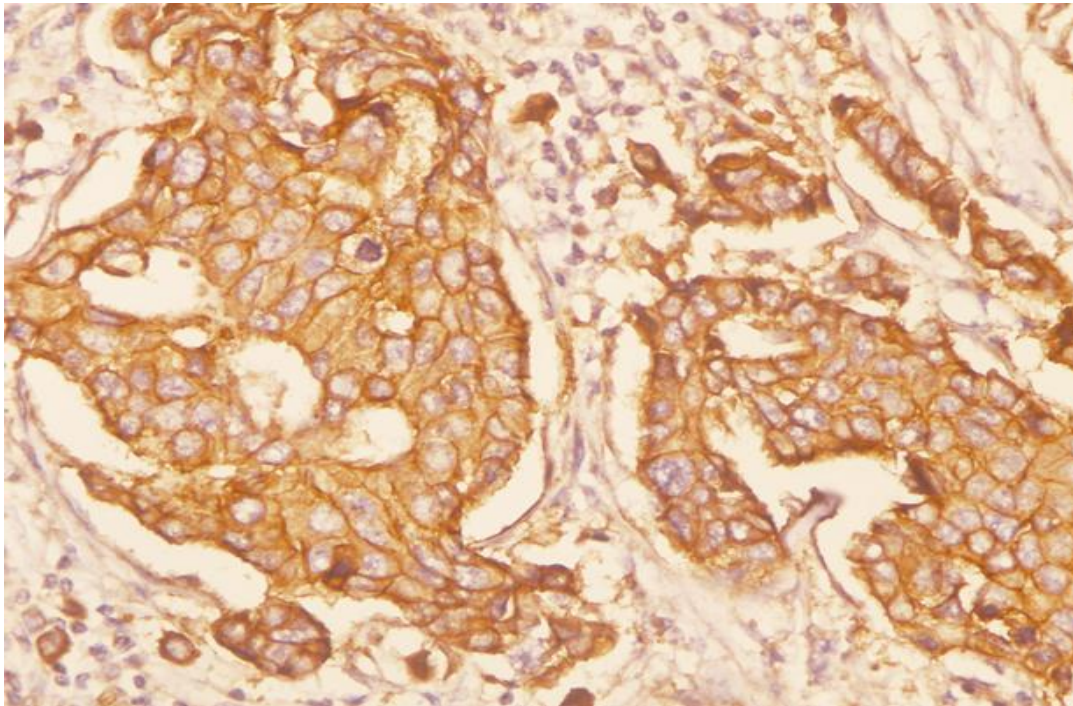


Figure 3: Strong positive membranous expression for HER 2 (score 3) in case of high grade invasive duct carcinoma. (x 400)

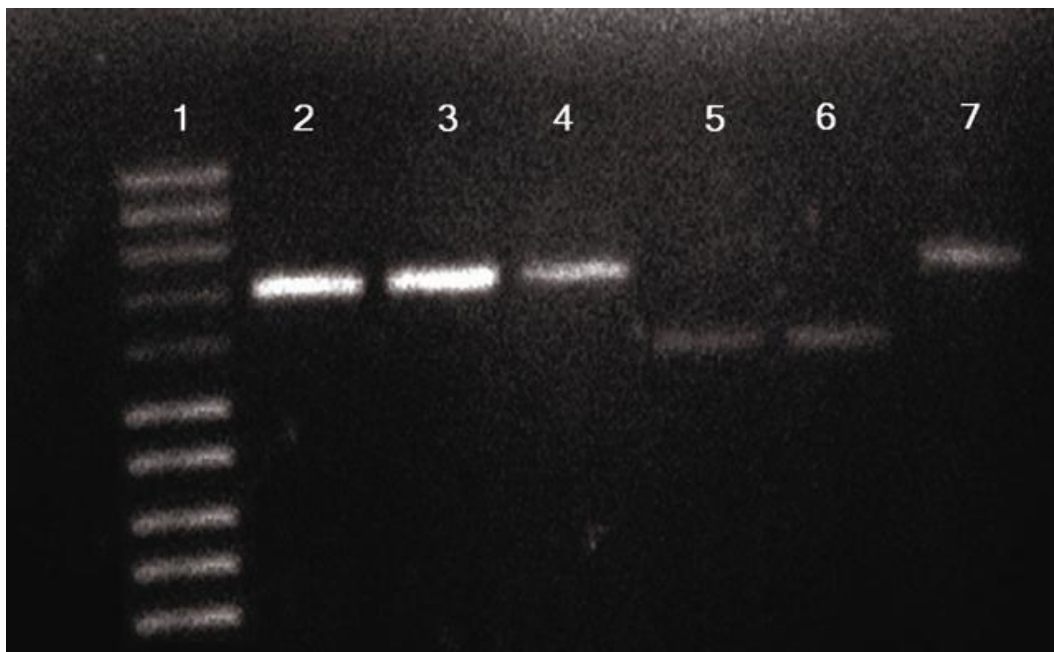


Figure 4: Post PCR products CK19 in breast cancer patients.

Lane 1 DNA molecular weight 100 base pairs plus ladder (100 lanes)

Lane 2 MCF7 positive control (720 base pairs)

Lanes 3- 4 -7 CK19 positive (720 base pairs)

Lanes 5-6 B -actin positive (540 base pairs)

References:

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire (1987): Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*, 235(4785): 177-82. DOI: [10.1126/science.3798106](https://doi.org/10.1126/science.3798106).
2. Omar S, Khalid H, Gaafar, R *et al.* (2003): Breast cancer in Egypt: a review of disease presentation and detection strategies. *East Mediterr. Health J.* May 9(3): 448-63.
3. Mokhtar N (1991): Cancer pathology registry 1985-1989, National Cancer Institute. NCI, Cairo University, Egypt.
4. El- Bolkainy M N (1998): Topographic pathology of cancer. First edition; NCI, Cairo University.
5. El- Bolkainy M N, Nouh M A, El-Bolkainy T M (2005): Topographic pathology of cancer, Third edition. NCI, Cairo University.
6. Pantel k, Barkenhoff RH(2004): Dissecting the metastatic cascade .*Nat Rev Cancer*; 4:448-56.
7. Slade MJ, Coombes RC(2007): The clinical Significance of disseminated tumor cells in breast . *Cancer.Nat Clin Pract Oncol.*;4:30-41,.
8. Ignatiadis M, Kallergi G, Ntoulia M, *et al.* (2008): Prognostic value of the molecular detection of circulating tumor cells using a multimarker reverse transcription-PCR assay for cytokeratin 19, mammaglobin A, and HER2 in early breast cancer. *Clin Cancer Res.* May 1; 14(9):2593-600.
9. Daskalaki A, Agelaki, Perraki M, *et al.* (2009):Detection of cytokeratin 19mRNA-Positive cells in the peripheral blood and bone marrow of patients with operable breast cancer .*British Journal of Cancer*; Jan101:583-597.
10. Chen Y, Zou TN, Wu ZP, *et al.* (2010): Detection of Cytokeratin 19,human mammaglobin and carcinoembryonic antigen-positive circulating tumor three marker reverse transcription PCR assay and its clinical outcome in early breast cancer. *Int J Biol Markers*; Apr-Jun;25(2):59-68.
11. Schoenfeld A, Kruger KH, Gomm J, *et al.* (1997): The detection of micrometastases in the peripheral blood and bone marrow of patients with breast cancer using immunohistochemistry and reverse transcriptase polymerase chain reaction for keratin 19. *Eur J Cancer*; 33:854-861.
12. Lacorix M(2006): Significance, detection and markers of disseminated breast cancer cells. *Endor Relat Cancer*; 13:1033-67.
13. Stathopoulou A, Vlachonikolis I, Mavroudis D, *et al.* (2002): Molecular Detection of Cytokeratin-19-Positive Cells in the Peripheral Blood of Patients with Operable Breast Cancer: Evaluation of Their Prognostic Significance *Journal of Clinical Oncology*, Vol 20, and Issue (16): (August: 3404-3412).
14. Brown NM, Stenzel TT, Friedman PN, Henslee J, Huper G, and Marks JR(2006): Evaluation of expression based markers for the detection of breast cancer cells. *Breast Cancer Res Treat.*; 97:41-47.
15. Stathopoulou A, Mavroudis D, Perraki M, Apostolaki S, Vlachonikolis I, Lianidou E, Georgoulas V. (2003): Molecular detection of cancer cells in the peripheral blood of patients with breast cancer: comparison of CK-19, CEA and maspin as detection markers. *Anticancer Res.*; 23:1883-1890.
16. Datta YH, Adams PT, Drobyski WR, Ethier SP, Terry VH, Roth MS. (1994): Sensitive detection of occult breast cancer by the reverse-transcriptase polymerase chain reaction. *J Clin Oncol.* ; 12(3):475-82.
17. Elston E and Ellis IO(1998): *The Breast*,Vol.13,NY,Churchill Livingstone, p 365.
18. American Joint Committee on Cancer (AJCC) (2002):AJCC cancer staging handbook from the AJCC cancer staging manual. 6th edition. New York (NY): Springer-Verlag; p 257-81,.
19. Allred D C, Harvey J M, Berardo M. *et al.* (1998): Prognostic and predictive factors in breast cancer by immunohistochemical analysis. *Mod Pathol.*, 11:155-168, 1998.
20. Jacobs TW, Gown AM, Yaziji H, *et al.* (1999): Specificity of HercepTest in determining HER-2/neu status of breast cancers using the United States Food and Drug Administration-approved scoring system. *J Clin Oncol.*; 17: 1983-1987,.
21. Pantel K, Cote RJ, Fodstad O(1999): Detection and clinical importance of micrometastatic disease. *J Natl Cancer Inst.*; 91:1113-1124,.
22. SUN Shan-ping,ZANG Yi-xiu,WANG Xiao,CHEN Lian-sheng,TIAN Bin,SUN Tao,JIN Guang-chao ...2012...:Detection of EMA, EGP-2 and CK19 and Their Clinical Significance in Peripheral Bloods of Breast Cancer Patients Posted in Cancer Research > Breast Cancer > on 2011-07-03 | 171
23. Xenidis N, Markos V, Apostolaki S, *et al.* (2007): Clinical relevance of circulating CK-19 mRNA-positive cells detected during the adjuvant tamoxifen treatment in patients with early breast cancer. *Ann Oncol.*; 18:1623-1631.
24. Emmanouil Saloustris , Maria Perraki , Stella Apostolaki ,*et al.* (2011):Cytokeratin-19 mRNA-positive circulating tumor cells during

- follow-up of patients with operable breast cancer: prognostic relevance for late relapse. *Breast Cancer Research*. 13:R60
[doi:10.1186/bcr2897](https://doi.org/10.1186/bcr2897).
25. Xenidis N, Perraki M, Kafousi M, *et al.* (2006): Predictive and prognostic value of peripheral blood cytokeratin-19 mRNA-positive cells detected by real-time polymerase chain reaction in node-negative breast cancer patients. *J Clin Oncol.* ; 24:3756–3762.
 26. Grunewald E, Totzke G, *et al.* (2000): High percentage of false-positive results of cytokeratin 19 RT-PCR in blood: A model for the analysis of illegitimate gene expression. *Oncology*, 59:81-88.
 27. Burchill SA, Bradbury MF, Pittman K, *et al.* (1995): Detection of epithelial cancer cells in peripheral blood by reverse transcriptase polymerase chain reaction. *Br J Cancer*, 71:278-281,
 28. He Q, Marjamaki M, Soini H, *et al.* (1994): Primers are decisive for sensitivity of PCR. *Biotechniques*, 17:82-87.
 29. Zheng HY, Chen Q, Ye YB, Huang WW (2008): mRNA expression of CK19 and CEA in peripheral blood of patients with breast cancer detected by real-Time -quantitative PCR. *Aug 19*; 88(32):2267-70[Article in Chinese]
 30. Wong IH, Yeo W, Chan AT, Johnson PJ(2001): Quantitative relationship of the circulating tumor burden assessed by reverse transcription-polymerase chain reaction for cytokeratin 19 mRNA in peripheral blood of colorectal cancer patients with Dukes' stage, serum carcinoembryonic antigen level and tumor progression. *Cancer Lett.*, 162:65-73.
 31. Weihrauch MR, Skibowski E, Koslowsky TC, Voiss W, Re D, Kuhn-Regnier F, Bannwarth C, Siedek M, Diehl V, Bohlen H(2002): Immunomagnetic enrichment and detection of micrometastases in colorectal cancer: correlation with established clinical parameters. *J Clin Oncol.*, 20:4338-4343.
 32. Braun S, Cevalti B, Assemi C., *et al.* (2001): Comparative analysis of micrometastasis to the bone marrow and lymph nodes of node-negative breast cancer patients receiving no adjuvant chemotherapy. *J Clin Oncol.*, 19: 1468-1475.
 33. CHIH-HSIEN CHANG, LAI-CHEN TSAI, SHUI-TEIN CHEN, CHIOU-CHUNG YUAN (2005): Radioimmunotherapy and Apoptotic Induction on Ck19-overexpressing Human Cervical Carcinoma Cells with Re-188-mAbCx-99. *Anticancer Research*, 25: 2719-2728.
 34. Dontu G, El-Ashry D, Wicha MS(2004): Breast cancer, stem/progenitor cells and the estrogen receptor. *Trends Endocrinol Metab.*, 15:193-197.
 35. Krismann M, Todt B, Schroder J, *et al.* (1995): Low specificity of cytokeratin 19 reverse transcriptase-polymerase chain reaction analyses for detection of hematogenous lung cancer dissemination. *J Clin Oncol.*, 13:2769-2775,
 36. Burchill SA, Bradbury MF, Pittman K, *et al.* (1995): Detection of epithelial cancer cells in peripheral blood by reverse transcriptase polymerase chain reaction. *Br J Cancer*, 71:278-281.

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Diastolic Dysfunction in Septic Patients in Correlation with Renal Function

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Abstract: Septic shock remains one of the most challenging medical conditions, with increasing incidence over the last years. One of the most important features of sepsis is myocardial dysfunction and renal impairment. **Objective** is to evaluate diastolic dysfunction in patient with septicemia and detect its relation to renal impairment in this subset of patients. **Methods** The study was conducted on 40 patients diagnosed to have various degrees of systemic sepsis admitted to Intensive Care Unit of Mansoura International Specialized Hospital. After exclusion of patients with structural heart diseases and renal impairment, each patient was subjected to the following: Full clinical evaluation, complete laboratory investigation -including serum troponin I & creatinine levels- and echocardiographic evaluation with measuring of left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), calculation of LVEF & assessment of diastolic function measuring mitral annulus E/A ratio, E deceleration time (DT) & isovolumic relaxation time (IVRT). **Results** A non-randomized non-controlled prospective study done between July 2009 to August 2010. The study included 40 patients, 24 males & 16 females, with mean age of 62±12. Renal impairment (defined as serum creatinine > 1.4 mg/dl following a normal creatinine level on admission associated with oliguria <0.5 ml/kg/6hours) was present in 78% (31 pts). These pts had significantly shorter IVRT & shorter DT than those with normal renal function. LVEDD and LVESD were significantly larger & LVEF was significantly lower in pts with renal impairment. Renal impairment was associated with significantly lower hemoglobin, higher liver enzymes, higher bilirubin and higher troponin levels. Eighteen patients had SIRS & sepsis (group A, 45%) & 22 had septic shock (group B, 55%). Patients with septic shock showed significantly higher creatinine & significantly higher troponin level than pts with sepsis. Regarding ventricular functions, LVEDD and LVESD were significantly larger & LVEF was significantly lower in septic shock pts than pts with SIRS & sepsis. In group B, both DT and IVRT were significantly shorter than group A. Overall mortality was 55% (100% in septic shock versus 0% in pts with SIRS & sepsis). **Conclusion** The presence of renal impairment was associated with a more severe form of diastolic & systolic dysfunction in septic patients. Septic shock patients showed larger ventricular dimensions and significant systolic and diastolic dysfunctions than patients with sepsis. Higher evidence of myocardial injury in septic shock.

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Key Words: Diastolic dysfunction, septic patients, renal function

1. Introduction

Sepsis, defined by Consensus Conference as “the systemic inflammatory response syndrome (SIRS) that occurs during infection,” is generally viewed as a disease aggravated by the inappropriate immune response encountered in the affected individuals^{1,2}. Although much has been learned about the pathophysiology of sepsis in the last decade, the mortality of this condition is still high.

One of the most important features of sepsis is myocardial dysfunction³.

The hemodynamic pattern in human septic shock is generally characterized by a hypercirculatory state including decreased systemic vascular resistance and a markedly increased cardiac index after adequate fluid resuscitation⁴. Nevertheless, several studies have revealed clear evidence of intrinsic depressed left ventricular performance in patients with septic shock^{5,6}.

Sepsis-induced myocardial dysfunction has traditionally been thought of as principally affecting systolic heart function. One of the primary reasons for this concept is that systolic dysfunction is relatively easy to conceptualize, visualize, and measure⁷. Recently, a evidence is beginning to emerge regarding impaired cardiac relaxation in sepsis^{8,9}.

Acute kidney injury (AKI) approximately develops in 11%-64% of septic patients and is associated with a higher morbidity and mortality¹⁰.

Several mechanisms have been proposed for the pathogenesis of AKI occurring in sepsis. In normal states, the kidney maintains renal blood flow and glomerular filtration through auto regulation dependant on the tone of the afferent and efferent arterioles, this auto regulation is disturbed in sepsis. The cytokines –induced systemic vasodilatation and relative hypovolaemia in sepsis are responsible for

renal hypoperfusion. The renal vasculature has been shown to participate variably to mediators of systemic vasodilatation and renal blood flow has been shown to be variable in septic models¹¹.

Secondary cardiorenal syndrome (CRS Type 5) is a systemic illness leading to simultaneous heart and renal failure. This is almost always in the setting of critical illness such as sepsis, multiple trauma, or burns¹².

Sepsis as a precipitator of CRS Type 5 is common and its incidence is increasing, with a mortality estimated at 20%-60%^{13,14}.

2. Patients and Methods:

Between July 2009 and August 2010, 40 patients diagnosed to have various degrees of systemic sepsis, Admitted to the Critical Care Unit of Mansoura International Specialized Hospital were enrolled in our study.

Inclusion Criteria:

1. SIRS defined as Two or more of the following parameters:
 - Temperature $>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$.
 - HR >90 bpm
 - RR $>20/\text{min}$ with $\text{paCO}_2 < 32$ mmHg.
 - TLC $>12000/\text{dl}$ or $<4000/\text{dl}$ or $>10\%$ staff cells.
2. Sepsis defined as SIRS + confirmed source of infection.
3. Severe sepsis defined as sepsis with organ dysfunction, hypoperfusion, or hypotension.
4. Septic shock defined as sepsis with refractory arterial hypotension (a systolic pressure <90 mmHg, or reduced from baseline by >40 mmHg) or hypoperfusion abnormalities in spite of adequate fluid resuscitation.

Exclusion criteria:

1. Patients with known renal impairment.
2. Patients with known structural heart disease.

The study group was subjected to:

- Full medical history.
- Baseline 12-lead ECG and daily follow up.
- Baseline arterial pressure followed by continuous monitoring of hemodynamics.
- Full blood chemistry including ;
 1. Complete blood picture
 2. Liver function tests
 3. Coagulation profile
 4. Serum troponin I
 5. Kidney function test: Urea and creatinine with value of serum creatinine > 1.4 mg/dl following a normal creatinine level on admission and oliguria is used to define impaired renal function.
- Echocardiography:

Each patient was subjected to 2-Dimensional, M-mode & Doppler study using ATL HDI 500

echocardiography machine using a 3.5 MHZ transducer, measuring the following parameters on admission:

1. Left ventricular end diastolic dimension (LVEDD)
2. Left ventricular end systolic dimension (LVESD)
3. Ejection fraction (EF)
4. Isovolumic relaxation time (IVRT)
5. E Deceleration time (DT)
6. E/A ratio

In our study we divided patients into two groups according to ACCP /SCCM Consensus Conference definition of sepsis;

Group A:

- Patients with SIRS & severe sepsis.
- This group included 18 patients (13 Males, 5 Females) with mean age 59.39 ± 8.65 years.

They had adequate hemodynamic response to fluids resuscitation

Group B:

- Patients with septic shock
- This group included 22 patients (11 males and 11 females) with mean age 65.05 ± 8.8 years.

They had hypotension which was not responding to fluid resuscitation and necessitate vasoactive drugs administration.

Statistical Method:

Data were collected and coded prior to analysis using the professional Statistical Package for Social Science (SPSS 10). All data were expressed as mean and standard deviation (SD). Frequency table for all categorical data. Student t- test (paired & un-paired) after checking normality for all continuous data and Standard Error (SE) of proportion was calculated AP value <0.05 was considered significant.

3. Results:

A non-randomized non-controlled prospective study on 40 patients diagnosed to have SIRS, sepsis & septic shock. Twenty four were males (60%) and 16 females (40%) with mean age 62 ± 12 years.

Eighteen patients (45%) were diabetics, and 12 (40%) had malignancy. Medical cases were 15 (38%) & surgical cases (63%). Renal impairment was diagnosed in 78% (31 patients).

Ten days mortality was 20% & overall mortality was 55% (22 pts).

Comparison between patients with normal and impaired renal function according to clinical & laboratory variables:

No age or sex difference between patients with normal & impaired renal function.

Significantly higher pulse rate, lower systolic &

diastolic BP in patients with renal impairment.

Statistically higher liver enzymes, serum bilirubin, international normalized ratio (INR), C-reactive protein (CRP) & serum troponin in patients with impaired renal function in comparison to

patients with normal kidney function.

Statistically significantly lower hemoglobin (Hb) & Ca levels in patients with impaired renal functions. Table (1)

Table (1): Comparison between pts with normal and impaired renal function.

Variables	Normal renal function (n=9, 22%)	Impaired renal function (n=31, 78%)	P Value
Age(Y)	56±12.1	64 ± 11.4	0.142
Gender (M)	6 (66.7 %)	18 (58.1 %)	0.16
(F)	3 (33.3 %)	13 (41.9 %)	0.18
Pulse (BPM)	107 ± 9	128 ± 12.4	0.03
SBP (mmHg)	106 ± 13.5	84.5 ± 11.2	0.005
DBP (mmHg)	57 ± 14	45 ± 11.2	0.001
TLC	20.2 ± 7	24 ± 9	0.245
HB	9.8 ± 2.4	8.45 ± 1.6	0.0174
HCT	23.3 ± 8.4	21.1 ± 10.3	0.036
Total Bil	1.14 ± 0.23	2.45 ± 1.6	0.042
SGOT	46.3 ± 22	92.4 ± 13.2	0.002
SGPT	69 ± 22	85 ± 31.4	0.03
INR	1.2 ± 0.18	2.13 ± 0.62	0.14
Urea	48.1 ± 41	87 ± 31	0.032
Creatinine	1.14 ± 0.23	2.98 ± 1.2	0.031
CR.CL	66.5 ± 28.2	32.4 ± 16.5	0.04
Calcium	9.2 ± 1.3	8.1 ± 1.3	0.09
Troponin	.045±.01590	.11585±.1160	0.035
CRP	9.8 ± 4.3	16 ± 8.5	0.0416

Comparison between patients with normal and impaired renal function according to Echocardiographic parameters.

Statistically larger EDD, ESD, and statistically lower EF in patients with impaired renal function. Patients with impaired kidney function showed

statistically shorter DT and IVRT than those with normal renal function.

No significant difference in the E/A ratio between pts with normal & impaired renal function. Table (2)

Table (2): Echocardiographic parameters in patients with normal and impaired renal function.

Variables	Normal renal function (n=9, 22%)	Impaired renal function (n=31, 78%)	P Value
LVEDD	4.56 ± 0.27	5.4 ± 0.37	0.0128
LVESD	3.34 ± 0.29	3.8 ± 0.28	0.01
EF	55.2 ± 5.8	46.4 ± 2.9	0.01
IVRT	87 ± 9.2	73.2 ± 12	0.004
E/A	1.05 ± 0.14	1.36 ± 0.37	0.6
DT	231.4 ± 22.2	172 ± 32.4	0.033

Comparison between sepsis & septic shock according to clinical & laboratory data:

No age or sex difference between both groups.

Significantly higher pulse rate, lower systolic & diastolic BP in patients with septic shock.

Statistically higher liver enzymes, serum bilirubin, INR, CRP & serum troponin in group B in comparison to group A.

Statistically lower Hb level in septic shock than in sepsis. Table (3)

Table (3): Comparison between sepsis (Group A) and septic shock (Group B).

