Modulatory Effects of Pomegranate Juice on Nucleic Acids Alterations and Oxidative Stress in Experimentaly Hepatitis Rats

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Abstract : The present study was designed to test whether the pre-treatment with pomegranate juice could attenuate the nucleic acids alterations and oxidative stress that produced in D-Galactosamine /lipopolysaccharide induced hepatitis in rats. Animals were dosed with D-Galactosamine / lipopolysaccharide (300 mg kg^{-1} b.wt , i.p / 30 µg kg^{-1} b.wt , i.p) with or without pretreatment of pomegranate juice. The protective role of pomegranate juice was evaluated on the aspects of the release of hepatic enzymes into serum, the nucleic acids alterations**,** the formation of malondialdehyde, and the histopathological changes in hepatic tissues. Obtained results revealed that D-Galactosamine / lipopolysaccharide led to increase in the activities of serum marker enzymes such as aspartate transaminase, alanine transaminase and alkaline phosphatase, while there was a significant inhibition in deoxyribonucleic acid, ribonucleic acid and protein contents in liver tissues. Oxidative stress was also increased in hepatic tissue represented by increased malondialdehyde and decrease of antioxidants (superoxide dismutase, catalase and reduced glutathione). which accompanied with histopathological changes in the hepatic tissue.In addition; pretreatment with pomegranate juice (20 ml kg^{-1} b. w. day^{-1} for 14 days) effectively hindered the adverse effect of D-Galactosamine / lipopolysaccharide and protect against hepatic damage via suppression of oxidative stress. Histopathological studies of the liver of different groups also support the protective effects exhibited by pomegranate juice through restoring the normal hepatic architecture. In conclusion, pomegranate extract could afford a significant protection in the alleviation of D-Galactosamine / lipopolysaccharide –induced hepatiis.

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1. Introduction

Hepatitis is a major public health problem worldwide, responsible for considerable morbidity and mortality from chronic liver disease [1]. Endotoxin from gram negative bacteria (lipopolysaccharide: LPS) induces septic shock and finally wide variety of several organ disorders in human and animals [2]. On the other hand, D-galactosamine (GalN) highly sensitizes animals to develop lethal liver injury mimicking fulminant hepatitis when given together with a sublethal dose of lipopolysaccharide (LPS) [3,4]. A growing body of evidence is emerging which suggests that reactive oxygen-derived free radicals play a crucial role in the pathogenesis of LPS/D-GalN induced liver injury [5,6].

There is an urgent need for the clinical development of safe and non-toxic cytoprotective agents for the adequate management of hepatitis. Hence crude drugs or natural food diet which possess antioxidant or free radical scavenging activity has become a central focus for research designed to prevent or ameliorate tissue injury and may have a significant role in maintaining health [7]. .*Punica granatum*, commonly known as pomegranate, is a shrub or a small tree native, to the Mediterranean region. Edible parts of pomegranate fruit (about 50% of total fruit weight) comprise 80% juice and 20% seeds. Fresh juice contains 85% water, 10% total sugars, and 1.5% pectin, ascorbic acid, and polyphenolic compounds such as anthocyanins, punicalagin, ellagic and gallic acid. The soluble polyphenol content in pomegranate juice (PJ) varies within the limits of 0.2% to 1.0% depending on the variety [8]. The antioxidant activity of pomegranate associated with its phytochemicals, such as, polyphenols, flavonoids, and anthocyanidins has gained importance [9]. Fruits are globally consumed fresh, in such processed forms as juice, jam, wine and oil and in extract supplements [10]. PJ has anti-arthrogenic [11]; anti-carcinogenic [12], chondroprotective [13], anti-nephrolithiasis [14], anti-bacterial [15] and anti-gastric ulceration effects [16]. In addition pomegranate could modify the risk of hypercholesterolemia [17] and has photoprotective properties on skin [18]. The aim of the present study was to evaluate the efficacy of pre-supplementation with PJ against nucleic acid alterations and oxidative stress in hepatitis induced by D- GalN / LPS in rats.

2. Materials and Methods

Pomegranate juice preparation

The fresh pomegranate fruits, free of blemishes or obvious defects were washed and stored at 4°C until use. The fruits were manually peeled, without separating the seeds. PJ was obtained by squeezing using a commercial blender (Braun blender, Germany) and was filtered to remove the residue. The juice was used within 1 h after squeezing and filtration [13].

Animals:

The study was conducted on forty adult male Sprague –Dawley rats with body weights 120- 150 g, were obtained from the Animal House in National Research Centre, Giza, Egypt. Animals were housed in plastic cages at an environmentally controlled room (constant temperature 25-27°C, with 12h light / dark cycle) for one week prior to starting the experiments and they were fed on standard laboratory diet and water *ad libitum*.

Experimental protocol:

The animals were divided into four groups of rats each of ten.

G 1: served as vehicle control and received distilled water through oral route (20 ml kg^{-1} b. w, day^{1}) for 14 days.

G 2: rats were given PJ orally by gavages at a dose of 20 ml kg^{-1} b. w, day^{-1} for 14 consecutive days. The used dose was selected on the basis of the previous studies [13]. **G3**: rats were induced with hepatitis by giving i.p injections of D-GaIN and LPS (300 mg kg^{-1} b.w and 30 μg/ kg^{-1} b.w, respectively) which well known to induce hepatitis damage [19].

G 4: rats were pretreated with PJ for 14 days prior to the induction with D- GaIN / LPS as in G3.

Blood collection and tissue homogenate:

After the end of the treatment period, rats were fasted overnight and the blood samples were collected using capillary tubes from the retro-orbital plexus of the individuals of all groups. Samples were left to clot then centrifuged at 3000 rpm for 15 min to separate the