Variables	Group A (n=18, 45%)	Group B (n=22, 55%)	P Value
Age (Y)	59.39±8.6	65 ± 8	0.324
Gender (M)	13 (72 %)	11 (50 %)	0.16
(F)	5 (27 %)	11 (50 %)	0.18
Pulse (BPM)	95.6 ± 6	106.6 ± 12	0.03
SBP (mmHg)	114.7 ± 18.9	79 ± 5	0.005
DBP (mmHg)	69.4 ± 12	43 ± 7.2	0.001
TLC	16.1 ± 4	18.59 ± 4	0.115
HB	10.1 ± 1.6	9.4 ± 1.5	0.042
HCT	33.17 ± 3.5	31.27 ± 3.7	0.028
Total Bil.	1.46 ± 0.47	1.63 ± .53	0.006
SGOT	49.5 ± 14	56.23 ± 17.5	0.002
SGPT	52 ± 13	55 ± 17	0.03
INR	1.48 ± 0.58	2.42 ± 0.33	0.001
UREA	50.1 ± 20	63 ± 26	0.032
Creatinine	1.89 ± .79	2.55 ± 0.84	0.0062
CR.CL	51.6 ± 19.2	43.9 ± 18.5	0.04
Troponin	0.08818	0.11981	0.043
Calcium	8.85 ± 0.91	8.98 ± 1.05	0.075
CRP	11.78 ± 4.9	14.73 ± 4.4	0.016

Comparison between both groups according to Echocardiographic parameters:

Statistically larger EDD, ESD, and statistically lower EF inpatients with septic shock. They also

showed statistically shorter DT and IVRT than those with sepsis. No significant difference in the E/A ratio between both groups. Table (4).

Table (4): Echocardiographic parameters in both groups.

parameters	Group A (n = 18)	Group B (n = 22)	P value
LVEDD (cm)	4.6 ± 0.27	5.3 ± 0.34	0.01
LVESD (cm)	3.22 ± 0.30	3.97 ± 0.27	0.018
EF (%)	56.26 ± 11.6	45.6 ± 13.5	0.01
IVRT(ms)	88. ± 8.1	74.5 ± 12	0.004
E/A ratio	0.981 ± 0.15	1.32± 0.37	0.6
DT (ms)	210.2 ± 13.2	171 ± 15.4	0.033

Mortality in both groups:

100% mortality in group B versus no mortality in group A. Eight patients (36%) died within 10 days and 14 patients (64%) died within 20 days. Table (5) & figure (1)

According to echocardiographic parameters,

statistically larger LVEDD in patients died within 20 days than those who showed early mortality (LVEDD was 54.50±2.822 versus 50.88±3.227 respectively, P = 0.012). No significant difference of other echocardiographic parameters (ESD, EF, E/A ratio, DT and IVRT).

Table (5): Mortality rate in both groups.

Outcome	Group A		Group B	
	N	%	N	%
Survivors	18	100	0	0
Non-survivors	0	0	22	100

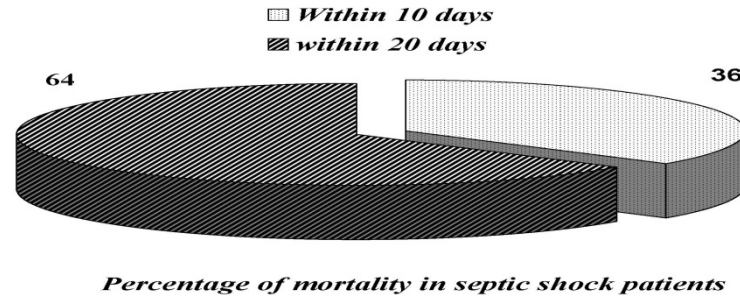


Figure (1): Percentage of mortality in septic shock patients

4. Discussion:

Sepsis is the number one cause of death among critically ill patients and accounts for more than 215,000 deaths every year in the United States alone^{2,13}. Randomized controlled trials emphasizing early resuscitation have improved the prognosis of sepsis by optimizing macrocirculatory parameters^{15,16}. Despite these advancements, as many as 21% to 28% of patients with severe sepsis or septic shock may die of the disease. The significant mortality that persists with maximization of global hemodynamic indices suggests that this approach may be insufficient as a total treatment strategy. Despite aggressive resuscitation, normal blood pressure, and adequate global oxygen delivery, septic patients often persist in exhibiting signs of tissue hypoperfusion, which may lead to acidosis and, ultimately, multiorgan failure^{2,13,15-16}.

In our study we found that patients with impaired renal function (78%, 31 patients) showed significantly decreased systolic & diastolic BP with significantly higher pulse rate than pts with normal renal functions.

These results could be explained by lower organ perfusion in this subset of patients who show more severe form of sepsis than patients with normal renal function.

Previous studies have reported that sepsis causes or contributes to AKI in 32–48% of patients¹⁷⁻¹⁸ & up to 64% in septic shock alone¹⁰.

In the PICARD study group¹⁹, who studied 611 critically ill patients in a multicenter observational study; 28% of patients had sepsis before AKI, 32% patients sepsis-free, and 40% developed sepsis 5 days after AKI diagnosis.

In the study by Bagshaw et al.,¹⁰ a total of 4,532 adult patients with septic shock were studied. 64.4% of patients with septic shock developed early AKI (i.e., within 24 h after onset of hypotension). By RIFLE criteria, 16.3% had risk, 29.4% had injury and 18.7% had failure.

The difference in percentage of AKI could be explained by the different definitions of acute renal

failure between studies.

Acute renal failure often accompanies sepsis due to acute tubular necrosis. The mechanism is complex but involve decrease effective intravascular volume due to systemic hypotension, direct renal vasoconstriction, release of cytokines, and activation of neutrophils by endotoxins and other peptides, which contribute to renal injury²⁰.

Patients with renal impairment and patients with septic shock had significantly higher liver enzymes, INR & bilirubin and significantly lower Hb & Hct compared to patients with normal renal function.

These markers included higher creatinine level, liver enzymes, bilirubin & higher INR. Patients with septic shock showed lower Hb than patients with SIRS or sepsis. These parameters were comparable to the laboratory results in septic shock patients in both studies conducted by Afifi et al.²¹, and Esmat et al.²². In both studies the parameters were pointing toward more severe organ dysfunction as compared to their control groups.

Hepatic dysfunction represents a common manifestation during the sepsis process, ranging from a mild elevation of serum bilirubin and/or liver enzymes to severe hepatic failure²³. The pathophysiology of liver injury in sepsis is multifactorial and involves infection, drugs, metabolic disturbances and a broad spectrum of inflammatory mediators²⁴.

In our study, troponin I was elevated in septic patients with renal impairment. In patients with severe renal dysfunction troponin T as well as troponin I, elevations are found that cannot be linked to myocardial injury. The reasons for these elevations are not yet convincingly explained. Reexpression of cardiac isoforms in skeletal muscles has been excluded by different analyses and investigators^{25, 26}. Loss of membrane integrity and constant outflow from the free cytosolic troponin pool as well as amplified elevation of normal low levels because of impaired renal excretion are more likely. The higher unbound cytosolic pool and higher molecular weight

may explain why troponin T is more frequently found elevated than troponin I²⁷.

In asymptomatic patients with renal dysfunction, troponins are not presently part of the routine diagnostic work-up because results with regard to their predictive value based on small series was controversially discussed²⁷.

In the prospective landmark study by Apple et al.,²⁸ serum was obtained from 733 end stage renal disease patients and measured for cardiac troponin I (cTnI) and T cTnI.

They documented 2- to 5-fold increase in all-cause mortality with increases in cTnT and cTnI in ESRD. Of particular interest is the gradual rise in risk with increasing troponin T levels independent of other variables at various discriminator levels. The level of troponins was associated with a significant increase in 1-, 2-, and 3-year mortality.

In our study, a more severe form of diastolic dysfunction was observed in patients with renal impairment. Those patients had higher LV dimensions and lower LVEF%.

The relation between cardiac & renal function in systemic illness is called the secondary cardiorenal syndrome or CRS type 5¹².

Sepsis as a precipitator of CRS Type 5 is common and its incidence is increasing, with a mortality estimated at 20%-60%^{13,14}. Approximately 11%-64% of septic patients develop AKI that is associated with a higher morbidity and mortality¹⁰. Abnormalities in cardiac function are also common in sepsis including wall motion abnormalities and transient reductions in left ventricular ejection fraction²⁹. Observational data have found approximately 30%-80% of individuals with sepsis have measurable blood troponin I or T that are above the 99th detection limits³⁰. These elevated cardiac biomarkers have been associated with reduced left ventricular function and higher mortality even in patients without known coronary disease^{31,32}. Importantly, volume overload as a result of aggressive fluid resuscitation appears to be a significant determinant of CRS Type 5. Among 3147 patients enrolled in the Sepsis Occurrence in Acutely Ill Patients (SOAP), there was a 36% incidence of AKI, and volume overload was the strongest predictor of mortality³¹. Iatrogenic volume overload appears to play an important additional role, possibly along passive venous congestion of the kidney, in the pathogenesis of AKI. At the same time, volume overload increases left ventricular wall tension and likely contributes to cardiac decompensation in those predisposed to both systolic and diastolic HF³². Cardiorenal syndrome Type 5, both AKI and markers of cardiac injury followed by volume overload are common in sepsis, with each being associated with

increased mortality. However, there is a current lack of integral information on the incidence of bidirectional organ failure and its pathophysiological correlates in a variety of acute care settings¹².

In our study, as regard cardiac functions measured by echocardiography, we found that LVEDD and LVESD were significantly higher & LVEF was significantly lower in group B (septic shock) compared to group A (sepsis or SIRS).

Reversible myocardial depression in patients with septic shock was first described in 1984 by Parker et al. using radionuclide cineangiography⁵. In a series of 20 patients, they reported a 65% incidence of left ventricular (LV) systolic dysfunction, defined by an ejection fraction <45%⁵.

In 1990, using transthoracic echocardiography, Jardin et al. reported the same results³³. The same authors published their series of 183 patients with septic shock³³⁻³⁶ a hypokinetic state at admission, as defined by a low cardiac index (< 3 L/minute/m²), was present in 64 patients (35%). Assessment of LV systolic function by echocardiography found this profile associated with a markedly hypokinetic LV (mean LV ejection fraction: 38 ± 17%)

More recently, Barraud et al. confirmed the presence of severe depressed intrinsic LV contractility using LV pressure/volume loops in lipopolysaccharide-treated rabbits³⁷. All of these studies, and many others demonstrate the reality of the impairment of intrinsic LV contractility in septic shock³⁸.

Many factors may contribute to cardiac depression during sepsis. Studies performed in humans have ruled out coronary hypoperfusion requiring coronary intervention as a cause of LV systolic dysfunction in sepsis^{39,40}.

On the other hand, the role of cytokines has been strongly advocated in the genesis of septic cardiomyopathy. In 1985, Parrillo et al. demonstrated in vitro that myocardial cell shortening is reduced by exposure to the serum of septic patients⁴¹. Later, the same team showed that the circulating factor responsible for this was tumor necrosis factor α (TNF- α)⁴², even though later studies have implicated other cytokines, such as interleukin-1 β ⁴³. Kumar et al. suggested that the effect of cytokines on cardiac myocytes results from an increase in intracellular cGMP and in nitric oxide⁴⁴. In addition, direct alteration in cellular respiration with mitochondrial dysfunction also was advocated⁴⁵, and, finally, Tavernier et al. suggested that increased phosphorylation of troponin I was involved by reducing myofilament response to Ca²⁺⁴⁶.

In our study we found significantly higher troponin level in group B in comparison to group A. This result matched with Arlati et al.,⁴⁷,

Kristren et al.,⁴⁸

In a recent large study by John et al.⁴⁹, elevated cTn I in patients with severe sepsis was associated with significantly high 28 days mortality.

In our study group B patients showed more severe form of diastolic dysfunction in the form of shorter DT & shorter IVRT E/A ration compared to patients with systemis sepsis.

These results matched with Munt et al.,⁵⁰ who measured deceleration time and E/A ratio in septic patients and they found significantly lower DT in non- survivors versus survivors while E/A ratio were lower in non-survivors (statistically not significant), and concluded that increased severity of diastolic dysfunction associated with increased mortality.

The severity of diastolic dysfunction in non-survivors compared to survivors could be explained by the same cause of myocardial dysfunction. The grading of diastolic dysfunction is very important in prognosis in patients with septic shock, as non-survivors showed more sever diastolic dysfunction which limited the adequate volume resuscitation which is essential for recovery in septic shock patients together with early inappropriate use of inotropic support (vasoactive drugs) in a relatively hypovolemic patients leading to increased tissue hypoperfusion and sever ischemia of vital organs⁵⁰.as they had patients with severe forms of septic shock in their study like patients of group B in our study.

In our study, 100% mortality in patients with septic shock compared to 0% mortality in sepsis and SIRS.

These results do not go with result of Shoemaker et al.,⁵¹ who showed 71.6 % mortality, and Vieillard et al.,⁵² who showed 60 % mortality in his study. Our results may be related to high degree of severity of septic shock in our subset of patients.

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References:

1. Bone RC, Balk RA, Cerra FB, Dellinger RP, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Chest* 1992;101:1644–1655.
2. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. *N Engl J Med*. 2003;348:138–150.
3. Riedemann NC, Guo R, Ward PA. Novel strategies for the treatment of sepsis. *Nat Med* 2003;9:517–524.
4. Maeder M, Fehr T, Rickli H, et al. Sepsis-Associated Myocardial Dysfunction. Diagnostic and Prognostic Impact of Cardiac Troponins and Natriuretic Peptides. *Chest* 2006;129(5),1349-1366.
5. Parker, MM, Shelhamer, JH, Bacharach, SL, et al.

6. Profound but reversible myocardial depression in patients with septic shock. *Ann Intern Med*. 1984;100,483-490.
6. Ognibene, FP, Parker, MM, Natanson, C, et al. Depressed left ventricular performance. Response to volume infusion in patients with sepsis and septic shock. *Chest* 1988;93,903-910.
7. Heitner SB and Hollenberg SM. The cardiac force-frequency relationship and frequency-dependent acceleration of relaxation are impaired in lipopolysaccharide - treated rats: is the phospholamban-SERCA axis a therapeutic target? *Critical Care* 2009, 13:132.
8. Joulin O, Marechaux S, Hassoun S, et al. Cardiac force-frequency relationship and frequency-dependent acceleration of relaxation are impaired in LPS-treated rats. *Crit Care* 2009, 13:R14.
9. Bouhemad B, Nicolas-Robin A, Arbelot C, et al. Isolated and reversible impairment of ventricular relaxation in patients with septic shock. *Crit Care Med*. 2008, 36:766-774.
10. Bagshaw SM, Lapinsky S, Dial S, et al. Acute kidney injury in septic shock: clinical outcomes and impact of duration of hypotension prior to initiation of antimicrobial therapy. *Intensive Care Med*. 2009;35:871–881.
11. Edelstein CL, Schrier RW, Pathophysiology of Ischemic Acute Renal Injury. In: Schrier RW (ed.), *Diseases of the Kidney and Urinary Tract* (vol 2), 8th ed., Philadelphia: Lippincott, Williams and Wilkins, 2007;930–61.
12. McCullough PA and Ahmad A. Cardiorenal syndromes. *World J Cardiol*. 2011 26; 3(1): 1–9.
13. Angus DC, Linde-Zwirble WT, Lidicker J, et al. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med*. 2001;29:1303–1310.
14. Martin GS, Mannino DM, Eaton S, et al. The epidemiology of sepsis in the United States from 1979 through 2000. *N Engl J Med*. 2003;348:1546–1554.
15. Lin SM, Huang CD, Lin HC, et al. A modified goal-directed protocol improves clinical outcomes in intensive care unit patients with septic shock: a randomized controlled trial. *Shock* 2006;26(6):551–7.
16. Jones AE, Brown MD, Trzeciak S, et al. The effect of a quantitative resuscitation strategy on mortality in patients with sepsis: a metaanalysis. *Crit Care Med*. 2008; 36(10):2734–9.
17. Bagshaw SM, Uchino S, Bellomo R, et al. Septic acute kidney injury in critically ill patients: clinical characteristics and outcomes. *Clin J Am Soc Nephrol*. 2007;2:431–439.
18. Bagshaw SM, George C, Bellomo R. Early acute kidney injury and sepsis: a multicentre evaluation. *Crit Care* 2008;12:R47.
19. Mehta RL, Bouchard J, Soroko SB, et al. Sepsis as a cause and consequence of acute kidney injury: Program to Improve Care in Acute Renal Disease (PICARD). *Intensive Care Med*. 2011; 37(2): 241–248.
20. Al-Khafaji AH, Pinsky MR. Multiorgan failure in sepsis, e-medicine, October 2011 update.
21. Afify M, Mokhtar S, El Naggar A, et al. Protein C in systemic inflammatory response syndrome, diagnostic and prognostic implication. Master thesis, Critical Care Department, Master Thesis, Critical care Department, Cairo University, 2003. (233)
22. Esmat A, Abdel Fattah A, Rizk A, et al. C reactive protein in view prognosis in sepsis. Master Thesis,

- Critical care Department, Cairo University, 2003.
23. Vermillion SE, Gregg JA, Baggenstoss AH, et al. Jaundice associated with bacteremia. *Arch Intern Med* 1969; 124: 611-618.
 24. Pastor CM, Billiar TR, Losser MR, et al. Liver injury during sepsis. *J Crit Care* 1995; **10**: 183-197.
 25. Haller C, Zehelein J, Remppis A, et al. Cardiac troponin T in patients with end-stage renal disease: absence of expression in truncal skeletal muscle. *Clin Chem*. 1998;44: 930-938.
 26. Fredericks S, Murray JF, Bewick M, et al. Cardiac troponin T and creatine kinase MB are not increased in exterior oblique muscle of patients with renal failure. *Clin Chem*. 2001;47: 1023-1030.
 27. Hamm CW, Giannitsis E, Katus HA. Cardiac Troponin Elevations in Patients Without Acute Coronary Syndrome. *Circulation* 2002; **106**: 2871-2872
 28. Apple FS, Murakami MM, Pearce LA, et al. Predictive value of cardiac troponin I and T for subsequent death in end stage renal disease. *Circulation* 2002; **106**: 2941-2945.
 29. Lopes JA, Jorge S, Resina C, et al. Acute renal failure in patients with sepsis. *Crit Care* 2007;11:411.
 30. Zanotti-Cavazzoni SL, Hollenberg SM. Cardiac dysfunction in severe sepsis and septic shock. *Curr Opin Crit Care* 2009;15:392-397.
 31. Favory R, Neviere R. Significance and interpretation of elevated troponin in septic patients. *Crit Care* 2006;10:224.
 32. Ammann P, Maggiorini M, Bertel O, et al. Troponin as a risk factor for mortality in critically ill patients without acute coronary syndromes. *J Am Coll Cardiol*. 2003;41:2004-2009.
 33. Jardin F, Brun-Ney D, Auvert B, Beauchet A, Bourdarias JP: Sepsis-related cardiogenic shock. *Crit Care Med*. 1990, 18:1055-1060.
 34. Jardin F, Valtier B, Beauchet A, et al. Invasive monitoring combined with two-dimensional echocardiographic study in septic shock. *Intensive Care Med*. 1994;20:550-554.
 35. Jardin F, Fourme T, Page B, et al. Persistent preload defect in severe sepsis despite fluid loading: a longitudinal echocardiographic study in patients with septic shock. *Chest* 1999; 116:1354-1359.
 36. Vieillard-Baron A, Schmitt JM, Beauchet A, et al. Early preload adaptation in septic shock? A transesophageal echocardiographic study. *Anesthesiology* 2001;94:400-406.
 37. Barraud D, Faivre V, Damy T, et al. Levosimendan restores both systolic and diastolic cardiac performance in lipopolysaccharide-treated rabbits: comparison with dobutamine and milrinone. *Crit Care Med*. 2007, 35:1376-1382.
 38. Vieillard-Baron A. Septic cardiomyopathy. Review. *Annals of Intensive Care* 2011, 1:6
 39. Cunnion RE, Schaer GK, Parker MM, et al. The coronary circulation in human septic shock. *Circulation* 1986, 73:637-644.
 40. Dhainaut JF, Huyghebaert MF, Monsallier JF, et al. Coronary hemodynamics and myocardial metabolism of lactate, free fatty acids, glucose, and ketones in patients with septic shock. *Circulation* 1987, 75:533-541.
 41. Parrillo JE, Burch C, Shelhamer JH, et al. A circulating myocardial depressant substance in humans with septic shock. Septic shock patients with a reduced ejection fraction have a circulating factor that depresses in vitro myocardial cell performance. *J Clin Invest*. 1985, 76:1539-1553.
 42. Hollenberg SM, Cunnion RE, Lawrence M: Tumor necrosis factor depress myocardial cell function: results using an in vitro assay of myocyte performance. *Clin Res*. 1989, 37:528-534.
 43. Kumar A, Thota V, Dee L, et al. Tumor necrosis factor-alpha and interleukin-1 beta are responsible for depression of in-vitro myocardial cell contractility induced by serum from human septic shock. *J Exp Med*. 1996, 183:949-958.
 44. Kumar A, Brar R, Wang P, et al. Role of nitric oxide and cGMP in human septic serum-induced depression of cardiac myocyte contractility. *Am J Physiol*. 1999, 276:R265-R276.
 45. Levy RJ, Piel DA, Acton PD. Evidence of myocardial hibernation in the septic heart. *Crit Care Med*. 2005, 33:2752-2756.
 46. Tavernier B, Li JM, El-Omar MM, et al. Cardiac contractility impairment associated with increased phosphorylation of troponin I in endotoxemic rats. *FASEB J* 2001, 15:294-296.
 47. Arlati S, Brenna S, Prencipe L, et al. Myocardial necrosis in ICU patients with acute non cardiac disease : a prospective study *Intensive Care Med*. 2000;26: 31- 37.
 48. Kristien M., Spopen H., Ngmyen D. Cardiac troponin I and T are biological markers of left ventricular dysfunction in septic shock . *Clinical Chemistry* 2000; 46 650-657.
 49. John J, Woodward DB, Wang Y, et al. Troponin-I as a prognosticator of mortality in severe sepsis patients. *Journal of Critical Care* 2010, 25(2); 270-75.
 50. Munt B, Jue J, Gin K, et al. :Diastolic filling in human severe sepsis :an echocardiographic study. *Crit Care Med*. 1998,26:1829-1833.
 51. Shoemaker WC, Apel, pl , Kram HB, et al . Sequence of physiologic patterns in surgical septic shock. *Critical Care Med*. 1993 12 (12);1876-1889.
 52. Vieillard A-prins S, Chergni K, Jardin F. Haemodynamic instability in sepsis bedside assessment by Doppler echocardiography. *Am J of Respiratory and Critical Care Medicine* 2003,168:1270-1276.

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The Hydrology of Wadi Ibrahim Catchment in Makkah City, the Kingdom of Saudi Arabia: The Interplay of Urban Development and Flash Flood Hazards

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Abstract: This paper investigates the development of a hydrological model for the dryland catchment of Wadi Ibrahim, which hosts the Holy Mosque of Makkah, in order to assess the interaction of urbanizing the alluvial channels and the flow discharges of occasional flash floods. The original landscape and landcover of the catchment have witnessed significant changes during the past few decades, where most of the alluvial channels and the mountain footslopes have been covered by urban. The infrequent threat of flash floods has prompted the development of a mitigation measure including; installation of rainfall-sewage system and subsurface culverts, in addition to the conveyance of flows from the upper sub-catchment into another drainage basin. However, the latest flash flood event of 30th of December 2010 has resulted in fatalities and demonstrated the insufficiency of the current mitigation system to control flash floods. The runoff coefficient was estimated from the opportunistic observations and measurements of the flow discharge parameters for the latest event, in addition to the recorded rainfall parameters. The digital elevation model (DEM) was analyzed using Geographic Information System (GIS) to determine the spatially distributed time-areas zones of the catchment, which were used to simulate the runoff hydrographs under certain runoff coefficients and designed storms of long return periods. The development of urban areas on expense of the alluvial channels resulted in a significant surge of runoff discharge, and therefore increasing the threat of flash floods on urban areas downstream. As a result the transmission loss is diminishing; thus raising the alarm on the potential recharge to the underlying alluvial aquifer of the sacred well of Zamzam. Therefore, it is suggested that several small dams to be constructed at the fingertip drainage channels; to retain considerable amount of water and sediment within the catchment and to act as point-source recharge to the alluvial aquifer.

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1. Introduction:

The drylands are characterized by droughts and the scarcity of water resources, occasional heavy storms often develop severe flash floods that devastate the inhabited areas (Cooke et al., 1982; Tooth, 2000; Foody et al., 2004). The records of destructive flash floods and the notable regional frequencies have largely raised the public awareness and motivated more scientific research on the hydrological processes within the fluvial system (e.g. Walling and Gregory, 1970; Chin and Gregory, 2001). Originally, flash flood frequencies and magnitudes are controlled by the interplay of different natural variables, including precipitation, antecedent conditions of the catchment, distribution of alluvium and water storage areas, etc. However, the growth of urbanization within the catchments can be added as an additional significant controlling factor for the development of flash flood. Urbanization, through the construction of impervious

surfaces; building, roads, storm sewers and paving usually decreases the infiltration capacities of the underlying soils, and it significantly increase runoff-discharge downstream. The estimated increase of runoff coefficients; higher flow peaks and the decrease of time to peak in urban catchment Depends on the extent of urbanization and the anthropogenic managements of runoff of these catchments (Suriya and Mudgal, in press (2011). In completely impervious and fully sewerred areas, peak discharge increases 6 times more than in non-urbanized areas (leveson, 1980), and 90 % of the total rainfall may be converted into urban runoff (Shang and Wilson, 2009). However, the assessment of urbanization impact on the hydrological response of the developed catchments is complex and hampered by the lack of hydrological parameters and measurements, and the non-systematic temporal changes of the landuse and landcover due to urbanization (Smith and Bedient, 1981).

Generally, the hydrological data collected in drylands remain insufficient and limited due to technical, political and economical factors, and therefore, the hydrological processes are not fully understood and the hydrological models are uncalibrated (El Bastawesy et al., 2009). Nonetheless, most of these data are also limited and gathered by individuals or entities during pilot projects and various independent case studies, which cannot represent the diversity within dryland setting and processes. It is also difficult to monitor flash floods that occur suddenly, and the potential to capture during classical-field experiments is very rare. Therefore, the investigation of most flash flood-events was mainly based on post-event survey, which plays a critical role in gathering essential observations and data (Borga et al., 2008). The spatial and temporal variability of flash flood events even within a single catchment clearly demonstrate the need for the development of distributed model; to assess vulnerability and to mitigate against future damage.

Remote sensing data and Geographic Information System (GIS) techniques are widely used to estimate various distributed hydrological parameters for the investigation of catchment hydrology following given techniques and interpretations (Schultz, 1987; Scipal et al., 2005; Milzow et al., 2008). The most straightforward use of remote sensing images is to identify geologic, geomorphologic and landuse-land cover features, which in turn have a strong influence on overland flow generation. Several remote sensing-products such as the Tropical Rainfall Monitoring Mission (TRMM) and the Global Precipitation Climatology Project (GPCP) are increasingly available to measure the precipitation at semi-global coverage with a grid spatial resolution of $0.25^0 \times 0.25^0$ (Huffman et al., 2007; Hossain et al., 2011). The remote sensing-rainfall estimates are widely utilized in the hydrological models, particularly over the areas with poor or no rain-gauges data (e.g. Milewski et al., 2009; Abu El Magd et al., 2010). However, the uncertainty of remote sensing-rainfall estimates on flood prediction has to be considered; the calibration with in situ data in different gauged areas showed non-systematic overestimation and underestimation (Almazroui, 2011). Although, the remote sensing-based rainfall estimates may represent the sole source of precipitation input to any hydrological model for the dryland catchments.

The digital elevation models (DEM) are mainly obtained from different sources and at different spatial resolution, and therefore are widely being used in the various hydrological models. The automatic delineation of catchment-hydrographic

parameters from the DEM has gradually replaced the traditional manual delineation of these parameters from the conventional topographic maps (Band, 1986; Chorowicz et al., 1992). The manual method is a tedious and error-prone technique in dryland alluvial areas, where the thalweg or active longitudinal channel courses are not marked on topographic maps and significant changes in these courses can take place over relatively short periods of time. The major issues associated with the derivation of surface drainage networks from DEM are related to the quality, source and resolution of the DEM and to the processing techniques and algorithms employed (Zhang and Montgomery, 1994; Wolock and Price, 1994). However, the delineation of various morphometric parameters (for a typical dryland catchment) was not very sensitive to the change of DEM resolution (from 20 m to 90 m) (El bastawesy, 2007).

The aim of this paper is to develop a hydrological model for a typical dryland catchment, which has undergone dramatic urban expansion, in order to assess the impact of flash flood hazard on urban areas, and also to determine the effect of constructed mitigation measures on the fragile surface and groundwater resources of the area. Herein the Wadi Ibrahim catchment of Makkah city in the Kingdom of Saudi Arabia is selected for this study, due to its importance as it hosts the Holy Mosque of Makkah and considerable in situ data are available.

The study area:

Makkah city is located in the southwestern part of Al Hijaz province of the Kingdom of Saudi Arabia, between the low-lying coastal plain (Tihamat Al Hijaz) and the escarpment of the rugged Sarawat mountains that has resulted from the uplift associated with the Red Sea rifting. The mountain ranges of Makkah are structurally controlled; they are aligned in northwest-southeast (e.g. Mena Mountains) or east-west directions (e.g. El Tarqi Mountains). However, few mountains are isolated and conform semi-circular shape such as Thour Mountain (755 m) and El Nour Mountain (642 m). The chronology of underlying rocks units in Makkah area is complex; the isolated outcrops of amphibolites, gneiss and schist are of uncertain stratigraphic relationship to other layered rock units of the mountain ranges, and thus they are left unassigned on the geological map of the area (Fig 1). The predominant rock mass underlying the catchment area of Wadi Ibrahim belongs to the Precambrian, and mainly composed of quartz diorite and tonalite (Kamil Suite). These outcrops are of moderate to steep relief, and also range from massive to well deformed and foliated plutons. The Wadis of Makkah dissecting the

mountain ranges are characterized by complex and interlocking patterns. This complex pattern of intersecting alluvial areas are very common in the dryland setting and reflect the morphotectonic evolution of these drainage basins, where the paleochannels were used to flow through different directions than the contemporaneous flow pathways (e.g. El Bastawesy et al., 2010). The Quaternary wadi alluvium consists of unconsolidated, moderately to poorly sorted sand and gravel. The alluvium of the upper reaches of wadi courses is relatively thin and very poorly sorted, but it becomes well sorted and reaches considerable thickness in the lower reaches.



Fig 1: location map of the study area.

The urban pattern of Makkah metropolitan city is unique and controlled by the geomorphological setting; the city has sprawled in radial direction on the limited surface areas of the complex alluvial channels separated by the steep mountain ranges. It is estimated that approximately 9000 hectares of alluvial channels and footslopes of the mountain were converted into urban areas from 1978 to 2000 (Al-Ghamdi and Al-Najjar, 2002). The development also encroached the alluvial corridors, which dissect the barrier mountain ranges, and thus the urban areas in different subcatchments are locally connected. Moreover, tunnels or rock-cut corridors are commonly constructed in mountain ranges that obstacle the connectivity of neighborhood. The Holy Mosque of Makkah (i.e. Al Al Masjed Al Haram) is located within the lower reaches of Wadi Ibrahim catchment; it embraces the Kaaba, and the sacred well of Zamzam (located in a basement room 20 m east of the Kaaba). The Zamzam well is about 30 m deep and it taps the groundwater from the wadi alluvium (i.e. the upper half) and the underlying

fractured and weathered basement rocks (i.e. the lower half). The well is heavily pumped to provide millions of visitors with sacred water which is available throughout the Holy Mosque via water fountains and dispensing containers.

The earliest available record of rainfall for Makkah area is dated back to 1966, when the Saudi Meteorological Authority installed a gauging station at Umm Al-Gud of Makkah. However another rainfall gauging station was installed by the University of Umm Al-Qura at the main campus in 1989, the rainfall data remains limited. Therefore, The Custodian of the Two Holy Mosques has installed 2 additional automatic network weather stations around Makkah since 2000 to deliver accurate, and reliable meteorological measurements that can be used in hydrological applications. Overall, the available rainfall data of Makkah area shows a very high spatial and temporal variability. For example the total annual rainfall in 1980 was less than 5 mm, while the total rainfall of 1969 was 318 mm of which more 269 mm was precipitated in one single storm (Fig 2). Although surge in local rainfall magnitudes over the catchments of Makkah city is vital to the recharge of the underlying aquifer, but it usually develops severe and unfavorable flash floods. For example, a destructive flash flood has occurred in 1969 following the precipitation of more than 269 mm of rainfall over Makkah; the water partially filled the holy mosque and stood 2.5 m higher above the floor in vicinity of the Kaaba (Yousef, 1992).

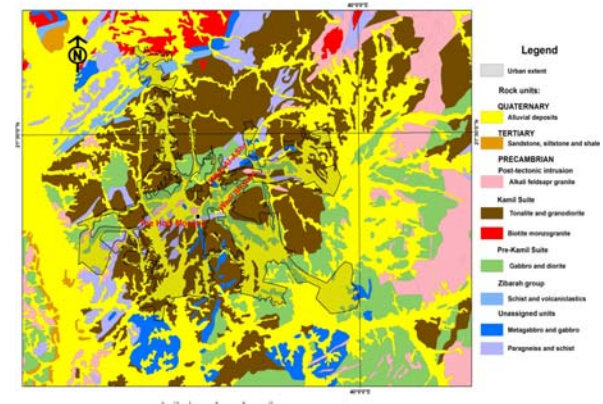


Fig 2: A simplified geological map shows the main rock units and the complex alluvial patterns, note the extent of developed urban areas

As a result of the repeatability of flash floods several mitigation measures and different engineering structures are constructed to collect and discharge the runoff from the urban and mountaneous areas in Wadi Ibrahim. An open channel with a maximum cross-sectional area of 2 m² constructed in the upstream zone of the catchment, in addition to

several other interconnected lines of box culverts (cross sections vary from 4.5 to 18 m²) constructed in the middle and lower parts of the catchment. A new culvert of 2.5 m depth and 3.5m width is being constructed to convey the runoff water from the urban areas within the upper sub-catchment northward into neighboring Wadi Al-Ashr via an alluvial channel, which breaches the barrier mountain range (Saudi Geological Survey, 2011). However, hazardous flash floods are developed and the most recent event of 30th of December 2010 has produced fatalities as large urban areas were flooded. Therefore, it is necessary to assess the impact of recent development in Wadi Ibrahim on the hydrological balance and the adequacy of the installed flash flood mitigation measures. Visual interpretation of recent satellite images shows that most of the alluvial bed of Wadi Ibrahim has been converted into urban areas, except few small and scattered parcels of bare alluvial soil in the upper sub-catchment. There is also a growing concern of the impact of these landcover changes on the recharge and quality of the groundwater water within Zamzam well surface catchment (Saudi Geological Survey, 2011).

2. Data and methods:

The morphometrical parameters of Wadi Ibrahim catchment was delineated from the available SRTM DEM following the ArcInfo multi-steps procedure of the D-8 method (Fig 3). The processing of a DEM to produce hydrologically correct and connected drainage networks requires that sinks be first removed (Jenson and Dominique, 1988; Mark, 1984; Wise, 2000). The naturally occurring sinks are not common within the study area. Only few cells were missing elevation data (i.e. voids) resulting from the generation technique of the SRTM DEM, and thus they have been assigned elevation values from the neighbouring cells via the 'filling' step. A given sink is filled to reach the nearest lowest elevation, the boundaries of the filled area may then be part of new sinks, which then need to be filled, and this iteration process is repeated until all the pits are removed. Once a hydrologically connected DEM is produced by sink removal, flow direction is calculated for each cell in the DEM into the most downslope of the neighbouring cells. The 'flow direction' grid is then used to calculate a grid of 'flow accumulation', and finally thresholding is applied to extract up-slope contributing areas for all the drainage channels in the DEM (Jenson and Domingue, 1988). The resulting drainage networks were overlaid on the satellite images to check that they correspond to visible wadis and to identify the

urban areas constructed in the pathways of these thalweg drainage lines.

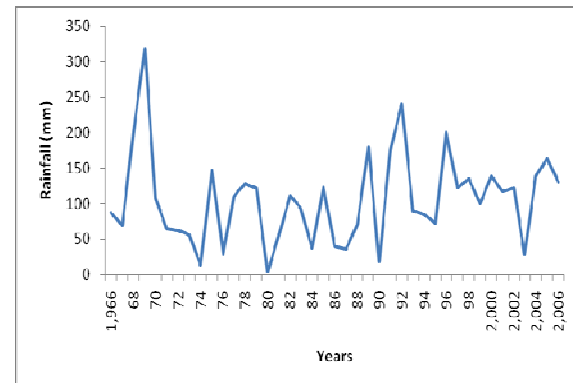


Fig 3: The total annual rainfall of Makkah area modified after Yousef (1992).

The DEM was also used to determine the spatially distributed time-area zones of the catchment, which conforms the runoff hydrograph when rainfall pattern and abstraction losses are uniform across the catchment (Maidment, 1993). It is interesting to note that the application of pure flow routing that neglects the volume of losses as the flow is transmitted from one zone into another, was not appropriate to the modeling of dryland hydrographs, as it omits one dominant hydrological processes (i.e. transmission loss into alluvium channels) (El Bastawesy et al., 2009). Here in the alluvium channels are no longer recognized and have been converted into urban. Therefore the Maidment technique of pure flow routing is acceptable; it will be used to estimate the runoff hydrograph resulting from the extreme rainfall events. Therefore, this time-area diagram for the catchment can represent a spatially distributed unit hydrograph without the need for empirical functions for the time of concentration. The flow routing is simulated as a purely translation process that neglects abstractions due to storage or loss over the flow pathway, and the runoff produced from any time-area zone will reach outlet of the catchment in a given time. Runoff will follow the same routes with the same velocity regardless of excess rainfall depth. The runoff velocities were estimated using the Manning Equation (equation 1), which is widely been used in modeling the flows of natural open-channels as well as storm-drainage from urban areas (e.g. Ramier et al., 2011).

$$V = (R^{0.67} * S^{0.5}) / n \quad \text{equation (1)}$$

Where V is the cross-sectional average velocity (m s⁻¹); n is the Manning coefficient of roughness; R is the hydraulic radius (m); S is the slope of the water surface, which is assumed to be parallel to the slope

of the channel bed. Municipal streets, whether planned or not, conveys large portion of runoff, and the flow- parameters of cross sectional areas (i.e. width * height of the platform) and wetted perimeter were measured. The Manning's n was averaged and set to 0.06 for hillslopes and 0.025 for urban areas; these values are typical of reported values in literature (e.g. Mignot et al., 2006). Slopes were estimated from the available DEM. Once these time-area zones was determined, the climatic data obtained for Makkah area were then analyzed to estimate the designed storm that will be used in computing the hydrograph for the catchment. A statistical probability technique was adopted; first the climatic data was ranked (i.e. from highest to lowest), then the return periods of the extreme storm was calculated using the following probability function (FAO, 1991):

$$P(\%) = (M - 0.375 * 100) / (N + 0.25) \quad \text{equation (2)}$$

Where; P = probability in (%) of the observation, M = the rank of observation, and N = the total number of observation used.

A satellite image of SPOT 5 acquired at 2010 was also investigated to determine the landcover and hydrological parameters within the catchment (Fig 4).

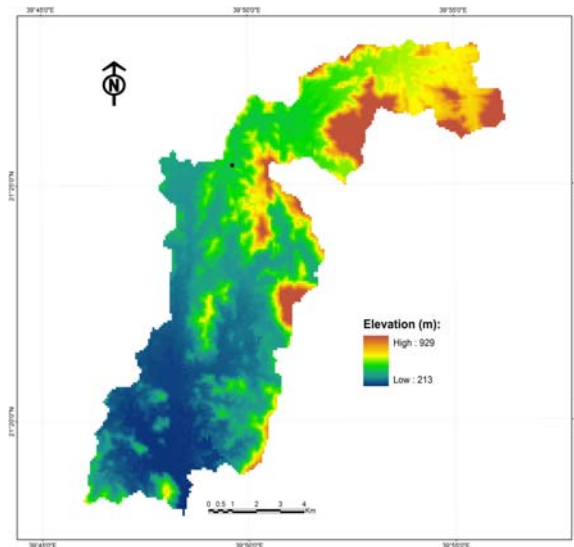


Fig 4: The SRTM DEM for Wadi Ibrahim catchment, location of The Holy Mosque is indicated by the black square.

3. Results:

Hydrology of the dry land catchment of Wadi Ibrahim is modeled to assess the impact of the occasional flash floods hazard, and to address the negative impact of the adopted mitigation measures. It is so important to note, therefore the drastic

changes of the landscape on the recharge to groundwater aquifer feeding the sacred well of Zamzam. The analysis of recent satellite images and field observations showed that urban development has sprawled on the limited alluvium soils and adjacent foot slopes of the mountains. Because the estimated infiltration rates at the point-scale by the 'double ring' are highly variable and associated with much uncertainty with up-scaling to the catchment level; the simulated hydrograph considered a uniformly distributed runoff coefficients estimated from the opportunistic observations and measurements of the 30th of December 2010 hydroclimatical parameters. The discharge was measured at a well-defined cross sectional area of the trapezoidal concrete channel in the upper part of Wadi Ibrahim (Fig 5). The upstream flow contributing area at this channel cross section is approximately 6.7 km², the rainfall total depth was approximately 51 mm as measured at the nearest meteorological station of The Custodian of the Two Holy Mosques institute for Hajj Research, and the estimated runoff coefficient of this specific sub catchment is 60%. The catchment-simulated runoff hydrographs for the 30th of December flash flood event is shown in Fig (6). It is important to consider that the urban development on the bare-soil parcels in the catchment will increase the runoff coefficient, as the alluvial surfaces will be obscured and mostly covered by urban features. Thus, the sensitivity of runoff hydrographs simulated at different runoff coefficients (i.e. for bare soil, hill slopes and urban areas) was investigated, and also the scenario of developing urban areas on these bare soil was tested for the impact on hydrograph magnitudes. These results show that the subtle changes in runoff coefficient was of significant impact on the magnitudes and attenuations of hydrographs (Fig 7). However, the changes of runoff coefficient for bare soil parcel were of the least impact on the developed hydrographs; it may be because these alluvium soils cover small surface areas of the whole catchment. In contrary, in large-area catchments the hydrograph (i.e. attenuation and duration) is mostly determined by the runoff coefficient and transmission loss into this alluvium (El Bastawesy et al., 2009). Frankly speaking, the flows developed within the upper reaches of large and undeveloped catchments may not reach the outlet as they totally seep into the underlying alluvium while moving downstream along these channels. In the catchment of Wadi Ibrahim urban areas are developed on almost all of the alluvial channel beds and these changes of the land cover has minimized the transmission loss. But on the other hand, it has amplified the peaks of hydrographs of the flash floods, and it thus increased

the threat of flash floods to urban areas in the downstream. Tens of vehicles were drifted during the 30th of December 2010 flash flood event; and as a result several people lost their lives. The torrential flows were accompanied with loads of sediments gullied from the remnant alluvial terraces hanging upon some hill slopes as well as some rock boulders of different sizes, which were dragged by flows from the surrounding hill slopes and the artificially-cut foot slopes areas. The sediment loads can reduce the competent of the installed rainfall-sewage system, which could be partially blocked by the trapped sediments. However, the captured photos on 30th of December 2010 clearly show that the capacity of the culverts-discharge was exceeded and the flow depth reached more than 50 cm on the main roads located in the downstream of Wadi Ibrahim. Furthermore, the flood water has gushed into air from a manhole cover of one of the sewage- culverts, which may have contributed to increasing the surface runoff on downstream urban areas (Fig 8). These undesired outflows may have developed by the intense pressure caused by excessive water than the designed-discharge capacity of the culverts, or due to the clogging of this culvert by washed debris at some point downstream. Therefore, the likely development of a flash flood from more intense rainstorm (i.e. similar to those of 1969 and 1998), may be of serious consequences, unless extra-mitigation measures are considered. The current installed culverts and rainfall-sewage system were not sufficient to control the flows of flash floods during the latest event.



Fig 6: A field photo of the channeled flash flood of the 30th of the 30th of December 2010 event.

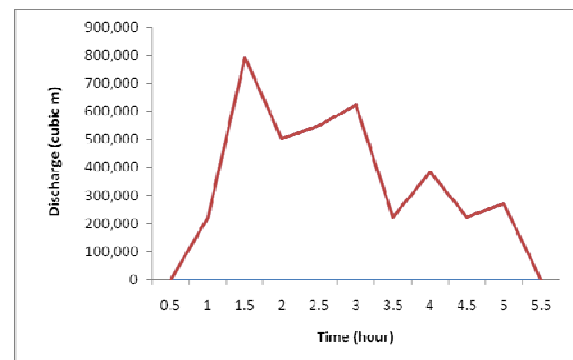


Fig 7: The simulated hydrograph for the 30th of December flash flood event in Wadi Ibrahim.

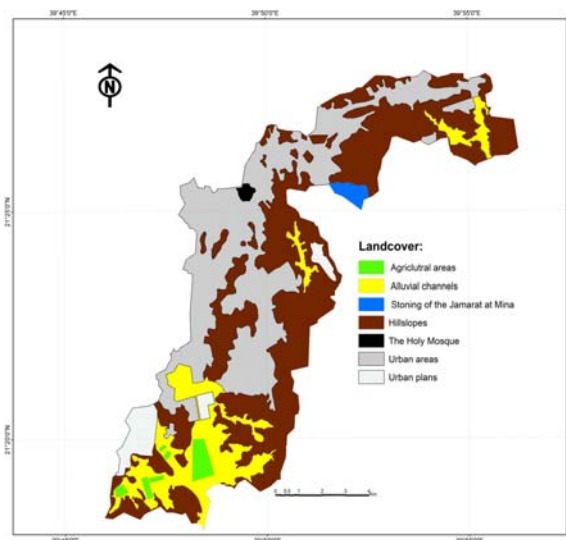


Fig 5: The determined landcover units for Wadi Ibrahim using a SPOT 5 satellite images of 2010.

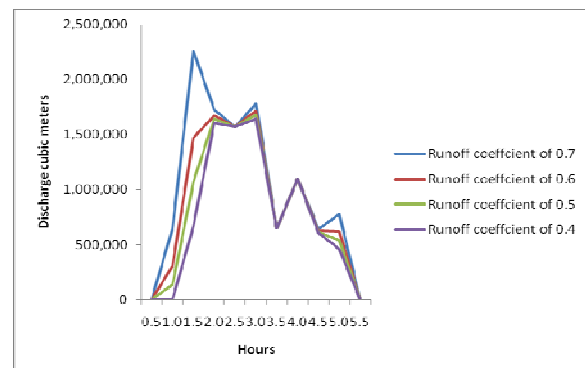


Fig 8: The sensitivity of simulated hydrographs for changes in the landuse and associate runoff coefficients, note the increase of peak discharge and the decrease of time to peak when runoff coefficient is increasing.



Fig 9: The outflows from sewers (top) and the runoff hazards on municipal streets of Makkah during the 30th of December 2010 flash flood (bottom).

4. Discussion:

The hydrology of flash floods developed within the dryland catchment is usually not fully understood. The observations and in situ data measurements are sparse; also the gauging of flash floods is very rare when compared with the seasonal or perennial flows of more humid areas. As such the modeling of flash floods can be grossly inadequate, particularly when it is based on calculated empirical parameters or data obtained from very few rainfall and runoff gauging stations. This data limitation may lead to erroneous conclusion, as the intricate meso-scale atmospheric patterns and complex terrain processes that control desert flash floods are neglected (Pilgrim et al., 1998; Greenbaum et al., 1998). The alternative approach to indirectly estimate flow discharge from the analyses of paleostage indicator (i.e. slack water deposits and

drift shrubs and wood lines) (e.g. Baker, 1987) is also insufficient to represent the complex spatial and temporal variability of flows and associated sediment erosion pattern. Therefore, the developed hydrographs of dryland catchments always contains considerable uncertainty. Lots of research has investigated the subtle sensitivity of the hydrological model results to the minor changes in values of used parameters, such as the empirical roughness coefficient of Manning's widely used to estimate channel-flow velocities (El Bastawesy et al., 2009).

Thus, the modeling of flash floods needs to be further improved to minimize uncertainty in order to better manage these torrential flows. Particularly for urban areas and facilities that are being rapidly developed within certain dryland catchments due to, but not limited to the availability of local valuable socioeconomic resources. In Wadi Ibrahim catchment various mitigation measures are currently adopted to control the impact of flash floods on the Holy Mosque as well as other urban areas. The efficiency of these installed measures was recently tested as a severe flash flood has occurred on the 30th of December 2010 following the precipitation of 51 mm on average. Unfortunately, the modeling approach behind these implemented projects are not available, and it is not known if these engineering structures had considered the scenarios of landuse and landcover change within the catchment for their impact on altering the hydrograph as well as recharge to the ground water aquifer of the area. It is clear that the problems created by converting alluvial areas into urban are complex, and the concept of 'complete' flood control yet does not exist. In Wadi Ibrahim two main methods are being adopted to control flash floods, 1); Rainfall waters are collected through a sewage networks and confined subsurface culverts 2); Conveying flash floods from the upper part of Wadi Ibrahim into Wadi Al Ashr via a subsurface culvert, which is being fed from a rainfall sewage system and an open-lined channel. It was noticed that a considerable amount of flows during the latest event were moving along the main municipal streets; thus adequacy of the current rainfall-sewage system can be questioned. Obviously flash floods will continue to be a threat for urban areas in Wadi Ibrahim unless a more competent management is addressed. Herein it is suggested that several small-size dams have to be constructed at the bottom of the fingertip drainage to retain flows and sediments. Consequently high-velocity runoff will be reduced and values of peak discharge will be lessened. Moreover, these small dams can act as a point-source recharge to the alluvial aquifer, where most of the harvested water will seep into the underlying alluvium.

Of course further investigation is required for this particular suggestion to assess the cost-benefit analysis, feasibility of construction and the hydrological connectivity of the first-order drainage segments to the main alluvial aquifer. In the mean time, the entire hydrological management of Wadi Ibrahim catchment needs a further assessment of the effluent pattern of rainfall-sewage downstream. Indeed, the prevailing arid conditions and the severe shortage of underground water resources in the area require a careful management of the fragile water resources. The drainage pattern of Red Sea region (i.e. including Makkah area) is complex, structurally controlled and underwent different phases of morphotectonic evolution related to the Tertiary-Quaternary motions in connection with Red Sea Rifting (Alwash and Zakir, 1992). As such the alluvium thickness is highly variable along the downstream profile of these wadis; several contact-springs used to discharge water along the transect fault Plains. The alluvial aquifers in Makkah area are mostly depleted by excessive pumping of the groundwater for local irrigation and potable water supplies (Alwash et al., 1986). The current outlets of the rainfall-sewage for Wadi Ibrahim as well as for the neighboring urbanized catchments, which are embracing the metropolitan city of Makkah, can be extended to a specific alluvial channel. This proposed collection of the harvested runoff would maximize the benefit from severe rainfall storms for groundwater recharge. Ideally, the selected alluvial reach has to be underlain by 'a trough' that attains considerable thickness, and also it should be hydrologically separate from contamination by other waste dump-sites and sanitary sewage outlets.

Conclusion:

The problem of flash floods in Wadi Ibrahim catchment is prominent, and it has been further complicated by the sprawl of urban development on the alluvial channels. Consequently, the significant reduction of transmission losses into the underlying alluvium has increased the runoff coefficient and discharge from this catchment. The adopted management strategies for the flash flood included the construction of a rainfall-sewage system in addition to conveyance culverts in the upper part of the catchment to transfer the flows into Wadi Al Ashr (i.e. a hydrologically separate drainage basin). The latest flash flood event of the 30th of December 2010 clearly showed the insufficiency of the current mitigation measures to deal with flash floods; the main municipal streets were flooded and fatalities occurred as several vehicles were drifted. It is evident that the urban areas of Wadi Ibrahim are prone to more serious threat of flash floods, particularly if

more severe rainfall events (e.g. The 1969 and 1998 storms) are re-occurred. Indeed the flows of these severe storms will far exceed the maximum discharge capacities of installed culverts, and will develop considerable hazards. Furthermore, gulying of sediments from the abandoned fluvial terraces on hillslopes can cause clogging problems to the rainfall-sewage and delimit their designed capacity. It is highly recommended to consider the construction of several small dams at the fingertip channels, in order to retain considerable amount of water and sediments. These dams could be acting as a point-source recharge to the underlying alluvium aquifer, as the original replenishment of this aquifer from channeled runoff was greatly reduced by urban development.

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References:

- Abou El-Magd, I. H., Hermas, E. A., Bastawesy, M. 2010. Integration of hydrological models and GIS for the assessment and prediction of natural hazards in Abu Dabbab area, Red Sea region, Egypt. The Egyptian Journal of Remote Sensing and Space Sciences, 13, 81 - 88.
- Al-Ghamdi, S. A., and Al-Najjar, Y. M., 2002. Analysis of urban growth and its patterns using remotely sensed data: The case study of the Holy City of Makkah Al-Mukkramah (1987-2000). Journal of King Abdul-Aziz University, Special issue of the 20th Anniversary of King Fahd Coronation, pp231-273.
- Almazroui, M., 2011. Calibration of TRMM rainfall climatology over Saudi Arabia during 1998–2009. Atmospheric Research, 99, 400-414.
- Alwash, M.A. Zaidi, S.M.S. and Terhalle, U., 1986. Description of arid geomorphic features using landsat-TM data and ground truth information (Wadi Fatima, Kingdom of Saudi Arabia). Catena, 13, 277-293.

- Alwash, M.A., and Zakir, F.A.R., 1992. Tectonic analysis of the Jeddah Taif area on the basis of LANDSAT satellite data. *Journal of African Earth Sciences (and The Middle East)*, 15,293-301.
- Baker, V. R. 1987. Paleoflood hydrology and extraordinary flood events. *Journal of Hydrology*, 96, 79-99.
- Band, L. E. 1986. Topographic partition of watershed with digital elevation models. *Water Resources Research*, 22, 15-24.
- Borga, M., Gaume, E., Creutin, J. D., and Marchi, L., 2008. Surveying flash floods: gauging the ungauged extremes. *Hydrological processes*, 22, 3883-3885.
- Chin, A., Gregory, K.J., 2001. Urbanization and adjustment of ephemeral stream channels. *Annals of the Association of American Geographers* 91, 595-608.
- Chorowicz, J., Ichoku, C., Riazanoff, S., Kim, Y. and Cervelle, B. 1992. A combined algorithm for automated drainage network extraction. *Water Resources Research*, 28, 1293-1032.
- Cooke, R., Brunnsden, D., Doornkamp, J., Jones, D., 1982. *Urban Geomorphology in Drylands*. Oxford University Press, Oxford.
- El bastawesy, M., 2007. Influence of DEM source and resolution on the hydrographical simulation of Wadi Keed catchment, Sinia, Egypt. *Egyptian Journal of Remote Sensing and Space Sciences*, 10, 53-62.
- El Bastawesy, M., White, K., and Nasr, A., 2009. Integration of remote sensing and GIS for modelling flash floods in Wadi Hudain catchment, Egypt. *Hydrological Processes*, 23, 1359-1368.
- El Bastawesy, M., Faid, A., and El Gammal, E., 2010. The Quaternary development of tributary channels to the Nile River at Kom Ombo area, Eastern Desert of Egypt, and their implication for groundwater resources. *Hydrological processes*, 24, 1856-1865.
- FAO. 1991. *Water Harvesting. A manual for the design and construction of water harvesting schemes for plant production*, Rome, Italy.
- Foody, G.M., Ghoneim, E M., and Arnell, N.W., 2004. Predicting locations sensitive to flash flooding in an arid environment. *Journal of Hydrology*, 292, 48-58.
- Gheith, H., Sultan, M., 2002. Construction of a hydrologic model for estimating Wadi runoff and groundwater recharge in the Eastern Desert, Egypt. *Journal of Hydrology* 263, 36-55.
- Greenbaum, N., Margalit, A., Schick, A. P., Sharon, D., Baker, V.R., 1998. A high magnitude storm and flood in a hyperarid catchment, Nahal Zin, Negev Desert, Israel. *Hydrological Processes*, 12, 1-23.
- Hossain, F., Anagnostou, E. N., and Dinku, T., 2011. Sensitivity Analyses of Satellite Rainfall Retrieval and Sampling Error on Flood Prediction Uncertainty. *IEEE Transactions On Geoscience and Remote Sensing*, 42, 130-139.
- Huffman, G.J., Adler, R. F., Bolvin, D. T., Gu, G., Nelkin, E. J., Bowman, K. P., Hong, Y., Stocker, E. F., Wolff, D. B., 2007. The TRMM Multi-satellite Precipitation Analysis: Quasi-Global, Multi-Year, Combined-Sensor Precipitation Estimates at Fine Scale. *Journal of Hydrometeorology*, 8, 38-55.
- Jenson, S. K., Domingue, J. O., 1988. Extracting topographic structure from digital elevation data for geographical information system analysis. *Photogrammetric Engineering and Remote Sensing*, 54, 1593-1600.
- Leveson, D., 1980. *Geology and the urban environment*. Oxford University Press, New York, 386pp.
- Maidment, D. R., 1993. Developing a spatially distributed unit hydrograph by using GIS. In: *Proceeding of the Vienna Conference on Application of geographic Information Syaten in Hydrology and Water Resources*, IAHS, 211, 181-192.
- Mark, D M., 1984. Automatic detection of drainage networks from digital elevation models. *Cartographica*, 21, 168-178.
- Mignot, E., Paquier, A., Haider, S., 2006. Modeling floods in a dense urban area using 2D shallow water equations. *Journal of hydrology*, 327, 186-199.
- Milewski, A., Sultan, M., Yan, E., Becker, R., Abdeldayem, A., Soliman, S., Abdel Gelil, K., 2009. A remote sensing solution for estimating runoff and recharge in arid environments, *Journal of Hydrology*, 373, 1-14.
- Milzow, C., Kgotlhang, L., Kinzelbach, W., Meier, P. and Bauer-Gottwein, P. 2008. The role of remote sensing in hydrological modelling of the Okavango Delta, Botswana. *Journal of Environmental Management*, 90, 2252-2260.
- Pilgrim, D. H., Chapman, T. G., and Doran, D. G. 1988. Problems of rainfall-runoff modelling in arid and semiarid regions. *Hydrological Science Journal*, 33, 379-400.
- Ramier, D., E. Berthier, E., and Andrieu, H., 2011. The hydrological behaviour of urban streets: long-term observations and modelling of runoff losses and rainfall-runoff transformation. *Hydrological Processes*, 25, 2161-2178.
- Rendell, H., Alexander, D., 1979. Note on some spatial and temporal variations in ephemeral channel form. *Geological Society of America Bulletin* 90, 761-772.

- Saudi Geological Survey, 2011. Wadi Ibrahim Environmental Management System (WIEMS), Progress report 6, pp 39.
- Schultz, G. A., 1987. Parameter determination and input estimation in rainfall-runoff modeling based on remote sensing techniques. *Water for the Future: Hydrology in Perspective (Proceedings of the Rome Symposium, April 1987)*. IAHS Publication number 164.
- Scipal, K., Scheffler, C. and Wagner, W., 2005. Soil moisture-runoff relation at the catchment scale as observed with coarse resolution microwave remote sensing. *Hydrology and Earth System Sciences*, 9, 173-183.
- Shang, J., Wilson, J. P., 2009. Watershed urbanization and changing flood behavior across the Los Angeles metropolitan region. *Natural Hazards* 48, 41–57.
- Smith, D. P., Bedient, P. B., 1981. Preliminary model of an urban floodplain under changing landuse. *Journal of Hydrology*, 51, 179-185.
- Suriya, S., and Mudgal, B. V., 2011. Impact of urbanization on flooding: The Thirusoolam sub watershed – A case study. *Journal of Hydrology* (in press).
- Tooth, S., 2000. Process, form, and change in dryland rivers: a review of recent research. *Earth-Science Reviews* 51, 67–107.
- Yousef, B. E. M. A., 1992. The climate of Makkah area. *Journal of Umm Al-Qura Social Sciences*, 15, 1-94.
- Walling, D.E., Gregory, K.J., 1970. The measurement of the effects of building construction on drainage basin dynamics. *Journal of Hydrology* 11, 129–144.
- Wise, S. M. 2000., Assessing the quality for hydrological applications of digital elevation models derived from contours. *Hydrological Processes*, 14, 1909-1929.
- Wolock, D. M. and Price, C. V. 1994. Effects of digital elevation map scale and data resolution on a topographically based watershed model. *Water Resources Research*, 30, 3041-3052 .
- Zhang, W. and Montgomery, D. R. 1994. Digital elevation model grid size, landscape representation, and hydrological simulations. *Water Resources Research*, 30, 1019-1028.

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Impact of Drought Stress on Germination and Seedling Growth Parameters of Some Wheat Cultivars

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Abstract: With a view to understand the parameters which can be used as a quick criteria for drought tolerance, the present investigation has been performed to evaluate eight wheat (*Triticum aestivum* L.) cultivars, four local cultivars (Madini, Kaseemi, Yamanei and Tabokei) and four introduced cultivars (Sakha 93, Giza 168, Seds 12 and Masr 1) from Agricultural Research Center, Giza, Egypt, to drought stress induced by polyethylene Glycol (PEG)6000 at different concentration 0.0, 60, 120, 180, 240 and 300 g/l PEG during germination and seedling growth stage of plant development. Five germination parameters; finally germination percentage, mean daily germination, germination index, mean germination time and coefficient of velocity of germination and eight seedling growth parameters; shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, seedling length and root number were measured under experiment conditions. Experiment units were arranged factorial completely randomized design with three replications. Mean comparison showed that the highest value for most of parameters were recorded for Sakha 93 and Madini cultivars followed by Yamanei, Kaseemi and Tabokei. With due attention to interaction cultivars x drought levels, cultivars Masr 1, Giza 186, Seds 12 under 120, 180, 240 and 300 g/l PEG6000 had the lowest value of noted parameters than other cultivars. Results of variance analysis made clear that different osmotic potential had significant effect on all parameters except root dry weight. In contrast, using all germination and seedling growth parameters, except root number, under study can be used as a selectable parameters to discrimination between tolerance and sensitive cultivars under drought stress in breeding programs and laboratory experiment would appear to be suitable for screening under drought stress.

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Key words: Wheat (*Triticum aestivum* L.). Abiotic stress. Drought. Germination. Seedling growth parameters. Polyethylene glycol.

Abbreviations: PEG - Polyethylene Glycol; FGP – Final Germination Percentage; MGT – Mean Germination Time; GI – Germination Index; CVG – Coefficient of Velocity of Germination; MDG – Mean Daily Germination

1. Introduction

Wheat (*Triticum aestivum* L.) is a staple food for more than 35% of the world population and it is also the first grain crops in most of developing countries [1]. Bread wheat is the main food of people in many countries and about 70 % calories and 80 % protein of human is supplied from its consumption [2]. Abiotic stress, especially drought stress is a world wide problem, seriously constraining global crop production [3]. It is one of the major causes of crop loss world wide, which commonly reduces average yield for many crop plants by more than 50% [4-5].

The high yield of plant in sufficient irrigated conditions is not necessarily related to high yield under drought stress [6]. Depending on which stage of growth a plant experiences drought stress, it reacts quite differently to the stress [7]. Plant may be affected by drought at any time of life, but certain stage such as germination and seedling growth are critical [8]. In most of developing countries, wheat is mainly grown on rainfed lands without supplementary

irrigation. About 37% of land area in these countries consists of semiarid environments in which available moisture constitutes a Primary constraint to wheat production [9].

Seed germination and seedling growth characters are extremely important factors in determining yield [10]. Dhandas *et al.*, [11] indicated that seed vigor index and shoot length are among the most sensitive to drought stress, followed by root length and coleoptiles length. The rate of seed germination and the final germination percentage as well as the amount of water absorbed by the seeds were considerably lowered with the rise of osmotic stress level [12]. There are many studies such as the selecting plant species or the seed treatments that are helpful for alleviating the negative effect of drought stress on plant [13, 14, 15, 16-17]. Selection of drought tolerance at early seedling stage is frequently accomplished using simulated drought induced by chemicals like poly ethylene glycol(PEG6000).

Poly ethylene glycol (PEG6000) can be used to modify the osmotic potential of nutrient solution

culture and thus induce plant water deficit in relatively controlled manner [18, 19-20]. Lu and Neumann [21]; Kulkarni and Deshpande [22] showed that Poly ethylene glycol molecules are inert, no-ionic, virtually impermeable to cell membranes and can induce uniform water stress without causing direct physiological damage. PEG as a factor causing drought stress by reducing water potential results in reducing growth in seed germinated and stopping seedling growth so that this effect has been observed more in the shoot than primary roots [23-24]. Dodd and Donovan [25] also suggested that PEG prevent water absorption by seeds, but penetrable ions by reducing potential inside cell results in water absorption and starting to germinated.

The present study was conducted to evaluate five wheat cultivars for drought resistance at germination and seedling stage. PEG-6000 was used as an osmoticum to induce stress conditions. The objective of this study was to evaluate wheat varieties for drought resistance at germination and seedling stage.

2- Material and Methods:

In order to study the effects of water stress, using polyethylene glycol, on germination indices and seedling growth parameters in wheat, an experiment was conducted in Department of Biology, Faculty of Science-North Jeddah, King Abdul-Aziz University, KSA in 2011. The form of experiment was factorial, using a completely randomized design (CRD) with three replications. In the present study seeds of eight cultivars from wheat (Madini, Kaseemi, Tabokei, Yamanei, Masr 1, Sakha 93, Giza 168 and Seds 12) were used. grains of first four cultivars were obtained from Agriculture company in KSA and the last four cultivars were obtained from Agricultural Research Center, Giza, Egypt.

Grains of eight cultivars were subjected to six stress level of PEG6000 (0.0, 60, 120, 180, 240 and 300 g/l) According to methods by Michel and Kaufmann [26]. PEG6000 was prepared by dissolving the required amount of PEG in distilled water at 30°C. Wheat grains were disinfected with 10% sodium hypochlorite solution for 30 seconds. After the treatment the grains were washed two times with distilled water. 10 grains from each cultivars were germinated on two layers of filter paper in 9-cm Petri dishes with respective treatment from PRG6000. The Petri dishes were covered to prevent the loss of moisture by evaporation under laboratory condition (24±2 °C) for 8 days.

Grains were considered germinated when they exhibited radicle extension of > 3 mm. Every 24 hours after soaking, germinated grains were made daily during the course of the experiment to determine following germination parameters. Where the number

of germinated seeds was recorded 8 days after planting as Final Germination Percentage (FGP) according to ISIA [27] and ISIA [28] where $FGP = Ng / Nt \times 100$, Ng=Total number of germinated seeds, Nt=Total number of seeds evaluated. Mean Germination Time (MGT) was calculated according to Sadeghi *et al.*, [29]. The Germination Index (GI) was calculated as described in the Association of Official Seeds Analysts (AOSA) [30] by following formula: $GI = \text{no. of germinated seed} / \text{Days of first count} + \dots + \text{no. of germinated seed} / \text{Days of final count}$. Coefficient of Velocity of Germination (CVG) determined by a mathematical manipulation $CV = \sum Ni / \sum NiTi \times 100$ according to Scott *et al.*, [31]. Mean Daily Germination (MDG) which is index of daily germination was calculated from the following equation $MDG = FGP / d$, FGP is final germination percentage and d is days to the maximum of final germination.

The experiment was terminated by harvesting seedlings 8 days after grains soaking and traits including shoot length, root length, shoot fresh weight, root fresh weight, shoot dry weight, root dry weight, root number and seedling length were measured. The data collected was analyzed statistically using Costat software to identify significant differences among wheat varieties and among treatments. Least significant difference test was applied at five and one percentage level of probability to comparisons among means as explained by Stell and Torrie [32].

3- Results and Discussion:

As screening technique, the survival ability of the eight wheat cultivars to tolerate chemical desiccation by PEG during germination stage is exhibited in Figure (1). In the present study, there was a significant two-way interaction (drought level and cultivars) ($P \leq 0.01$) for all germination parameters. Data pertaining the effect of PEG induced stress on final germination percentage, germination index, main daily germination, mean germination time and coefficient of velocity germination is shown in Table (1). In all cultivars, the final germination percentage was highest at control treatment and started to decrease as the drought level increasing using PEG. The cultivars differences in response to drought stress for final germination were highly significant (Table 2). The cultivars Sakha 93 and Madini had higher final germination percentage than the other cultivars regardless of drought stress (Table 1). However, the cultivar Masr 1 generally had the lowest final germination percentage regarding of drought stress. For other germination parameters, an inverse relationship was observed between drought stress and mean germination time, daily germination

time, germination index and coefficient of velocity germination. The average value for MDG, GI, CVG and MGT decreased from 4.895, 3.2329, 82.5 and 1.548 in control treatment to 0.2329, 0.2475, 21.471 and 0.7916 under 300 g/l from PEG, respectively (Table 1 and Fig. 1).

Hegarty [33] indicated that water stress at germination stage can result in delayed and reduced germination or may prevent germination completely. Also, once a grain attains a critical level of hydration it will precede with out cessation toward full germination. However, physiological changes do occur at hydration levels below this critical level that can cause an inhibition of germination. **Dodd and Donavon [25]** observed that reduction in germination percentage can result from PEG treatments that decrease the water potential gradient between seeds and their surrounding media. Different cultivar response to these osmotic stress treatments suggests a great deal of genetic variation among cultivars that could be utilized to develop new wheat cultivars adapted to arid and semiarid regions. **Alaei et al., [34]; Jaijarmi [35], Bayoumei et al., [5] and Metwali et al., [1]** reported variable response of wheat cultivars for germination indices to various

abiotic stress levels. Results presented here are consistent with previous finding that certain germination criteria can be used for selecting drought-resistant cultivars [9].

Seedling development under laboratory conditions have been accepted an suitable growth stage for testing the drought tolerance in wheat it could be speculated that the presence of increased concentrations of PEG during the growth of seedling inhibits the developmental traits and survival of wheat seedling (Table 3). The shoot length of different cultivars differed under different osmotic potential of PEG. In normal condition the maximum value of shoot length was recorded for Yamanei cultivar (11.5 cm), while Madini cultivar recorded lowest value (9.16 cm) followed by Seds 12 (9.5 cm). With increasing concentration of the PEG decline in shoot length occurred. Under treatment with PEG (300 g/l) shoot growth was observed only in Sakha 93 and Madini cultivars, it is recorded 0.66 and 0.26 cm, respectively. Shoot growth was observed for Masr 1 and Sed 12 under 0.0, 60, 120 and 180 g/l from PEG6000, while under 240 and 300 g/l PEG6000 these cultivars were not able to generate any shoot growth.

Table 1: Effect of different drought levels on germination indices of eight wheat cultivars

Parameters	PEG (g/l)	Cultivars								Mean	LSD 0.05	
		Madini	Yamanei	Kaseemi	Tabokei	Masr 1	Sakha 93	Giza 168	Seds 12			
Final Germination Percentage (FGP)	0.0	93.30	100	96.6	96.6	100	100	100	100	97.083a	6.5509	
	60	100	96.9	100	93.3	93.3	100	93.3	100	97.083a		
	120	96.6	93.30	90.00	100	86.6	93.30	90.00	93.3	90.00b		
	180	76.6	73.30	76.60	73.3	80.00	86.60	86.60	73.3	78.33c		
	240	53.30	23.30	40.00	53.3	20.00	50.00	23.30	3.33	37.08d		
Mean Daily Germination (MDG)	0.0	1.00	0.00	6.66	10.00	0.00	16.6	0.00	0.00	5.41e	0.2324	
	60	4.66	5.00	4.83	4.83	4.5	5.00	5.00	5.00	4.89a		
	120	5.00	4.83	5.00	4.66	4.66	5.00	4.66	5.00	4.85a		
	180	4.83	4.66	4.50	5.00	4.66	4.66	4.5	4.66	4.70a		
	240	3.83	3.66	3.83	3.50	4.00	4.33	4.33	3.66	3.81b		
Germination Index (GI)	0.0	0.50	0.00	0.33	0.50	0.00	0.83	0.00	0.00	0.270d	0.1685	
	60	3.11	3.33	3.22	3.22	2.99	3.33	3.33	3.33	3.23a		
	120	3.33	3.22	3.33	3.10	3.10	3.33	3.22	3.33	3.22a		
	180	3.22	3.10	2.99	3.33	3.11	3.11	3.00	3.40	3.12a		
	240	2.55	2.44	2.55	2.44	2.33	2.55	2.88	2.44	2.60b		
Coefficient Velocity Germination (CVG)	0.0	1.77	0.77	1.33	1.77	0.66	1.55	1.33	1.11	1.28c	6.5908	
	60	0.33	0.00	0.44	0.66	0.00	0.55	0.00	0.00	0.24d		
	120	81.60	83.00	82.30	82.30	81.30	83.00	83.00	83.00	82.50a		
	180	82.30	82.30	83.00	82.00	82.00	83.00	82.30	83.00	82.45a		
	240	82.30	78.00	81.30	83.00	81.60	81.00	81.00	81.66	81.25a		
Mean Germination Time (MGT)	0.0	82.30	84.60	87.60	84.00	70.30	81.00	63.00	72.33	78.29a	0.2105	
	60	66.30	69.00	66.00	69.00	50.00	70.60	65.33	55.33	63.70b		
	120	50.00	0.00	44.00	66.00	0.00	55.30	0.0	0.000	21.47c		
	180	0.0	1.21	1.20	1.22	1.22	1.15	1.20	1.20	1.20		1.54a
	240	1.20	1.20	1.22	1.20	1.21	1.21	1.20	1.2	1.20		1.24b
	0.0	1.22	1.28	1.22	1.20	1.22	1.24	1.22	1.22	1.22b		
	60	1.21	1.09	1.13	1.16	1.32	1.23	1.46	1.31	1.20b		
	120	1.50	1.44	1.49	1.55	2.00	1.41	1.57	1.38	1.20b		
	180	1.21	1.09	1.13	1.16	1.32	1.23	1.46	1.31	1.20b		
	240	1.50	1.44	1.49	1.55	2.00	1.41	1.57	1.38	1.20b		
	300	2.00	0.00	1.00	1.50	0.00	1.16	0.00	0.00	0.79c		

Table 2 : Analysis of variance for effect of cultivars and drought levels on germination indices of eight wheat.

SOV	df	MS				
		Final Germination Percentage	Mean Daily Germination	Germination Index	Coefficient Velocity Germination	Mean Germination Time
Cultivars	7	339.682**	0.6736**	0.3092**	800.196**	0.3324**
Drought Levels	5	34336.660**	87.4819**	37.099**	13841.31**	1.3955**
Cultivars x Drought Levels	35	203.015**	0.2724**	0.1377**	451.908**	0.4454**
Error	96	105.555	0.1267	0.0686	17.0030	0.0231
Total	143					
Coefficient of Variation (%)		15.221	10.441	11.459	6.039	12.649

SOV: Source of variance, MS: Mean Square, df: degree of freedom * and ** significant at 5 % and 1 %, respectively.

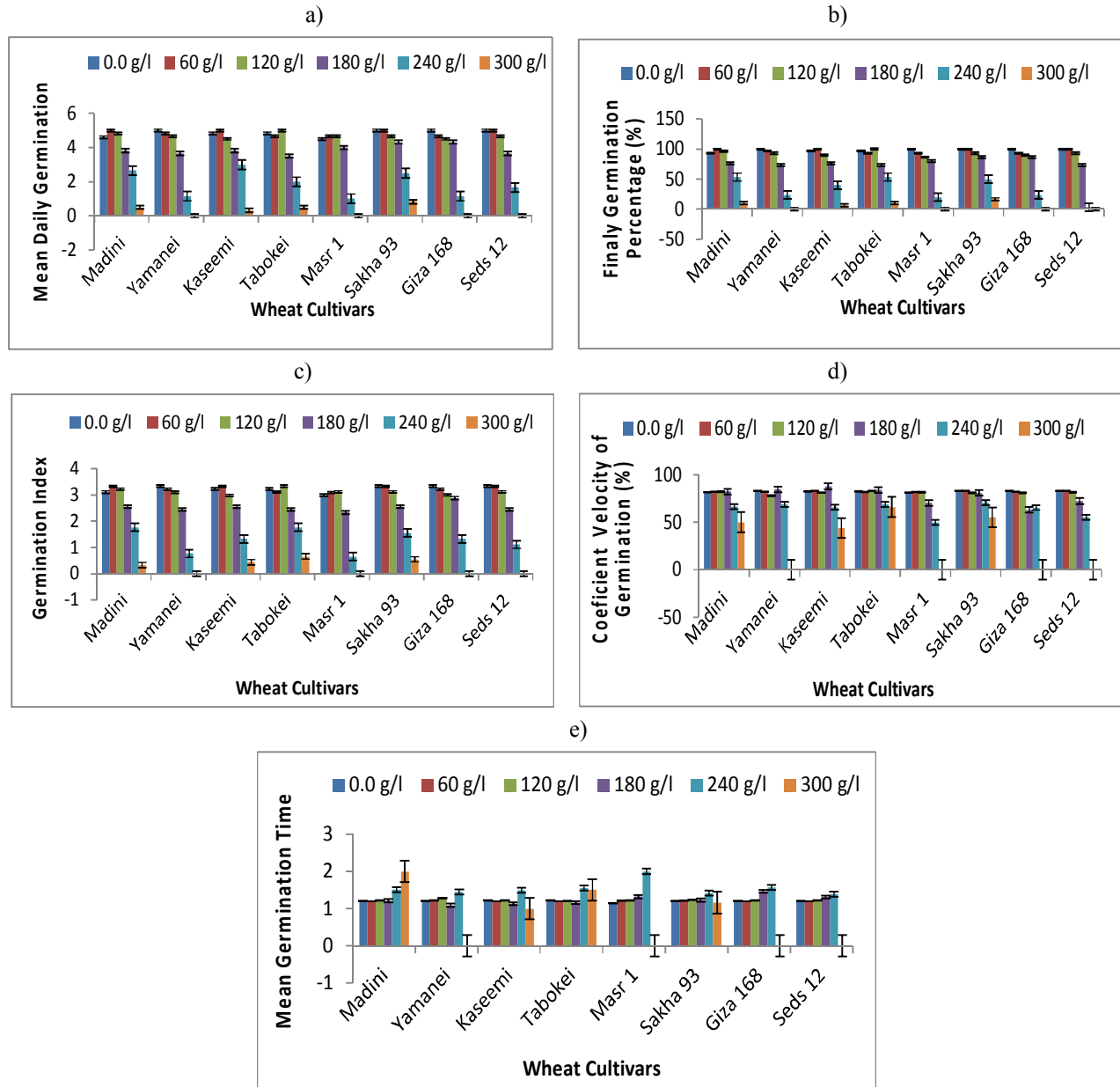


Figure (1): Interaction cultivars x drought levels (0.0, 60, 120, 180, 240 and 300 g/l) PEG6000 for a) Final germination percentage; b) Mean daily germination; c) Germination index; d) Coefficient of velocity of germination and e) Mean germination time of wheat cultivars. Bars represent standard error (±S.E) of means.

Table 3: Effect of different drought levels on growth parameters of eight wheat cultivars

Parameters	PEG (g/l)	Cultivars								Mean	LSD 0.05
		Madaini	Yamanej	Kaseemi	Tabokei	Masr 1	Sakha 93	Giza 168	Seds 12		
Shoot Length (cm)	0.0	9.16	11.50	9.83	9.66	10.50	11.66	10.83	9.50	10.330a	0.3973
	60	7.30	6.83	5.50	5.5	6.00	4.16	6.50	5.16	5.870b	
	120	4.30	4.33	5.10	4.66	3.66	4.00	4.63	3.66	4.330c	
	180	3.33	3.16	2.16	2.83	1.83	2.00	1.00	0.83	2.145d	
	240	1.93	1.00	1.46	2.03	0.00	1.76	0.0	0.00	1.037e	
Root Length (cm)	0.0	11.83	10.00	9.00	6.50	4.33	10.16	10.00	5.66	8.645a	0.7267
	60	7.00	5.00	7.16	6.83	5.83	4.66	6.00	4.83	6.620b	
	120	8.16	7.00	7.83	4.33	5.33	5.83	7.3	6.16	6.166b	
	180	5.16	4.5	3.16	3.33	4.00	3.66	1.83	4.66	3.791c	
	240	3.66	3.00	2.16	2.00	1.36	2.16	2.00	2.00	2.283d	
Shoot Fresh weight (gm)	0.0	1.68	1.52	1.75	1.34	1.48	2.03	1.63	1.26	1.615a	0.0315
	60	1.04	1.05	0.68	0.94	0.76	1.07	0.63	0.74	0.863b	
	120	0.45	0.23	0.25	0.23	0.17	0.46	0.28	0.32	0.303c	
	180	0.30	0.16	0.19	0.183	0.16	0.38	0.12	0.22	0.218d	
	240	0.24	0.100	0.13	0.30	0.00	0.24	0.00	0.00	0.138e	
Root Fresh Weight (gm)	0.0	1.63	1.62	1.86	1.09	1.19	1.97	2.00	1.55	1.616a	0.0545
	60	0.60	0.56	0.726	0.653	0.51	0.72	0.51	0.82	0.666b	
	120	0.37	0.18	0.18	0.27	0.14	0.37	0.190	0.20	0.246c	
	180	0.22	0.12	0.16	0.14	0.12	0.31	0.1	0.15	0.206c	
	240	0.18	0.07	0.10	0.233	0.076	0.25	0.11	0.06	0.137d	
Shoot Dry Weight (gm)	0.0	0.07	0.08	0.20	0.17	0.05	0.099	0.069	0.068	0.105a	0.0112
	60	0.23	0.06	0.05	0.06	0.06	0.06	0.04	0.04	0.052b	
	120	0.04	0.04	0.05	0.04	0.04	0.05	0.04	0.04	0.041b	
	180	0.03	0.02	0.03	0.02	0.022	0.03	0.02	0.02	0.023c	
	240	0.009	0.02	0.011	0.006	0.00	0.012	0.00	0.00	0.006d	
Root Dry Weight (gm)	0.0	0.055	0.078	0.142	0.1106	0.050	0.078	0.069	0.056	0.077a	0.0125
	60	0.039	0.048	0.059	0.050	0.040	0.0470	0.037	0.048	0.046b	
	120	0.033	0.040	0.032	0.028	0.024	0.039	0.023	0.029	0.031c	
	180	0.07	0.019	0.017	0.016	0.013	0.017	0.016	0.0183	0.023c	
	240	0.006	0.004	0.005	0.005	0.012	0.011	0.001	0.043	0.010d	
Root Number	0.0	4.00	5.00	5.00	4.00	4.00	5.00	5.00	4.66	4.666a	0.3667
	60	4.6	5.00	4.33	4.66	3.66	4.66	5.00	5.33	4.583ab	
	120	3.33	4.66	5.33	4.33	3.33	4.66	4.00	4.66	4.291b	
	180	4.33	3.00	3.00	4.00	3.00	3.00	3.66	2.66	3.333c	
	240	3.00	3.00	3.00	3.66	2.33	3.00	3.00	3.00	3.000c	
Seedling Length (cm)	0.0	16.6	20.16	18.5	16.16	14.8	21.8	20.8	14.83	18.229a	1.1582
	60	14.16	11.83	13.00	12.3	12.8	8.83	12.50	11.00	12.083b	
	120	12.50	11.33	13.00	9.00	9.00	9.83	13.13	8.83	10.954b	
	180	8.50	6.66	5.33	6.16	5.83	5.66	2.83	5.5	5.937c	
	240	5.60	4.00	3.63	4.03	1.37	3.93	2.00	2.00	3.320d	
	300	2.26	0.56	0.66	0.533	0.06	2.66	0.76	1.00	1.033e	

Values in mean column sharing same letter are statistically no-significant at 5%.

Table 4 : Analysis of variance for effect of cultivars and drought levels on growth parameters of wheat

SOV	df	MS							
		Shoot Length	Root Length	Shoot Fresh Weight	Root Fresh Weight	Shoot Dry Weight	Root Dry Weight	Root Number	Seedling Length
Cultivars	7	3.007**	11.262**	0.214**	0.149**	0.002**	6.481 ^{ns}	2.0277**	17.337**
Drought Levels	5	340.95**	198.016**	0.691**	7.830**	0.034**	0.016**	38.1333**	972.55**
Cultivars x Drought Levels	35	1.777**	4.665**	0.033**	0.068**	0.001**	8.166**	0.8444**	8.200**
Error	96	0.4808	1.608	0.003	0.009	2.264	3.655	0.4097	2.623
Total	143								
Coefficient of Variation (%)		17.452	26.630	10.671	19.962	12.258	58.102	18.073	18.850

SOV: Source of variance, MS: Mean Square, df: degree of freed and ** significant at 5 % and 1 %, respectively.

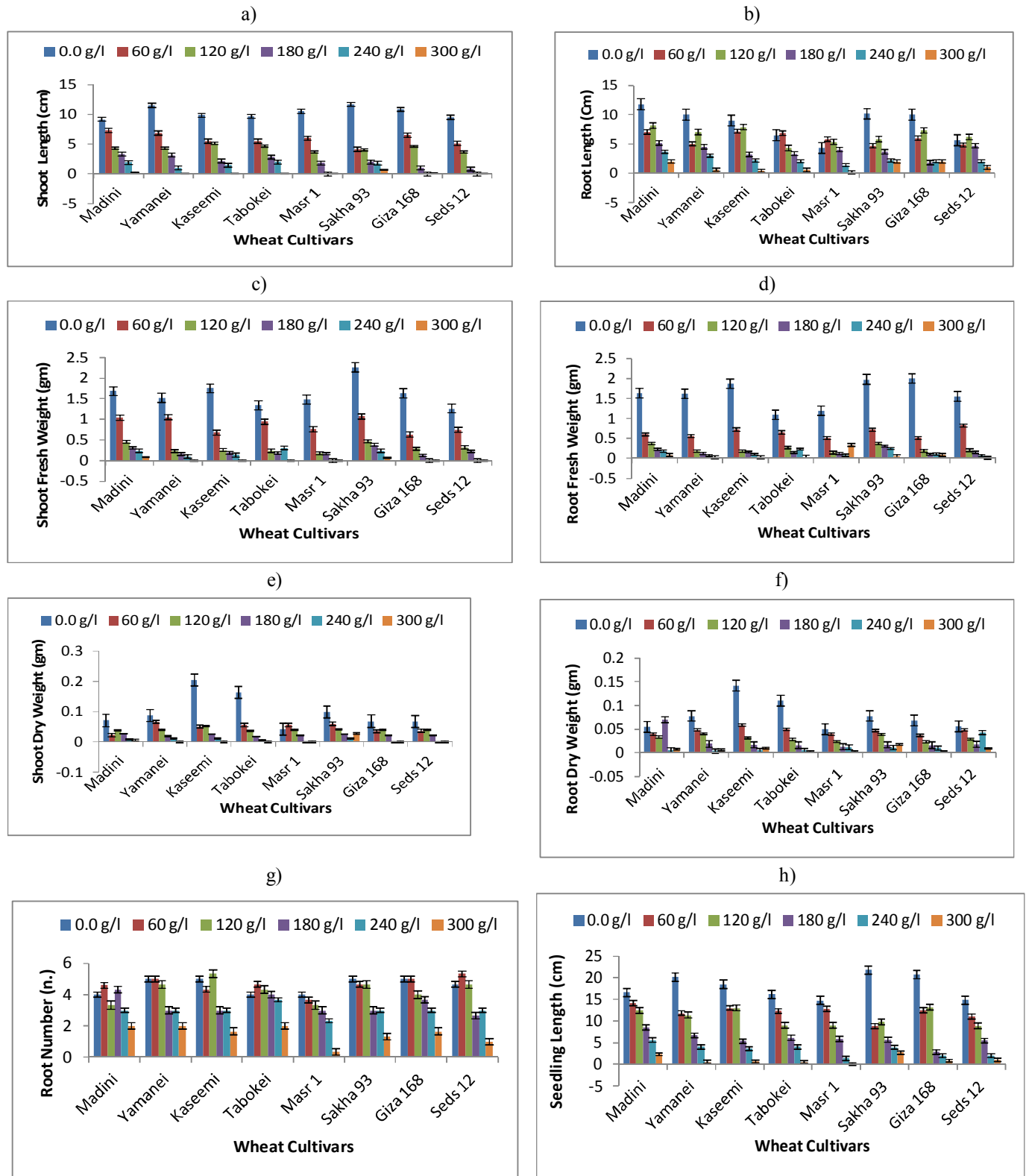


Figure 2: Interaction cultivars x drought levels (0.0, 60, 120, 180, 240 and 300 g/l) PEG6000 for a) Shoot length; b) Root length; c) Shoot fresh weight; d) Root fresh weight; e) Shoot dry weight; f) Root dry weight; g) Root number and h) seedling length of wheat cultivars. Bars represent standard error (±S.E) of means.

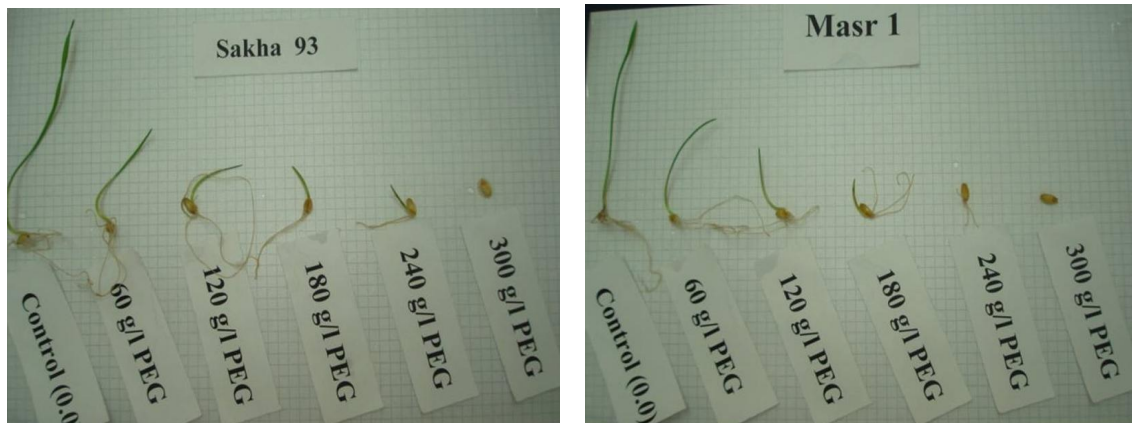


Figure 3: Effect of Different concentration of PEG on germination of Sakha 93 as a tolerant cultivars and Masr 1 as a Sensitive cultivar.

For root length parameter, there was an increase in root length associated with 120 g/l PEG6000 treatment for cultivars Madanei, Yamanei, Tabokei, Sakha 93, Giza 68 and Seds 12. This reflects on adaptive response involving an increase in root length to reach deeper water. Similar observation was reported by **Leila [36]**. In general root length was decreased significantly with increasing of PEG concentration (Table 3). **Fraser et al., [37]** concluded that the reduction in the root length under drought stress may due to an impediment of cell division and elongation leading to the Kind tuberization. This tuberization and the lignifications of the root system allow the conditions to become favorable again.

The PEG induced a drop in the shoot and root fresh weight which were the greatest (1.61 and 1.55 gm) under control treatment, respectively. While under 300 g/l PEG 0.0187 and 0.0472 g/l were recorded for shoot and root fresh weight, respectively. Greatest shoot and root fresh weight were recorded in Sakha 93 and Madini (Table 3). While the smallest value for shoot and root fresh weight was recorded in Seds 12 (0.0 and 0.013 gm, respectively). The reduction in shoot fresh weight was attributed to lower number and development of smaller leaves with increased PEG concentration of the growth media. It is important that drought resistance is characterized by small reduction of shoot growth under drought stressed condition (**Ming et al., [38]**; **Moucheshi et al., [39]** and **Saghafikhadeu [40]**).

PEG caused a greater reduction in dry weight of shoot and root at higher concentrations compared to control condition (Table 3). However, in Sakha 93, Madini, Yamanei and kaseemi, root dry weight value was increased with high concentration of PEG (300 g/l) (0.173, 0.008, 0.01 and 0.001), respectively,

comparing with the concentration of PEG (240 g/l) (0.011, 0.006, 0.005 and 0.004), respectively. On the other hand, there was a progressive decrease in root number with increased osmotic stress. Higher value of root number (5) was found under control treatment for cultivars Sakha93, Kaseemi, Yamanei and Giza 168 comparing with other different concentration of PEG. No significant different was recorded between cultivars, this indicated that root number could not be useful in the studies of genetic diversity and classification of adopted cultivars, thereby the improving the efficiency of wheat breeding programs. Seedling length decreased significantly with increasing osmotic stress (Table 4). the highest seedling length under PEG (300 g/l) was related to cultivars Sakha 93 and Madini with average 2.66 and 2.26 cm, respectively; and lowest value was related to Masr 1 and Yamanei with average of 0.06 and 0.56 cm, respectively. Interaction of genotype x drought treatment was meaningful at $P \leq 0.01$ (Table 4). The tested cultivars varied significantly in their reaction to PEG for all seedling growth parameters except root dry weight. **Baddiaw et al., [41]** indicated that the development of the root system in response to water deficit suggests that the expression of certain genes controlling root formation is stimulated by drought conditions. In addition to dominant alleles controlled the length of roots and the feature could be easily incorporated in breeding for drought resistance (**Vijendradas, [42]**).

4- Conclusion:

Generally, our results firstly clearly showed that different wheat cultivars differently responded to water stress at germination stage and early seedling growth. Second, the confined seedlings environmental

of laboratory experiment would appear to be suitable for screening large population to improve drought tolerance prior to yield testing. Third, all other germination and seedling growth traits, except root number, under study can be used as a selectable character to discriminate between resistance and sensitive cultivars under drought stress in breeding programs. Fourth, to find the best tolerant cultivar to drought condition, taking all traits into account in this study, we found that the eight cultivars can be classified into four groups depends on the ability to tolerate the osmotic stress as follows: first (High resistant group) include Sakha 93 and Madini; second (resistant) include Tabokei; third (Moderate group) include Yamane and Kassemi and fourth (sensitive group) include Seds 12, Masr 1 and Giza 168 (Figure 3). From this category, we observed that the Saudi cultivars were more tolerant than Egyptian cultivars, this may refer to the Saudi cultivars may be exposed to more natural selection for many years under semi-arid and arid conditions than Egyptian cultivars.

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References:

- Metwali, M.R. Ehab; Manal, H. Eid. and Tarek, Y. Bayoumi., 2011. Agronomical traits and biochemical genetics markers associated with salt tolerance in wheat cultivars (*Triticum aestivum* L.). Australian J. of Basic and Applied Science. 5(5): 174-183.
- Taregh, G., Mostafa, Valizadeh, S. and Hossein, S., 2011. Effect of drought on germination indices and seedling growth of 12 bread wheat genotypes. Advances In Environmental Biology, 5(6):1034-1039.
- Pan, X.Y., Wang, Y.F., Wang, G.X., Cao, Q.D. and Wang, J., 2002. Relationship between growth redundancy and size inequality in spring wheat population mulched with clear plastic film. Acta Phytoecol. Sinica, 26:177-184.
- Wang, X.Y., Vinocur, P. and Altman, A., 2003. Plant responses to drought, salinity and extreme temperatures: towards genetics engineering for stress tolerance. Planta., 218:1-14.
- Bayoumi, T. Y., Manal, H. Eid and E.M. Metwali., 2008. Application of physiological and biochemical indices as a screening technique for drought tolerance in wheat genotypes. African Journal of Biotechnology, 7(14):2341-2352.
- Vahidi, J. 2009. Effect of water stress on germination indices in seven wheat cultivar. World Academy of Science, Engineering and Technology, 49.
- Galies, J. and Ho, T.D., 1983. Multiple molecular forms of the gibberellin-induced α -amylase from the aleurone layer of barley seeds. Arch. Biochem. Biophys., 224.
- Pessaraki, M. 1999. Handbook of plant and crop stress. 2nd Ed., New York: Marcel Dekker Inc., 247-259.
- Abdul, Qayyum., Razzaq, A., Muhammad, A. and Matthew, A., 2001. Water stress causes differential effects on germination indices, total soluble sugar and proline content in wheat (*Triticum aestivum* L.) genotypes. African Journal of Biotechnology, 10(64):14038-14045.
- Rauf, M., Munir, M., Ul-Hassan, M., Ahmed, M. and Afzai, M., 2007. Performance of wheat genotypes under osmotic stress at germination and early seedling growth stage. African J. of Biotechnology, . 8:971-975.
- Dhanda, S.S., Sethi, G.S. and Behl, R.K., 2004. Indices of drought tolerance in wheat genotypes at early stages of plant growth. J. Agron. Crop. Sci., 190:6-12.
- Heikal, M.M., Shaddad, M.A. and Ahmed, A.M., 1981. Effect of water stress and gibberellic acid on germination of flax, sesame and onion seed. Biological Plantarum, 24 (2): 124-129.
- Ashraf, M., Bokhari, H. Cristiti, S.N., 1992. Variation in osmotic adjustment of lentil (*Lens culinaris* Medic) in response to drought. Acta Bot. Neerlandica. , 41:51-62.
- Almansouri, M., Kinet, J.M. and Lutts, S., 2001. Effect of salt and osmotic stresses on germination in durum wheat (*Triticum aestivum* L.). Plant Soil., 231:243-254.
- Okcu, G., Kaya, M.D. and Atak, M., 2005. Effect of salt and drought stresses on germination and seedling growth of pea (*Pisum sativum* L.). Turk J. Agric. For., 29:237-242.
- Kaya, M.D., Okcu, G., Atak, M., Cikili, Y. and Kolsarici, O., 2006. Seed treatments to overcome salt and drought stress during germination in sunflower (*Helianthus annuus* L.). Eur. J. Agron., 24:291-295.
- Iqbal, M and Ashraf, M., 2007. Seed treatment with auxins modulates growth and ion partitioning in salt-stressed wheat plants. J. Integr. Plant Biol., 49:1003-1015.
- Carpita, N., Sabulase, D., Monfezinos, D. and delmer, D.P., 1979. Determination of the pore size of cell walls of living plant cells. Science, 205:1144-1147.
- Money, N.P., 1989. Osmotic pressure of aqueous polyethylene glycols. Relation between molecular

- weight and vapor pressure deficit. *Plant Physiology*, 91:497-500.
20. Zhu, J.K., Hasegawa, P.M. and Bressan, R.A., 1997. Molecular aspect of osmotic stress in plants. *Critical Rev. In Plant Science*, 16;253-277.
 21. Lu, Z. and Neumann, P.M., 1998. Water-stressed maize, barley and rice seedlings show species diversity in mechanisms of leaf growth inhibition. *J. Exp. Bot.*, 49:1945-1952.
 22. Kulkarni, M. and Deshpande, U., 2007. *In-vitro* screening of tomato genotypes for drought resistance using polyethylene glycol. *Afr. J. Biotechnology*, 6:691-696.
 23. Khaheh, H., Bingham, M. and Powel, A., 2000. The effects of reduced water availability and salinity on the early seedling growth of soybean. *Proceeding of the Third International Crop Science Congress, Humburg, Germany*.
 24. Zhu, J., 2006. Effects of drought stresses induced by polyethylene glycol on germination of *Pinus sylvestris* var. *mongolica* seeds from pollination forests on sandy land. *Natural and Pollination Forests on sandy Land Journal of Forest Research*, 11(5);319-328.
 25. Dodd, G.L. and Donovan, L.A., 1999. Water potential and ionic effects on germination and seedling growth of two cold desert shrubs. *Am. J. Bot.*, 86:1146-1153.
 26. Micheal, B.E. and Kaufman, M.R., 1973. The osmotic potential of polyethylene glycol 6000. *Plant Physiol.*, 51:914-916.
 27. ISTA., 1993. *Hand book for seedling evaluation*. International Seed Testing Association, Zurich, Switzerland.
 28. ISTA., 1999. *International rules for seed testing*. International Seed Testing Association (ISTA), *Seed Science and Technology*, 287, supplement.
 29. Sadeghi, H., Fardin, K., Liela, Y. and Saman, S., 2011. Effect of seed osmopriming on seed germination behavior and vigor of soybean (*Glycin max* L.). *ARPN Journal of Agricultural and Biological Science*, 6(1):39-43.
 30. Association of Official Seed Analysis (AOSA). 1983. *Seed Vigor Testing Handbook*. Contribution No.32 to the handbook on seed testing.
 31. Scott, S.J., Jones, R.A. and Williams, W.A., 1984. Review of data analysis methods for seed germination. *Crop Science*, 24:1192-1199.
 32. Stell, R.G. and Torrie, J.H., 1980. *Principles and procedure of statistics. A Biometrical Approach*. 2nd Inter. Ed., Tokyo McGraw Hill, Book Co. New York. USA.
 33. Hegarty, T.W., 1977. Seed activation and seed germination under moisture stress. *New Phytol.*, 78:349-359.
 34. Alaei, M., Zaefizadeh, M., Khayatnezhad, M., Alaei, Z. and Alaei, Y., 2010. Evaluation of germination properties of different durum wheat genotypes under osmotic stress. *Middle-East Journal of Scientific Research*, 6;642-646.
 35. Jajarmi, V., 2009. Effect of water stress on germination indices in seven wheat cultivar. *World Academy of Science, Eng. Technol.*, 49:105-106.
 36. Leila, R., 2007. Response of Tunisian autochthonous pearl millet (*Pennisetum glaucum* L.) to drought stress induced by polyethylene glycol (PEG) 6000. *African Journal of Biotechnology*, 6:1102-1105.
 37. Fraser, T., Silk, W. and Rosr, T., 1990. Effect of low water potential on cortical cell length in growing region on maize roots. *Plant Physiology*, 93:648-651.
 38. Ming, D.F., Pei, Z.F., Naeem, M.S., Gong, H.J. and Zhan, W.J., 2012. Silicon alleviates PEG-induced water-deficit stress in upland rice seedling by enhancing osmotic adjustment. *Journal of Agronomy and Crop Science*, 198:14-26.
 39. Moucheshi, A., Heidari, B. and Assad, M.T., 2012. Alleviation of drought stress effects on wheat using arbuscular mycorrhizal symbiosis. *International Journal of Agriscience*, 291:35-47.
 40. Saghafikhadem, A., 2012. The effect of drought on growth and yield of wheat. *American Journal of Scientific Research*, 44:110-115.
 41. Badiow, F.A., Diouf, D., Sane, D., Diouf, O., Goudioby, V. and Diaalo, N., 2004. Screening cowpea (*Vigna unguiculato* L.) walp. Varieties by inducing water deficit and RAPD analyses. *African Journal of Biotechnology*, 3(3):174-178.
 42. Vijendradas, L.D. 2000. *Problems facing plant breeding*. CBS Publishers and Distributors. New Delhi. India.

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A Path Towards IP-V6 Transition Strategies for Scientific Research: An Overview

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Abstract: This research work explores various aspects regarding the introduction and analysis of transition strategies to the Internet Protocol Version 6 (IP-v6). We firstly analyze the different ways of transition as of IP-v4 to IP-v6. These ways are not integral parts of IP-v6 itself, rather they have administrative nature or they reside on lower layers of ISO/OSI model. We give an overview of non-technical issues and layer-2 options (targeted to distinct types of organizations and networks) which will aid in implementing the transition strategies supporting the Internet Protocol Version 6 (IP-v6). We also present a transition roadmap for each of these strategies in terms of addressing scheme; layer-2 technologies; routing, application and tunneling protocols; security problems relevant to transition and network protocols. Moreover, a comparative analysis of these strategies are presented using different criteria such as scalability, methods used for transition to native IP-v6 connectivity, degree of deployment difficulty and others. We hope this work will be found useful by both network operators and designers, and will be used to design strategies for transition to IP-v6 protocol based on the presented strategies, or at least as a reference.

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Keywords: BGP-Border Gateway Protocol; IP-v6; QoS-Quality of Service; VPN-Virtual Private Network

1. Introduction and Problem Statement

The most visible drawbacks of IPv4 (Perkins, 2002) in regard to current demand are insufficient security support and lack of end-to-end reach-ability. We will try to analyze these drawbacks in following sections by presenting arguments why they are harmful to different aspects of the current Internet growth. Some of these issues are as follows:

1.1 Address Space Shortage

The main reason why IP-v6 (Internet, 2001) was designed, the answer is because increasing scarcity of IP addresses (Srisuresh et al., 2001). With more and more organizations deploying NAT (Network Address Translation) (Rosenberg et al., 2003) as a measure to reduce this overhead, next generation application layer protocols which require end-to-end reach-ability and inherent security are harder to implement. Currently, it is clear that most of the predictions about when IPv4 address space will run out of allocate-table address prefixes were wrong.

The main reason why is that they primarily focused on density of address space population and not on how allocation process has changed over the years. Nowadays, the main constraint for Internet growth regarding address space is not its practical capacity, but rather overhead linked to allocation and assignment process. There is roughly 30% of address space left which can be used for allocation of global unicast IPv4 addresses to Regional Internet Registries (RIRs). In

practice however, IPv4 address blocks are hard to get because of strict policies imposed by RIRs.

1.2 End-to-End Reach-ability

NAT (Rosenberg et al., 2003) is widely deployed on current Internet. This technique breaks the initial idea of Internet saying that every two hosts connected to Internet should be able to reach each other directly. This brokenness is being softened by using additional mechanisms such as proxy servers, protocol extensions such as NAT detection and NAT traversal added to software logic, but in fact they only add another level of complexity - for both users and programmers/protocol designers.

1.3 Ad-hoc Mobility

The presence of IP-enabled mobile devices is more and more frequent. Since network stack implementations cannot cope with IP address changes, support for mobility in IP protocol (Perkins, 2002) is needed. The Support for IP mobility in IPv4 protocol was retrofitted and this affects the possibility to deploy it. On the other hand, IPv6 protocol was designed with mobility support in mind (Johnson et al., 2003). The most serious problems of mobility support in IPv4 are the following:

1). No end to end reach-ability: The implementation of IP mobility in IPv4 would have to solve NAT traversal problem – this is too big obstacle compared to transition to IPv6 (Bi et al., 2007).

2). Not coherent with IP protocol features:

The design of mobility support in IPv6 was built upon inherent features of IPv6 protocol (Deering et al., 1998) such as Neighbour Discovery (Narten et al., 1998), auto-configuration (Narten et al., 1998) and mandatory IPsec support, whereas in IPv4 it was not. This can bring some issues when implementing IPv4 mobility, mainly weaker security and greater complexity.

3). Lack of implementations: Mobility support for IPv6 has already (as of beginning 2005) (Johnson et al., 2003) working implementations of entities needed for IPv6 mobility. Some of these implementations are distributed with source code. Contrary to the state of mobility for IPv6, IPv4 mobility support can be found mostly in proprietary form made for specific deployment cases.

4). IPv4 mobility support mandates: This entity is necessary for mobile node to receive packets sent from its home-agent. On the contrary, in IPv6 mobility support, foreign agent is not necessary and thus infrastructure does not need to be upgraded in order to accept mobile IPv6 nodes.

5). IPv4 address space restricts (Report, 2002): The mobile IP deployment on a very large scale.

Efficiency: IPv4 stores data needed for routing a packet to destination node in one header. Format of IPv4 header (Perkins, 2002) has two main disadvantages - its length is not fixed and it contains Header checksum field which has to be recomputed on each router along the path to destination node. IPv6 solves this problem by splitting the data needed for routing into basic header and several extension headers (Deering et al., 2003). The size of basic header is constant which should theoretically result in faster packet processing in hardware. IPv6 also pushes checksum computation to upper layer protocols.

Security: For mobility support, IPsec (Internet Protocol Security) support is crucial because mobile node needs to secure the maintenance of bindings with its Home Agent which represents them while its absence in home network (Johnson et al., 2003). IPv4 specification does not provide any reference to whether IPsec should be included in IPv4 stack implementation or not. In this sense, IPv6 specification is more demanding and security aware. Enhanced security in IPv6 also coheres with end-to-end reach-ability; because nodes are able to communicate without an intermediary who would provide realization of security services for them (e.g., IPsec gateway), they are able to indulge in end-to-end, authorized, tamper-proof, private communication (Narten et al., 2001). IPv6 offers these services by specifying IPsec support as mandatory. It is important to note that although support for IPsec is mandatory in IPv6, it does not mean that

any IPv6 implementation not doing IPsec extension headers processing is not compliant to basic IPv6 RFC documents (Deering et al., 2003). Therefore there can be nodes implementing only small subset of IPv6 protocol without IPsec support.

2. Comparison of Transition Strategies

Although strategies presented in this section differ from each other they can be eventually modified and applied even to networks of different size than those on which these strategies were presented. Thus it is important to create a methodology for comparing the strategies in general. The methodology should provide information about how much effort will be needed to implement given strategy, what performance characteristics will it show, what services will be provided and how long it is sustainable to continue using this strategy.

Progress: Tunnel Broker strategy (Cisco, 2005) progresses slowly to the core and the transition to native connectivity are completed when it reaches core of the network. Similar approach was applied in IPv6 over MPLS Strategy (Rosen et al., 1997), which makes transport of IPv6 packets without any form of layer 3 encapsulation possible first in the backbone network and at the same time it proceeds from CPE devices to the edge of native backbone.

In Tunnel mesh strategy, the upgrade of network into native one was done in all phases with the emphasis on providing IPv6 connectivity to end users. If ordering all described strategies according to the time when native connectivity was first deployed in backbone network, the list would be as follows:

- IPv6 over MPLS Strategy
- Tunnel Mesh Strategy
- Tunnel Broker Strategy

Scalability: Scalability of given system is usually defined as ability of a system to adapt to increasing number of input variables. This definition holds as well for a strategy.

The scalability of a strategy can be thought of in following terms:

- Network performance
- Limit of difficulty of administration

The first point conveys how overall performance of a network is to change when the strategy is implemented in increasingly bigger part of the network. Each step of a strategy could be taken into account but usually generic strategy can be divided into two stages - proliferation of interim means of transmission of IPv6 packets and replacement of these means by native IPv6 connectivity.

Estimation of scalability could be only done for the first stage because the limiting factors are not present in the second stage. For the second point, some

coefficient analogous to HD-ratio can be specified, which would reflect how much "pain" will be endured by administrators when number of elements (tunnels, tunnel server, AAA servers, etc.) will grow above bound from which the coefficient would be computed (Durand et al., 2001).

Dual-Stack Backbone versus 6-PE: Deploying IPv6 in MPLS network can be solved by one of following four proposals:

- Use manually configured (static) tunnels between PE routers by creating a "tunnel mesh"
- Deploy IPv6 natively in the whole backbone
- Upgrade whole signalling plane (P routers) to support IPv6
- Deploy 6PE

The idea behind 6PE technology was that most of MPLS operators would not be willing to perform upgrade of P routers, which constitute the core of MPLS network. The other reason is that by disrupting the service of the fast forwarding in the core, the operation of whole MPLS would be lowered.

The first option would create a "static tunnel mesh" between 6PE routers. Its negatives are apparent – first, its performance compared to common operation of MPLS packet transport is low.

Second, creating another layer of boundaries between PE routers would lead to additional layer of complexity, which could make debugging network problems very difficult, especially in dense MPLS cloud. Thus, deploying IPv6 in MPLS network by setting up static tunnels is far worst solution for deploying IPv6 in MPLS-enabled network because its scalability is very limited - static tunnels have very low performance limit.

The comparison of dual-stack operated backbone and 6PE regarding performance is such as that in middle-sized network, these solutions should prove equal. However, on large scale, MPLS could perform better because it will probably take an advantage in fast forwarding in the core of MPLS network. Also, 6PE is a lot easier to deploy - only PE routers need to be upgraded, compared to every router in the path when upgrading backbone to dual-stack.

Degree of Deployment Difficulty: In regard to deployment difficulty each strategy can be looked at from two points of view:

- How much difficult is to deploy the techniques used in individual steps of the transition
- Overall efficiency of a strategy

There can be another issue of technical difficulties when implementing transition strategy: Looking at IPv6 over MPLS strategy, it is very straightforward and painless strategy regarding 6PE deployment for networks already using MPLS in backbone network. However, it is not very reasonable

to deploy MPLS in backbone just for the reason of transition to IPv6 using 6PE. In this case, this strategy will be technically difficult to implement.

Vicious Cycle: In most of the strategies described in this chapter it is presumed that customers will demand IPv6 connectivity by themselves which would start the next stage of transition strategy. While this might be true in some of the networks used for illustration of the strategies, in reality where ISP provides connectivity especially directly to end users, it is not likely the ISP will get big number of requests for IPv6 connectivity.

These obstacles cause kind of vicious cycle - until the users will be motivated they would not demand IPv6 connectivity from the providers and on the contrary, until providers will come up with technical solution users will not be motivated to proceed with transition. In order to make the transition to IPv6 as smooth as possible the providers should push native IPv6 connectivity to the edges of their networks because with the help of integral components such as auto configuration end users would not notice they are using IPv6 in ideal case. Therefore, the activity should be on provider's side.

Transition to Native Connectivity: The goal of each transition strategy is not only to provide IPv6 connectivity to all nodes which actually make some use of it but rather to provide native IPv6 connectivity to all end nodes in the network capable of using it.

Clear winner in this category is IPv6 over MPLS strategy because it incorporates only very limited number of tunnels during the whole process of transition to IPv6 connectivity. As opposed from other strategies, there is no tunnel present in the backbone of the network in every step of the transition roadmap.

The Tunnel mesh strategy can be put on same level because their approach to transition of core of the network is almost the same, although Tunnel Mesh Strategy does not push the transition of the core much forward in list of steps needed to complete the transition.

The Tunnel Broker strategy keeps tunnels in the network longest of all described strategies, but this is merely result of assumptions imposed on the particular network type, on which the strategy was shown. On the other hand, it is at least designed so that transition from tunneled to native connectivity should be gradual.

Addressing Schemes: The addressing scheme used in Tunnel mesh strategy can be called "classical" because it tries to map addressing scheme to network topology as close as possible. This allows greater aggregation but less conservation. Since it is presumed that the ISP mentioned in this strategy will get subsequent prefix allocation of the same size as initial allocation, the degree of aggregation will decrease because e.g. in one POP there will be prefixes from

initial as well as subsequent allocation, which cannot be aggregated at the POP level. At highest level, the aggregation imposed by a RIR will be preserved, thanks to the way how RIRs allocate prefixes.

The IPv6 over MPLS Strategy (Awduche et al., 1999) for addressing scheme construction is similar to that used in Tunnel Mesh Strategy because it also maps network topology to addressing scheme. The difference is merely in structure of the networks.

The addressing scheme constructed for Tunnel Broker strategy resigned on creating addressing scheme after 38th bit boundary. This decision was done mainly for two reasons – because of the transition technique used and also because of the assumption that the network will expand internally.

3. Conclusions

In the most of the current networks, the transition to IPv6 is yet to be accomplished on many fronts. Not only technical issues such as ISO/OSI layers or application-development perspective need to be refined but also administrative procedures for transition and business cases for IPv6 will have to be developed. This work aimed at both technical and strategic aspects of the transition.

At first, we have presented a motivation to the development of IPv6 protocol and transition to IPv6. We have also discussed basic concepts of IPv6, IPv4, OSI/ISO Model, and TCP/IP etc.

In the next, we have given an overview of non-technical issues and layer 2 options which will aid in implementing a transition strategy.

Finally, we have described several transition strategies, targeted to distinct types of organizations and networks. Each of these strategies was described in terms of addressing scheme, layer 2 technologies and network protocols. Also, a transition roadmap for each particular strategy was given. Moreover these strategies were compared using different criteria such as scalability, methods used for transition to native IPv6 connectivity, degree of deployment difficulty and others.

We hope this work will be found useful by both network operators and designers, and will be used to design strategies for transition to IPv6 protocol based on the presented strategies, or at least as a reference.

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References

Perkins, C., Ed, IP Mobility Support for IPv4, RFC 3344, (August, 2002).

Internet Architecture Board, Internet Engineering Steering Group, IAB/IESG Recommendations on IPv6 Address Allocation to Sites, RFC 3177, (September, 2001).

Srisuresh, P., and Egevang, K., “Traditional IP Network Address Translator (Traditional NAT)”, RFC 3022, (January, 2001).

Rosenberg, J., Weinberger, J., Huitema, C., and Mahy, R., STUN - Simple Traversal of User Datagram Protocol (UDP) Through NATs, RFC 3489, (March, 2003).

Johnson, D., Perkins and C., Arkko, J., Mobility Support in IPv6, RFC 3775, (June, 2004).

Bi, J., Wu, J., and Leng, X., IPv4/IPv6 Transitions and Univer6 Architecture, IJCSNS, Vol. 7, No. 1, (January, 2007).

Deering, S., and Hinden, R., Internet Protocol Version 6 (IPv6) Specification, RFC 2460, (December, 1998).

Narten, T., Nordmark, E., and Simpson, W., Neighbor Discovery for IP Version 6 (IPv6), RFC 2461, (December, 1998).

Narten, T., and Thomson, S., IPV6 Stateless Address Auto-configuration, RFC 2462, (December, 1998).

IPv4 Address Report, <http://bgp.potaroo.net/ipv4/>

Deering, S., and Hinden, R., Internet Protocol Version 6 (IPv6) Addressing Architecture, RFC 3513, (April, 2003).

Narten, T., and Draves, R., Privacy Extension for Stateless Address Auto configuration in IPv6, RFC 3041, (January, 2001).

Durand, A., and Huitema, C., The H-Density Ratio for Address Assignment Efficiency an Update on the H ratio, RFC 3194, (November, 2001).

Rosen, E., Viswanathan, A., and Callon, R., A Proposed Architecture for MPLS, Internet draft, (July, 1997).

Awduche, D., MPLS and Traffic Engineering in IP Networks, IEEE Commun. Mag., vol. 37, no. 12, 42-47, (December, 1999).

Cisco IOS Release 12.0 (7) T, MPLS Virtual Private Network enhancements, Cisco, (2005).

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Cross-Platform Service for Nomadic Devices in Biodiversity Research

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Abstract: The synergy between cloud and virtualization has become popular in the recent years. Different handheld devices come with versatile and heterogeneous hardware and operating systems, resulting in incompatibility issues for application users. We extend the concept of virtualization to provide a set of virtual machines capable of emulating major operating systems. These virtual machines run on a Virtual Box Web Services in a SOA and are designed to be tailored to meet the specific requirements of a user through which they can run any software on any handheld device. Our service provides the user with the ability to make and run major operating systems using cloud of virtual machines. This allows unlimited portability between different hardware and software architectures if some minor requirements are met. We present the result of initial testing of running Nokia Symbian application on an android device.

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Keywords: Handheld Device, Cloud Computing and Virtual Machine

1. Introduction

Cloud computing has somewhat succeeded in its ability to provide versatile services to users irrespective of the device hardware (e.g., storage, computation, and architecture constraints) and software resources like operating system. The system running inside the cloud is responsible for all computing intensive work [1]. Since, cloud computing is not processor intensive and yet provides all services of a business or personal needs, this has encouraged hardware manufactures to make lightweight, inexpensive, and low cost devices with just enough capability to run a web browser, the best examples are Netbooks and Tablets. In the future, we expect devices to become even smaller and there would be an increasing trend towards using internet on handheld devices like Tablets [2]. Cloud usually follows the economic model in which a user can add and remove resources dynamically on the cloud and therefore pay only as much as he needs [3].

Scope of problems that can be solved by cloud computing is immense for e.g. with cloud, we can have unlimited storage capacity by adding clouds of storage services as necessary and as our business grows. Similarly, we can have the illusion of super computer on our device by using cloud as a utility computing. Amazon EC2 [4] provides dynamic scalability of the cloud within minutes. Besides numerous advantages, cloud computing also provides mobility and collaboration to end users [5]

Traditional software like Word and Paint have now online cloud counter parts that can be used freely using just a web browser like Google Docs, adobe Photoshop express, online conversation services like doc to PDF and back, Online tools to create virtual machines, etc. When software is used in

this way it is called “software as a service (SAS)”, an integral part of the cloud architecture.

Virtualization is an art of dividing the computer into many virtualized systems each with its own hardware and software architecture. Hardware virtualization tools such as Intel Virtualization Technology [6] assist virtualization on Intel machines. Virtualization was once confined to large machines and now it is being available on a micro scale. Handheld users having some hardware or software constraints can now get benefit from a cloud virtual machine in order to perform any resource extensive task.

Till now virtualization for handheld devices has not acquired widespread due to low computation power and limited capability of a typical handheld device. But, gradually we have seen an increasing number of handheld devices with huge computation power for e.g. smart devices.

The problem that we tackle in this paper is of using cloud as a virtual platform for running heterogeneous mobile platforms.

There are many existing cloud services like Amazon EC2 [4], Google App Engine [6], Facebook Platform [7]. Amazon E2 provides a business cloud platform while Google App Engine provides its users with a free limited space to run their application written using python or java language on the Google engine. Facebook Platform allows users to run applications on the Facebook platform and taking advantage of the Facebook API. Facebook platform is not suitable for developing enterprise and utility applications.

Currently in most of the popular handheld devices like Android [8] and iOS [9] a user could only run the application for which the device has been

programmed for. This introduces several problems for users and also makes it difficult for them to switch to other device with a different architecture and platform. Legacy software like critical business and enterprise applications can't be ported to other handheld device with a different architecture. These problems have motivated us to solve it using a cloud architecture which is same for any handheld device.

Different mobile devices like Blackberry, Android and iPhone are in intense competition with each other. Though, the present trend is towards Android [10], other platforms will remain significant. There are millions of people using those platforms and in the future, we expect some other mobile platform. All these platforms are not at all compatible with each other as they are strong rivals of each other.

Millions of applications exist for each of these platforms and they are not compatible with each other. The ultimate burden is on the user community to live with this limitation. For example, a user of android device has no way to run iPhone application except by hacking the android platform or by creating a virtual iPhone device machine. We follow the second approach to implement our solution. Unfortunately it's not possible to create a virtual machine on a cellular device since it has its own memory and process limitation. But we can implement a virtual machine on a cloud that we called an emulator since its purpose is to emulate an operating system for running some handheld device file. Instead of creating a virtual machine for iPhone or Android Phone, we have created a generic virtual machine which can host any major operating system depending on the needs and requirement of a user.

Each of the virtual machine in our cloud acts as an emulator for a particular handheld device. The service theoretically allows users to run major mobile operating system files like Android, Blackberry, and iPhone IOS. Our virtual machine has been enhanced specifically to work with mobile platforms.

By using our solution an android user would be able to run iPhone applications on their device without even the web browser. Our cloud service would consist of two parts, a client and a server. A client run on the device and server reside on the cloud. Whenever, an attempt is made to run the iPhone application in an android phone, our client application detects this and asks the user about whether they want to run this application by using the online cloud. By approval from the user, application would be compiled and run on the VM residing in cloud and its GUI would be transferred at run time to the mobile device. It is important to note that only the computation part runs on the virtual machine since an application which requires camera can't be simulated on the virtual machine. Therefore, a client application

must act as a bridge to provide transparent camera interface to the virtual machine and to the user. In other words, all the hardware of the phone like camera, microphone, and accelerometer must be simulated on the client side. This adds extra complexity in implementing such a cloud which we shall see in the proposed solution section later on.

The motivation for RunIT Cloud comes after searching the Google for a service which can run cross-platform mobile code e.g. sis file, so when we Goggled an online service to run sis files, we couldn't find any online service which can assist users in running mobile files. This is how we developed the idea in the first place.

The rest of this paper is organized as follows. In section 2 we highlight and compare some existing cloud services similar to ours. In section 3 we present our proposed solution and subsequent details in subsections. In section 4 we present some of the results of using our service to run Symbian Operating system sis file on an Android device. Finally in section 5 conclusions are presented followed by references.

2. Related Work

GoPC [11] is an online service that provides its users the facility to access their systems using clouds. They can use their machines as well as use the software that is available on the cloud. The users can access the service using their handheld devices as well. Together with online storage, scheduled backups, and the same 128 Bit encryption used by most banks ensures the highest level of security. You can build your own cloud platform from GoPC's products and services. However it provides access to the complete desktop and may cost more than services which provide access to only one service like the online spread sheet. Additionally GOPC consumes more bandwidth than the service which provides limited functionality. RunITCloud service provides the same interface as available on the handheld device and is accessed only when a non-native program is executed on the device. This gives handheld device users the illusion that they are able to run any program on their device. The RunITCloud is an attempt to run non native programs on the handheld devices with the comfort of the native GUI whereas GOPC provides a desktop environment to access common services

Amazon Web Services (AWS) [12] also provide the Elastic Compute Cloud (EC2), in which customers pay for compute resources by the hour, and Simple Storage Service (S3), for which customers pay based on storage capacity.

Other utility services include IBM Blue Cloud [13] which provides services very similar to

the Amazon E2, EMC's recently launched storage cloud service, and those offered by startups such as Joyent and Mosso.

Layered Tech's virtual machines (VM) [14] provide high-performance, high-availability computing resources that are not confined to a single, vulnerable server. With a virtual machine (VM) your site or application is implemented across many nodes. SnowFlock [15] is another system that uses the fork command for creating multiple virtual machines. A user can create machines according to their needs. We have used SnowFlock for implementing virtual machines in our system.

Intel virtualized computer [16] is another technology that provides the functionalities of creating VM's. It provides facility of creating servers with security and flexibility.

As we can see cloud services and platforms are on the rise. Many services and platform exist which cover the common services from document editors to graphics application to pc desktop sharing but none of these services and platform provide an explicit ability to provide a cloud emulator to run non-native mobile files.

2.1 Google Trends

If we look at Google trends for the popular mobile operating systems like Android, Symbian, IOS, BlackBerry and Java Me we get the results shown in Fig 1.



Fig. 1: Google Trends for Popular Mobile Operating Systems

It is obvious from the Google Trend that Android is the most popular mobile OS in 2011 with a sharp steep curve. Very close to Android is Blackberry with a curve steadily going up from 2004 to 2010 but decreasing abruptly in 2011 and just below the Android. It should also be noted that the popular IOS operating system has not seen any worthy growth over the years and lags behind

Android and Blackberry by a significant factors. Finally Java Me seems to in its last breath as the interest of user community in Java ME has become negligible.

3. RunItCloud

Instead of running the program on the handheld device, we propose that the program should run on a cloud service and only its interface should be accessible from the handheld device. This can theoretically give illusion to a mobile user that the software is actually running on the embedded device. However this solution comes with its various technical and feasibility challenges for e.g. how different events should be propagated from the mobile device to the cloud service and vice versa. Can the user interact with the software interface being streamed from a cloud service? If so what would be the screen refresh rate for a smooth interface playback. Can we stream video quality refresh rated on a limited handheld limited bandwidth, memory and computation power. How would the system compensate for the different handheld display sizes and resolutions? Before we attempt to answer these important questions, we want the reader to have some reality check. Our solution is not the ideal one, it's in no way alternative of the real device, and it attempts to provide a solution so that software written for platform A can run successfully on platform B provided there exist a Virtual Box service for it.

We propose a service oriented architecture to provide a set of services each running an instance of VirtualBox Web Services as shown in Fig 2.

VirtualBox is a popular open source virtual machine solution through which we can run many different operating systems on a single host computer. Solutions like VirtualBox abstract the hardware and operating system of the host computer into several executing environments. Very similar to VirtualBox is a commercial software solution called VMware. However VMware is not an open source solution and is therefore not suitable for customizing it to our needs. VirtualBox on the other hand provides comprehensive support and access to its internal Virtual Box Main API. Using VirtualBox Main API an application user could develop a program which can create, run and delete virtual machines on the fly and also interact with the virtual machine guest operating system by providing a full set of API. Besides that Virtual Box provides Web Services which provide full access to the Virtual Box Main API.

Fig 2 shows the architecture of the RunItCloud, a handheld device user downloads a portion of the RunItCloud client program which

provides mobile specific implementation to access the RunItCloud service. A mobile specific program is responsible for discovering the services of the mobile like GPS, Accelerometer, and Cam etc and determines whether they are compatible with the service. In other words the client portion of the RunItCloud aims to provide a bridge between the mobile and the RunIt Service. In Fig 2 three layers of services are shown and only the top layer “Service 3” is visible. A mobile user can’t directly access any of these services. These layers of services are added for flexibility and load sharing purpose. Client program can only access the main server which contains the core implementation of the RunItCloud Service. The main server is responsible for dynamically allocating available web service to the mobile user and keeping track of which mobile web service is being used by a specific mobile device. This provides the additional architecture flexibility of the SOA as new VirtualBox web services could easily be added without any significant implementation and architecture changes. Each VirtualBox web service container can hold one or more virtual machines each running a specific operating system.

VirtualBox web service provides access to the major desktop operating systems like Windows, Mac, and Linux and mobile operating systems like Android, iOS. Most of the mobile versions running inside VirtualBox are only a stripped up version containing only the major features with core kernel, applications and GUI.

3.1 System Architecture

Our cloud architecture (see Fig. 2) is based on Service oriented Architecture. There are number of services each with its own VirtualBox web services instance. A client such as an Android device or a PC can only access the service through a RunItCloud main server. TCP/IP indicates that connections could be either wired or wireless. Initially a client connects to the Main Server which is running the main instance of the RunIt system. Main Server is hosted on a public IP server and can be accessible by using the host name and port number. This system is responsible for selecting the appropriate service and routing the client connection to the appropriate service. A client is unaware of the service it is using. This architecture is flexible; more services can be added on demand to satisfy a large number of users. Each service can only contain a limited number of virtual machine instances and each virtual machine instance can only run a single operating system such as Android OS or windows. Fig 2 shows detailed cloud architecture in which some consumer device (mobile phones, laptops, etc.) are consuming one of

the cloud services each running with its own virtual machine and running on the cloud container.

Each service has its own instance of the Virtual machine which is shown by rectangular layers of screens and works independently.

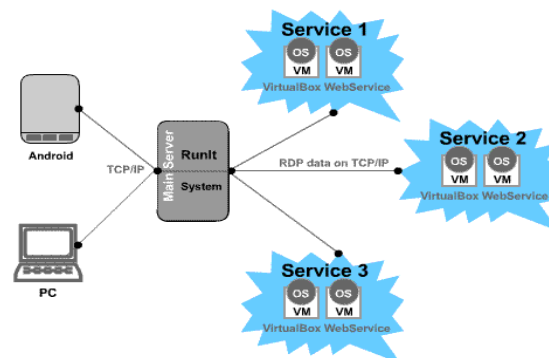


Fig. 2: RunItCloud Architecture Diagram

3.2 System Components

RunItCloud is divided into components as shown in Fig 3. The top layer is the Main API interface and provides access to core features of the system. Clients and users could only access this top layer to use the RunIt services.

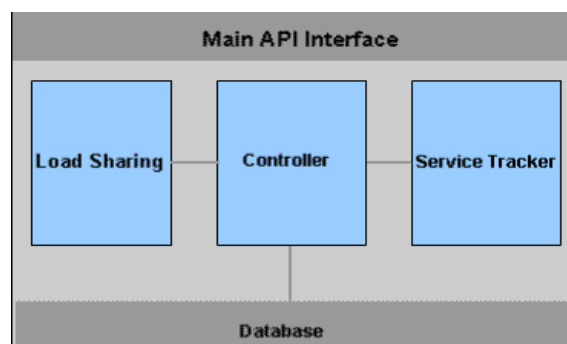


Fig. 3: RunItCloud Components

Main system consists of Load Sharing, Controller and Service Tracker. Load Sharing is responsible for dynamically sharing the clients load over the services so that the system appears to run smoothly when the traffic increases. Running a virtual machine on hardware is an extremely expensive process and it’s not possible to run many virtual machines on the same physical hardware. Load sharing provides the necessary flexibility so that users can enjoy a continuous reliable service by dynamically consuming the next free service to provide the maximum throughput.

The Controller is the center hub and performs the controlling functions on the service tracker and load sharing. It is responsible for

managing other components. Finally Service Tracker is responsible for profiling and keeping information about which service has been assigned to which client. Service Trackers also provide cache of the programs that the client has used previously and Controller first checks with the Service Tracker to determine whether the program should be transferred from the client to the web service. Database is used to provide storage, service related and configuration information and is directly accessible from the controller.

3.3. The Display

The RunIt service can be used to emulate different kinds of operating systems and versatile type of software such as real time systems and games can be requested by the client devices. Since the program that user is trying to run is incompatible with the user native device, therefore it must run on one of the RunIt services. If the RunIt service recognizes the software and runs it successfully, it must send the result back to the client. Naturally, the result is an image containing the GUI of the program. However it's not just an image, it's a series of images that are sent at regular intervals based on the following algorithm.

- i). Determine the type of the program game, simulation or a static application.
- ii). Intelligently calculate the refresh rate of the virtual machine screen by noting the movements at a video rate. This is done by continually scanning the program after every 50 milliseconds and noting down the difference from the earlier image sequences. If no difference is found the program is probably an application and only has static content waiting for the user to interact with it.
- iii). For a static software, RunIt service only send a single image and waits for some action from the client side such as click of a button. For video, it continually transmits 25 frames per second or less depending on the buffering rate of the client internet connection. This rate can be manually adjusted by the mobile user or determined by benchmarking the user mobile internet bandwidth.

The display is therefore based on the illusion that the application is running in real time on the client device when actually it's just a streaming. This approach may seem quite complicated, but in reality it is quite feasible due to the faster internet mobile internet connections.

3.4. The Client Part

The client part is small software written specifically for each of the mobile platform and

contains the platform specific logic. The client part acts as a bridge between the cloud service and the user mobile device. It's a connector to the advance features of the RunIT service as it provides interface to the user mobile storage access, I/O device access like camera, microphone, GPS and accelerometer. Device hardware for e.g. GPS is associated with a java interface and a platform specific client program must implement the interface to allow the main server to access the hardware features. Standard interfaces for hardware devices have been provided to on the service as well as on client side. To illustrate it let us consider a user tries to run a non-native program which needs the GPS to work properly. For example program may need a GPS location to pinpoint the location of the nearest restaurant. Obviously GPS can't be emulated on service side. Therefore, such information must come directly from the consumer hardware device in this case a GPS. The server ends a GPS request to the client, the client either denies or accept the request if GPS is available and has been implemented by the client program. The server uses the GPS specific interface to request any feature of the GPS hardware on the client side. The result of the GPS hardware is sent back to the server periodically. The client actually starts a separate thread as a callback function and also uses the timer to periodically sent the device specific data. A separate client/server connection is established for each hardware device to be used. The client part handles all the low level details and provides the GPS data to the service whenever the service requests it to do so. Currently client part is available for only two mobile platforms that is Android and BlackBerry.

3.5 Remote Desktop Streaming

Once a client program requests a non-native program to run on the cloud service, the main server runs the program on a free service and starts the virtual machine. The interface of the virtual machine then must be streamed over a TCP/IP connection back to the client. Fortunately Virtual Box web control provides a component called VRDB Server whose purpose is to provide a remote desktop access to the RDB client. Virtual Box VRDB also supports the keyboard and mouse events interaction from the RDB client. We implemented the RDB client on the client part of the RunIt cloud to stream the remote desktop.

3.6 Program Run Information

Program run information is a set of fields extracted by RunIt service and contain general information about the device and Platform. The device information includes the type of device, the

manufactures of the device, the SIM information for the device, etc. Platform information consists of operating system, supported file types, version, and supported hardware features like GPS, CAM. It also includes information about the SDK of the platform for which the program is written. This information is used to prepare the system to determine which OS emulator should be called upon.

3.7 Mechanism

What exactly happens when a user tries to run a non-native application? Simply starting a virtual machine with a specific operating system running is not very useful as the operating system can't access the user mobile program and therefore can't run it. Ideally a user should be able to click on the non-native application and somehow the application should start running automatically on the user device. This is accompanied using the following steps.

- i). User clicks on the non-native application or browse it using the RunIt client software.
- ii). RunIt client tool determines the device, program run information etc.
- iii). Client tool connects with the RunIt Main server and checks with the Service Tracker component if the program code is available on the service. If the program code is available move to point 5.
- iv). Client tool establishes a file transfer session with the main server and transmits the program code on the main server. Main server saves the program name and version information in its internal database.
- v). Main Server then shares the program code with the service Virtual machine so that it is visible to the guest operating system. This uses the VirtualBox shared folders feature which are provided exclusively to shared data between guest and host operating systems.
- vi). Using VirtualBox SDK Events Management, the main server simulates the click event on the program file in the shared folder to start the program on the virtual machine.
- vii). Main Server configures the VirtualBox VRDB server to transmit the remote desktop of the virtual machine to the client device.

If all of the above steps are successful a user of the client device gets the illusion that the program is actually running on the client device when it's in fact running on the cloud VM.

4. Use-Case: Run Android On Iphone

We have tested our system for mobile platform interoperability. The device which was used

as the host platform was HTC Android G2 cell phone as shown in Fig 4.



Fig 4: RunIT Cloud Client Application

The goal was to run the Symbian mobile application with extension "SIS" on this phone. The phone had a 400-600Kbit/s EDGE based internet access enough to stream a 3 inch video at low detail. As shown in the Fig 4, user clicks on the browse button and selects the sis file. By default any user who tries to run this file gets an error message that device does not support this file.

The client part of our service can be downloaded for each different mobile operating system and is optimized to run on that platform. After installation of the client part and assigning it necessary internet download permission, we again try to run the sis file. This time we get a prompt asking the user that running this file may cost you air time. With permission from the user, the client part prepares HTTP connection and connected to our RunIT service passing it the device information and program code. The RunIT service determines that the device operating system is Android and program code can be run in Symbian emulator, it emulates the suitable version of the Symbian emulator and calculates the refresh rate of the screen from which it determines that it doesn't contain much screen refreshes and runs it in application mode by transmitting one file after every 2 seconds threshold which can be adjusted. It important to note that the screen refresh calculation which is simply a standard motion detector between two or more consecutive screen frames is done entirely on the server side and is not visible to the client. However client part must be able to receive data at any time as in order to handle varying chunks of data from the server. This is due to when most of the application starts, this first screen is mostly a static interface waiting for user to

interact with the .Due to this reason, and our system fails to classify the application as an interactive process and transmits a single screen after every 2 seconds.

However there is a catch that we have incorporated in the system. After 50ms the system again checks the refresh rate of the screen using a difference between previous screens and the new screen for a specified time to calculate the screen repaint rate. This time it classifies the application as an interactive process based on the varying screen repaint rate. As a result the system sends a stream of screens after 33 ms to meet the 30 frames/sec requirement of the smooth video. Then, after every 50ms the system again evaluates the program to see if it has changed its behavior to a static or dynamic program. This is important because we want to make the system efficient and send the screen only when it is required based on principle of least privilege resulting in less bandwidth usage.

So based on the application the system intelligently determines the repaint rate and accordingly classifies the running file. However there is no formal classification of the application other than interactive or background processes. The goal is to determine the FPS of the application so that only necessary frames should be sent back to the handheld device saving bandwidth and computation.

5. Conclusion

RunIT Cloud service allows users of different platforms to run each other software using a set of virtual machines running on the cloud supporting major operating systems. For instance, an android user can run Symbian applications on his device without a dedicated Nokia set. Our cloud service consists of two parts, a client and a server. The client runs on the device and server reside on the cloud. This is a new approach as it allows the user to run non-native application from the comfort of the native GUI of the device. However there are obvious challenges to this approach, the most notable is to find a suitable version of the operating system image which can run the file user is trying to execute. Also the Operating system image should be compatible with the Virtual box system. The use case demonstrated our solution can be used to solve real world problems. Additionally the experience of people who were deliberately not told about the emulator was noted and necessary steps were taken to make the system feel more real. In future, the support of more operating, statistics and usage feedback of the service would be added.

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References

- [1] Michael A., Fox, A., Griffith, R., Anthony D., Randy, J. K., Konwinski, A., Lee, G., Patterson, D., Rabkin, A., Stoica, I., Zaharia, M., "Above the Clouds: A Berkeley View of Cloud Computing", Technical Report EECS-2009-28, EECS Department, University of California, USA., Feb. 10, 2009.
- [2] Dikaiakos, M. D., Katsaros, D., Mehra, P., Pallis, G., Vakali, A., "Cloud computing: Distributed internet computing for it and scientific research, Int. J. of IEEE Internet Computing, Vol. 13, No. 5, pp. 10–13, published by IEEE Computer Society, USA., 2009.
- [3] Dean, D., Saleh, T., "Capturing the value of Cloud Computing", white paper available at <http://www.bcg.com>, November, 2009.
- [4] <http://aws.amazon.com/ec2/>
- [5] Hayes, B., "Cloud computing", Communications of the ACM, vol. 51, no. 7, pp. 9–11, July 2008.
- [6] Ciurana, E., "Developing with Google App Engine" published by Apress, Berkely, CA, USA, 2009.
- [7] Hickey, M., "Facebook launches Facebook Platform; They are the Anti-My Space", available at <http://www.techcrunch.com/2007/05/24/facebook-launches-facebook-platform-they-are-the-anti-myspace/> consulted on September 07, 2011.
- [8] Rogers, R., Lombardo, J., Mednieks, Z., Meike, B., "Android Application Development: Programming with Google SDK", edition 1, published by O'Reilly Media, UK., May 20, 2009.
- [9] Patel, N., " iPhone OS 4 renamed iOS", available at www.engadget.com retrieved on June 09, 2010.
- [10] Chakraaborty, P., "Android to Become No. 2 Worldwide Mobile Operating System in 2010 and Challenge Symbian for No. 1 Position by 2014", blog posted at <http://www.pctelecoms.blogspot.com> on Sep. 12, 2010.
- [11] <http://www.gopc.net> accessed on April 2011.
- [12] <http://aws.amazon.com> accessed on Feb. 19 2009.
- [13] <http://www.ibm.com/cloud-computing/us/en/> access on April 2011.
- [14] <http://www.layeredtech.com/cloud-computing/virtual-machine-hosting/> retrieved on April 25, 2011.
- [15] Andres, H. L. C., Whitney, J. A., Scannell, A. M., Patchin, P., Rumble, S. M., Lara, E., Brundo, M., Satyanarana, M., "SnowFlock: Rapid Virtual Machine Cloning for Cloud Computing. In proceeding EuroSys' 09, 4th ACM European Conference on Computer System, held in Nuremberg, Germany, March 2009.
- [16] Uhlig, R., Neiger, G., Rodgers, D., Santoni, A. L., Martins, F. C. M., Anderson, A. V., Bennett, S.M., Kagi, A., Leung F.H., Smith, L., "IntelVirtualization Technology", IEEE Computer, vol. 38, no. 5, pp. 48-56, May 2005.

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Bond Strength of Poly (methyl methacrylate) Denture Base to cast Titanium and cobalt-chromium Frameworks of Different Designs

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Abstract: The lack of a chemical bond between conventional denture base materials and framework elements represents a significant problem in removable prosthodontics. Poor chemical bonding of a denture base resin to cast metal frameworks often introduces adhesive failure and increases microleakage. **Purpose.** The purpose of this study was to examine the shear bond strength of a denture base acrylic resin to commercially pure titanium alloy (CP Ti) and a cobalt-chromium alloy (Co-Cr) using a hybrid bonding system. **Material And Methods.** Square plates (of different designs) were cast from the 2 alloys. The plates were grit-blasted with 50 μm of alumina and treated with the Rocatec™ bonding system. A denture base heat-cured acrylic resin was then applied to the plates. Specimens without bonding were also prepared as controls. Both alloys were configured as frameworks with different retentive designs: flat plate, lattice retention, mesh retention and bead retention. Shear bond strength values were determined at a crosshead speed of 0.5 mm/min. **Results.** For CP Ti plates, shear bond strength was the highest for acrylic resin adhered with Rocatec™ to flat plates, followed by the bead, lattice and mesh designs. The shear bond strength for different retentive Co-Cr frameworks, with or without Rocatec™, was the highest for bead retention, followed by mesh, flat plate and lattice. There was a statistically significant difference ($P < .05$) in bond strength between the 2 alloys for both flat plate and lattice retentive frameworks bonded with Rocatec™ to acrylic resin. **Conclusion.** The application of the Rocatec™ bonding system significantly improved the shear bond strength of denture base resin using both cast (CP Ti) and Co-Cr alloys.

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Key Words: Denture base, Framework, bond strength, Rocatec bonding, Titanium framework, Cobalt chromium //

1. Introduction:

In 2011, Carr and Brown described removable partial dentures (RPDs) as combinations of cast metal and acrylic denture bases consisting of cast metal bases that were fitted over residual ridges, and acrylic resin that is processed to the metal to enhance esthetics, restore lost tissue contours, and retain artificial teeth¹. Cobalt-chromium (Co-Cr) alloys are frequently used to fabricate denture frameworks due to their favorable mechanical properties, whereas commercially pure titanium (CP Ti) and titanium alloys are preferred for their biocompatibility as well as desirable mechanical properties. The denture framework surface to be bonded with the denture base material should be effectively conditioned as a reliable bond between the denture base material and metal framework is required for the denture to function properly.² Co-Cr alloys may contain elements (Co and or Cr) causing sensitivity or allergic reactions in some patients. Due to the potential of titanium to eliminate some of these problems associated with Co-Cr alloys, titanium has been increasingly used in clinical practice for the fabrication of removable prostheses³.

Allergic reactions, sometimes encountered in treatment with RPDs, may be a problem due to the

presence of nickel in base metals. In a study comparing conventional base metal alloy and Ti in terms of biocompatibility, sensitivity or allergic reactions were found to decrease with the use of Ti. Base metal alloys show high shear bond strength values, which might be due to the thickness of the oxide layer and the surface roughness of the alloy surface⁴.

The use of titanium for the production of cast RPD frameworks has gradually increased. There are no reports about metallic allergy apparently caused by (CP Ti) dentures. There are still some laboratory-related drawbacks associated with the use of Ti alloy, such as the lengthy burn-out, inferior castability and machinability, reaction layer formed on the cast surface, difficulty of polishing, and high initial costs. However, clinical problems, such as the discoloration of titanium surfaces, unpleasant metal taste, decrease of clasp retention, tendency for plaque to adhere to the surface, detachment of the denture base resin, and severe wear of titanium teeth, have gradually been resolved with the use of Ti alloys⁵.

The mechanical retention for a denture resin (poly(methyl methacrylate), PMMA) in removable prostheses is usually provided by the framework design in the denture base, such as through the use of

beads, posts, an open lattice, a mesh, or some other macroscopic retentive design⁶. The 3 most commonly used acrylic retentive designs are open lattice, preformed mesh, and a metal base with bead retention. The lattice design has a high susceptibility to permanent deformation,⁷ and the open lattice design produces the greatest amount of retention for acrylic resin. Mesh can be used interchangeably with lattice in any given clinical situation. Beads or nail beads are used with metal base alloys that are cast to fit against edentulous ridges. This attachment is the weakest of the three types of acrylic retentive designs⁸.

External and internal finishing lines should be placed on the cast metal framework of all three types of acrylic retentive designs, wherever the acrylic resin joins the cast framework⁹. If there is a separation between the acrylic resin and the cast metal, especially at the finishing line, cracks or crazing may occur in the acrylic resin, leading to microleakage that is accompanied by staining¹⁰⁻¹². Microleakage from the metal-PMMA interface can lead to discoloration, deterioration of the resin, and the creation of a reservoir for oral debris and microorganisms. Incomplete fracture or total separation of the resin can also occur. The lack of a chemical bond can directly affect the metal-resin interface. The difference in the coefficients of thermal expansion between the metal and the resin might create a gap at the interface, leading to microleakage. Therefore, conventional adaptation between the acrylic resin denture base and the metal framework may not be sufficient to prevent microleakage (Kim *et al.*)³.

Significant research has focused on improving the chemical bond strength between the acrylic resin and the metal to withstand intermittent occlusal forces and endure the constant moisture from saliva and temperature variations in the oral environment^{13,14}.

Metal-resin bonding systems are classified into 2 categories: surface modification to create a thin layer of metal on the substrate metal alloys and direct application of a functional monomer to create a chemical bond^{15, 16}. Silica-coating and tribochemical coating systems are considered surface modification methods¹⁶. Tribochemistry involves the creation of chemical bonds by the application of mechanical energy that may take the form of rubbing, grinding or sandblasting. There is no heat or light application, which is normally used with chemical reactions¹⁷. In the silicoating system, a silica layer is pyrolytically applied to the surface over which a silane coupling agent is applied¹⁵.

The purpose of this study was to evaluate the bond strength of heat-cured PMMA to CP Ti and Co-Cr alloys, when pretreated with or without the

Rocatec™ bonding agent, in a laboratory model system.

2. Material and Methods

Two groups, each consisting of 48 frameworks made of commercially pure titanium alloy (CP Ti) or cobalt-chromium alloy (Co-Cr), were prepared. Frameworks in each group were further subdivided into 4 subgroups (n=12). The framework in each subgroup was designed as a flat plate, lattice retention; mesh retention or a flat plate with beads retention.

For each alloy, forty-eight square wax patterns (20 x 20 x 2mm) were prepared. Wax patterns of group-I were kept flat. The remaining patterns in groups II, III and IV incorporated a different central area (10 x 10mm) according to the other three framework designs (lattice, mesh and beads) using traditional wax specimens.

For Ti alloy specimens, 48 wax patterns were invested in casting rings (six in each ring) using Rematitan® Plus (Dentaurum J. P. Winkelstroeter KG, Pforzheim, Germany) as the investment material. The ring was then burned out by following the manufacturer's recommendations and then cast with CP Ti (grade II, ASTM-Rematitan®-Dentaurum) ingots using a vacuum-pressure machine (Rematitan System, Dentaurum J. P. Winkelstroeter KG, Pforzheim, Germany).

The casting machine automatically evacuated the chamber, which was filled by argon gas in 70 s. The argon supply continued for approximately 120 s after the molten metal had dropped into the mould. The machine then stopped automatically to allow air to enter the chamber. Castings were carefully removed from the mould, scrubbed under running water and then cleaned in an ultrasonic cleaner. The surface was ground and polished with sandpaper and alumina using rotary equipment (Metaserv 2000, Buehler UK Ltd., Coventry, England). Radiographic screening of the castings for internal defects excluded specimens showing porosities.

For comparison, 48 wax patterns (of the same size as those made of CP Ti alloy) of the Co-Cr alloy (Biosil F, DeguDent, York, PA, US) were conventionally cast using a silica-based investment (Univest Silky, Shofu Dental Corp., San Marcus, CA, US) in a centrifugal induction melting machine (Neutro-dyn-Easyti Manfredi, Italy). The 48 cast Co-Cr plates were also made with the same configurations representing the 4 framework designs. After bench cooling, castings were retrieved from the casting rings, and then cleaned with distilled water in an ultrasonic bath for 30 min to remove most of the adhering investment.

Metal plates (CP Ti and Co-Cr) were separated from the sprue using a diagonal cutter nipper while avoiding contact with the test central areas. Metal plates were then sandblasted with 110 μm of alumina delivered by air pressure applied for one minute.

In the central area of each metal specimen where the retentive framework designs were effected, rectangular wax blocks of 25mm height and 10mm X 10mm area were built to ensure that the acrylic resin portions of all test frameworks were of the same size and contour. These wax blocks were covered with high viscosity polyvinylsiloxane (Silagum Putty®, DMG, Hamburg, Germany) then invested in conventional flasks with Type 3 dental stone (Moldano; HeraeusKulzer, Hanau, Germany).

The 96 metal specimens with the wax blocks were invested in 16 denture flasks (six specimens per flask), and then placed in boiling water. The wax was boiled out and the flasks were allowed to cool. This procedure provided molds for processing heat-cured acrylic resin to all the test metal plates. In each group, metal plates were removed from the flask and sandblasted again with 50 μm alumina for 60 s before acrylic resin packing.

Acrylic resin (Lucitone L.D. Caulk Co., Milford, DE, USA) was polymerized on the sandblasted metal plates, with or without Rocatec™ bonding agent (Rocatec, ESPE GmbH, Seefeld/Oberbay, Germany), according to the manufacturers' recommendations. Sandblasted metal plates in each group were subdivided into two subgroups according to this surface treatment with Rocatec™ (6 plates in each flask): sandblasted metal plates without Rocatec™ (subgroup A) and sandblasted metal plates with Rocatec™ (subgroup B). Rocatec™ bonding agent was applied to the central area of the sandblasted metal plates (subgroup B) in three steps¹⁷:

- 1- Preabrasive cleaner was used to create a matte finish on the metal plates.
- 2- An adhesive (Rocatec Plus) was then applied as a thin coating to provide a chemically reactive surface.

- 3- Finally, a silane coupling agent (Rocatec-Sil) was applied to provide the bond with the acrylic resin block.

Specimens were deflashed and cleaned, the acrylic blocks were smoothed with burs, and all the samples were stored in distilled water at 20 °C for 7 days prior to bond testing.

The bond strength of the acrylic resin blocks that had adhered to the metal plates was determined by loading the bonded specimens to failure on an Instron Universal Testing Machine (Instron Corp. Canton, MA, USA). Specimens were held in a metal fixture (grasping unit). The test holder was oriented such that the shear force could be applied to the resin-metal interface with a knife-edged rod. Bond strength was calculated by dividing the load at fracture by the surface area¹⁸. Specimens were loaded at a cross head speed of 5 mm/minute. The bond strength was calculated in kg/cm^2 for each specimen. The means and standard deviations were calculated for each test group. The data were analyzed using 3-way ANOVA, followed by Scheffé's multiple range test at a significance level of 0.05.

3. Results

Table (1) shows the tensile bond strength (kg/cm^2) of acrylic resin blocks adhered to CP Ti plates at the time of failure for each of the tested specimens. There was a statistically significant difference between the treated versus non-treated groups ($P < 0.05$). For CP Ti plates that were not treated with Rocatec™ in subgroup (A), the highest bond strength was observed for the lattice design ($77.35 \pm 13.20 \text{ kg}/\text{cm}^2$). The mesh and beads retention areas incorporated in the CP Ti plates without treatment showed comparable bond strength, $75.42 \pm 6.83 \text{ kg}/\text{cm}^2$ and $72.37 \pm 10.43 \text{ kg}/\text{cm}^2$, respectively. The lowest tensile bond strength was noted for the plain-flat CP Ti plate, $40.48 \pm 7.64 \text{ kg}/\text{cm}^2$, which was statistically significant when compared with all types of retentive framework designs ($t = 11.667$).

Table 1: Bond strength (kg/cm^2) of acrylic resin to different designs of Ti frameworks

Group	Group I Acrylic resin bonded with Ti-flat plate design		Group II Acrylic resin bonded with Ti-lattice design		Group III Acrylic resin bonded with Ti- mesh design		Group IV Acrylic resin bonded with Ti- beads design	
	Subgroup		Subgroup		Subgroup		Subgroup	
	A	B	A	B	A	B	A	B
Mean	40.48	136.39	77.35	105.53	75.42	98.54	72.37	123.48
SD	7.64	18.63	13.20	14.70	6.83	9.30	10.43	14.63
t-value	11.667		3.494		4.907		6.958	
p-value	<0.001		0.005		0.001		<0.001	

Subgroup (A): Sandblasted CP Ti design without treatment

Subgroup (B): Sandblasted CP Ti design treatment with Rocatec™ bonding agent

Table 2: Comparison of bond strength (kg/cm²) of acrylic resin to different designs of Ti frameworks with and without Rocatec™ bonding agent

Group	Group I	Group II	Group III	Group IV	F
Without treatment (A)	40.48 ± 7.64	77.35 ± 13.20	75.42 ± 6.83	72.37 ± 10.43	18.736 P<0.001
Treatment with Rocatec™ (B)	136.39 ± 18.63	105.53 ± 14.70	98.54 ± 9.30	123.48 ± 14.63	8.209 P=0.001

In contrast, CPTi plates pretreated with Rocatec™ bonding agent in subgroup (B) demonstrated improved adhesion to the acrylic resin blocks. The highest bond strength was noted when using plain flat CPTi plates in group I, 136.39±18.63 kg/cm², followed by the beads design (group IV, 123.48±14.63 kg/cm²). The lowest bond strength was observed between acrylic resin and the CPTi plates of the lattice and mesh retentive framework designs, 105.53±14.70 kg/cm² and 98.54 ±9.30 kg/cm²,

respectively. Tensile bond strength comparison between the test specimens (Table 1) also showed that the tensile bond strength of acrylic resin blocks adhered to different Ti designs was significantly improved after using the Rocatec™ bonding agent (t=11.667, 3.494, 4.907 and 6.958, respectively; P<0.5). Table 2 shows the bond strength of the titanium frameworks to be significantly different, either with or without treatment using the Rocatec™ bonding agent (t=18.736 and 8.209, respectively; P<0.5).

Table 3: Bond strength (kg/cm²) of acrylic resin to different designs of Co-Cr frameworks

Group	Group I Acrylic resin bonded with Co-Cr flat plate design		Group II Acrylic resin bonded with Co-Cr lattice design		Group III Acrylic resin bonded with Co-Cr mesh design		Group IV Acrylic resin bonded with Co-Cr beads design	
	Subgroup		Subgroup		Subgroup		Subgroup	
Sample No.	A	B	A	B	A	B	A	B
Mean	65.59	107.28	63.39	94.18	78.76	135.33	91.72	147.01
SD	16.11	15.12	17.60	11.41	13.15	24.14	17.33	20.65
t-value	4.621		3.596		5.042		5.024	
p-value	0.001		0.005		0.001		0.001	

Subgroup (A): Sandblasted Co-Cr design without treatment

Subgroup (B): Sandblasted Co-Cr design treatment with Rocatec™ bonding agent

Table 4: Comparison of bond strength (kg/cm²) of acrylic resin to different designs of Co-Cr frameworks with and without Rocatec™ bonding agent

Group	Group I	Group II	Group III	Group IV	F
Without treatment (A)	65.59± 16.11	63.39± 17.60	78.76± 13.15	91.72± 17.33	3.966 P=0.023
Treatment with Rocatec™ (B)	107.28± 15.12	94.18± 11.41	135.33± 24.14	147.01± 20.65	10.466 P<0.001

Tables 3 and 4 show that the shear bond strength for different retentive frameworks without treatment (subgroup A) was highest for Co-Cr plates with bead retention (91.72±17.33 kg/cm²), followed by mesh retention (78.76±13.15 kg/cm²) and the flat plate (65.59±16.11 kg/cm²) designs. The lattice retentive framework yielded the lowest shear bond strength (63.39±17.60 kg/cm²). Greater bond strength was observed when Co-Cr frameworks were bonded

with Rocatec™ to the acrylic resin (subgroup B). The bond strength for Rocatec™-treated frameworks was highest for Co-Cr plates with the bead retentive design (147.01±20.65 kg/cm²), followed by the mesh retention (135.33±24.14 kg/cm²) and flat plate (107.28±15.12 kg/cm²) designs. Lattice retention showed the lowest bond strength (94.18±11.41 kg/cm²).

Table 5: Comparison of bond strength (kg/cm²) of acrylic resin to different designs of Ti and Co-Cr frameworks with Rocatec™ bonding agent

Retentive Framework Designs	Group I	Group II	Group III	Group IV
Ti	40.48 ± 7.64	77.35 ± 13.20	75.42 ± 6.83	72.37 ± 10.43
Co-Cr	65.59 ± 16.11	63.39 ± 17.60	78.76 ± 13.15	91.72 ± 17.33
t-value	3.449	2.972	1.555	1.494
p-value	0.006	0.014	0.151 NS	0.166 NS

NS= not significant at the 5% level

Table 6: Comparison of bond strength (kg/cm²) of acrylic resin to different designs of Ti and Co-Cr frameworks without Rocatec™ bonding agent

Retentive Framework Designs	Group I	Group II	Group III	Group IV
Ti	136.39± 18.63	105.53± 14.70	98.54± 9.30	123.48± 14.63
Co-Cr	107.28± 15.12	94.18± 11.41	135.33± 24.14	147.01± 20.65
t-value	0.551	3.484	2.343	2.277
p-value	0.494 NS	0.006	0.041	0.046

NS= not significant at the 5% level

Table 5 showed a statistically significant ($P<0.05$) difference in bond strength between the 2 alloys for both flat plate and lattice retentive frameworks bonded with Rocatec™ to the acrylic resin. No significant ($P>0.05$) differences in bond strength were found between the 2 alloys for the mesh or bead retentive frameworks, as they both adhered to the acrylic resin with Rocatec™.

There was a statistically significant ($P<0.05$) difference in bond strength between the 2 alloys for all retentive frameworks bonded without Rocatec™ to acrylic resin, except for the flat plate retentive frameworks, which was not significant ($P>0.05$) (Table 6).

4. Discussion

Recent developments in resin bonding have provided the means for direct chemical bonding of acrylic resin to a metal framework. The investing alveolar and gingival tissue replacement components can be attached without the use of loops, mesh or surface mechanical locks¹. Many studies have been conducted to determine the retentive design that can establish better bonding¹⁴. Chemical bonding between the metal framework and the denture base resin is also important. Poor chemical bonding in that area is a significant clinical problem, often introducing an adhesive failure and increasing microleakage of oral fluids into the finish lines, which causes an accumulation of oral debris, microorganisms and stains. As a result, the propagation of microorganisms contributes to an unfavorable soft tissue response¹⁹. In the present study, the Rocatec™ had a significantly positive effect on the bond between the heat-cured denture base resin and both CP Ti and Co-Cr alloys. May *et al.*, found that airborne particle abrasion of grade IICP Ti frame works did not improve the bond strength to PMMA when compared with those without any treatment^{9, 10}. They also found that surface pre-treatment of grade II titanium with 110µm of alumina airborne particle abrasion and silica coating significantly enhanced the shear bond strength to PMMA. The bonding method applied here required mechanical cleaning by air abrasion with alumina prior to the bonding procedure, which also increased the bonding area. Air abrasion can create suitable surface conditions of roughness and increase

the wettability of the metal surface. Thus, air abrasion with alumina should be performed prior to chemical modification¹⁶.

Airborne particle abrasion creates surface roughness by cleaning the surface of metal oxides and other substances and increases the chemo-mechanical bond strength between the metal and the acrylic resin. Specifically, the bonding generated by alumina air abrasion is mechanical, whereas the bonding generated by the Rocatec system is chemical-mechanical². In 2010, Bulbul and Kesim reported that the shear bond strength of base metal, titanium, and noble alloy to acrylic resins was improved by primer application²⁰.

The current study determined that surface pretreatment of CP-Ti and Co-Cr alloys improved bonding adhesion of the denture base to acrylic resin. The bond strength of heat-cured PMMA retained on both alloys pre-treated with Rocatec™ was evaluated. This agent is considered to be a hybrid bonding system that uses mechanical (embedded silane) and chemical retentive mechanisms that function together to enhance retention of acrylic resin. Both CP-Ti and Co-Cr alloys were configured as frameworks with four acrylic retentive designs: flat plate, lattice retention, mesh retention and bead retention. The dimensions of the retentive frameworks were selected to represent a clinical situation. CP-Ti and Co-Cr plates were either pretreated or not pretreated with Rocatec™ bonding agent. PMMA showed the highest bond strength to both flat plates and beads of pretreated titanium plates. The results of this study were not in agreement with those reported by Lee *et al.*, who demonstrated that the metal plate with bead retention showed significantly higher mean separation forces compared with smooth metal plate and lattice retention. The metal plate with bead retention proved to be effective in mechanically bonding acrylic resin to cast metal frameworks¹³.

The lowest bond strengths were observed with both lattice and mesh types. Bond strength of acrylic resin to titanium not pretreated with the bonding agent gradually decreased in the following order: flat surface, lattice, mesh, and bead retentive designs. Denture base resins and metal frameworks are substantially joined by mechanical retentive devices. An adhesive bonding agent may be useful to prevent marginal leakage as well as fracture of the resin

material at the border of the resin-to-metal joint². Denture deflection during mastication can result in debonding between the denture base resin and the framework, eventually leading to resin fracture. Thus, titanium frameworks should be designed to be stiff enough to keep deflection to a minimum²⁴.

In contrast, bond strength of acrylic resin to Co-Cr plates, whether pretreated with Rocatec™ or not, gradually decreased in the reverse order as that observed with titanium not pretreated with bonding agent: beads, mesh, flat surface, and lattice retentive designs. Any difference between bond strength values reported in the literature and in the present report may be due to the difference in chromium content of the tested alloys²¹. The Co-Cr plates with bead retentive design showed significantly higher bond strength than the mesh, flat plate and lattice retentive designs. This finding was inconsistent with a previous study that showed that bead retention did not offer strong retention for acrylic resin, whereas the open lattice design provided the strongest retention due to the bulk of the acrylic resin²². Mesh retention showed significantly higher bond strength than the lattice and flat plate designs. This was consistent with findings reported by Brown *et al.*, and Canay *et al.*, who demonstrated that retentive mesh was more effective in retaining acrylic resin than the lattice design^{6,23}. Additionally, flat Co-Cr plate designs showed significantly higher bond strength than lattice retentive designs, which supported the results obtained by Lee *et al.*, as smooth primed metal plates displayed significantly higher mean separation forces than those of primed lattice retention¹³.

Microleakage at the junction between the metal alloy and the acrylic resin in RPDs may result in discoloration, fluid percolation, and acrylic resin deterioration. Enhancing resistance to microleakage at this interface may improve the long-term union between the 2 materials²⁵. The application of an adhesive bonding agent may prevent marginal leakage as well as fracture of the resin material at the border of the resin-to-metal joint². May *et al.*, evaluated bond strength using air-abrasive, pretreated titanium adhered to PMMA but found no significant differences between the bond strength for Ti and PMMA when treated (or not treated) with an air abrasive. The tensile bond strength test allowed initial bond failure at the metal-PMMA interface to be quantified. The results of this study estimated that the highest bond strength was observed between acrylic resin and both plain-flat and beaded titanium plates when pretreated with Rocatec™ bonding agent. This finding may be explained by the bond strength being proportional to the surface area of titanium-PMMA interface¹⁴. May *et al.*, suggested that shear bond strength of heat-processed PMMA bonded to the

machined surface of wrought CP titanium with 110 µm of alumina air abrasion and silane coating was 63% greater when compared with specimens with no pretreatment. Canay *et al.*, studied 3 different retentive designs (mesh, ring-shaped, flat plate) that were subjected to a shear test and observed that the bond strength was highest between the 4-META adhesive acrylic resin and the flat plate design. 4-META is a recently developed, metal-bonding denture base material that is reported to possess excellent bond strength to metal^{9,12}. The metal plate with bead retention proved to be effective in mechanically bonding acrylic resin to cast metal frameworks. The size and number of beads were important, as the acrylic needed to flow evenly around the bead undercuts¹³.

In this study, a decrease in bond strength was observed when either lattice or mesh was incorporated in the center of pre-treated Ti plates. This may be due to the open spaces that reduced the surface area of the bond, resulting in lower strength. May *et al.* showed that the treatment of Ti with 4-META leads to a bond strength to acrylic resin denture base as consistent as when Ti was treated with the Rocatec™ bonding material⁹.

In this study, the bond strength between the acrylic resin and Ti plates not pre-treated with Rocatec™ bonding agent were highest with the lattice design followed by mesh then the beads. However, the difference among these designs was not statistically significant. This sequence supported the design considerations for minor connectors in which more open areas contained within the retentive minor connectors permit increased bulk of resin and, thereby, increase strength^{7,8}. In contrast, the weakest bond was noted when using flat Ti plates as the initial sandblasting may achieve micro-retentive topography and increase the surface area²⁶. The need of mechanical retentive elements such as lattice, mesh, loops, beads or posts, is essential for retaining denture base acrylic resin to minor connectors. These retentive elements may weaken the acrylic resin base by creating stress and by reducing the bulk on which the resin depends for strength. Failure of acrylic resin at the interface is a common problem when forces exceed the capacity of the retentive mechanisms.⁶ To minimize or eliminate these mechanical retentive elements that weaken the acrylic resin base, the strength of the bonding of the acrylic resin to the metal framework is an important aspect of prosthodontic research when compared with the emerging trends for fabricating implant-supported fixed or removal prostheses using Co-Cr or Ti metal frameworks.

Conclusion:

Within the limitations of this study, the shear bond strength of PMMA denture base material to cast titanium or cobalt-chromium alloy can be improved significantly by the application of the Rocatec™ bonding system.

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References

- 1- Carr AB, Brown DT(2011): McCracken's Removable Partial Prosthodontics, 12th ed. Elsevier, Mosby, pp: 110-112.
- 2- Matsuda Y, Yanagida H, Ide T, Matsumura H, Tanoue N(2010): Bond Strength of Poly(methyl methacrylate) Denture Base Material to Cast Titanium and Cobalt-Chromium Alloy. *J Adhes Dent.*; 12: 223-229.
- 3- Kim S, Vang M, Yang H, Park S, Lim H(2009): Effect of adhesive primers on bonding strength of heat cure denture base resin to cast titanium and cobalt-chromium alloy. *J Adv Prosthodont.*;1:41-46
- 4- Powers JM, Sakaguchi RL(2006): Craig's restorative dental materials. 12th ed. St. Louis: Elsevier;. p. 360, 375, 468.
- 5- Ohkubo C, Hanatani S, Hosoi T(2008): Present status of titanium removable dentures – a review of the literature. *J Oral Rehabil.*, 35:706–714
- 6- Brown DT, Desjardins RP, Chao EY(1987): Fatigue failure in acrylic resin retaining minor connectors. *J Prosthet Dent.*;58:329-35.
- 7- Boucher LJ., Renner RP. (1982): Treatment of Partially Edentulous Patients. St. Louis, Mosby, pp: 20:52-55.
- 8- Phoenix RD, Cagna DR, DeFreest CF(2008): Stewart's Clinical Removable Partial Prosthodontics, 4thed., Quintessence, pp:37-42
- 9- May K. B., Putten, M. C., Bow, D. A., and Lang, B. R(1997): 4-META polymethyl methacrylate shear bond strength to titanium. *Operative Dent.*, 22: 37-40.
- 10- May KB, Russell M M, Razzoog ME, Lang BR(1993): The shear strength of polymethyl methacrylate bonded to titanium partial denture framework material. *J Prosthet Dent.*;70: 410-413.
- 11- May, K.B., Fox, J., Razzoog, M.E., and Lang, B.R(1995): Silane to enhance the bond between polymethyl methacrylate and titanium, *J. Prosth. Dent.*, 73: 428-431.
- 12- Jacobson TE, Chang J C, Keri PP Watanabe LG(1988): Bond strength of 4-META acrylic resin denture base to cobalt chromium alloy. *J Prothet Dent.*; 60: 570-576.
- 13- Lee G, Engelmeier RL, Gonzalez M, Powers JM, Perezous LF, O'Keefe KL(2010): Force Needed to Separate Acrylic Resin from Primed and Unprimed Frameworks of Different Designs. *J Prosthodont.*;19:14-19
- 14- Canay S, Hersek N, Tulunoglu I, Uzun G (1997): Effect of 4-META adhesive on the bond strength of different metal framework designs and acrylic resins. *J Oral Rehabil.*;24:913-919.
- 15- Yoon SH, Pae A, Lee SH, Lee H(2007): Comparative study of shear bond strength between CP-TI / CO-CR alloy and composite resins. *J Korean Acad Prosthodont.* ;45:805-814
- 16- Yanagida H, Tanoue N, Ide T, Matsumura H (2009): Evaluation of two dual-functional primers and a tribochemical surface modification system applied to the bonding of an indirect composite resin to metals. *Odontology*; 97:103–108
- 17- 3M ESPE scientific product profile Rocatec. Scientific Affairs 12/01.
- 18- Sarafianou A, Seimenis I, Papadopoulos T(2008): Effectiveness of different adhesive primers on the bond strength between an indirect composite resin and a base metal alloy. *J Prosthet Dent.*; 99:377–387
- 19- Jacobson TE(1989): The significance of adhesive denture base resin. *Int J Prosthodont.*;2:163-72
- 20- Bulbul M, Kesim B(2010): The effect of primers on shear bond strength of acrylic resins to different types of metals. *J Prosthet Dent.*;103:303-308)
- 21- Watanabe E. Powers JM, Lorey LE (1988): *In vitro* bonding of prosthodontic adhesives to dental alloys. *J Dent Res.*, 67; 479-483
- 22- Dunny JA, King GE(1975): Minor connector designs for anterior acrylic resin bases: a preliminary study. *J Prosthet Dent.*;34:496-502
- 23- Canay S, Hersek N, Tulunoglu I, *et al.*(1997): Effect of 4-META adhesive on the bond strength of different metal framework designs and acrylic resins. *J Oral Rehabil.*;24:913-919
- 24- Ohkubo C, Watanabe I, Hosoi T, Okabe T(2000): Shear bond strengths of polymethyl methacrylate to cast titanium and cobalt-chromium frameworks using five metal primers. *J Prosthet Dent.*;83:50–57.
- 25- Sharp B, Morton D, Clark AE(2000): Effectiveness of metal surface treatments in controlling microleakage of the acrylic resin-metal framework interface *J Prosthet Dent.*;84:617-22.)
- 26- Kern M., and Thompson, V.P. (1994): Effects of sandblasting and Silica Coating procedure on pure titanium. *J. Dent.*, 22: 300-306.

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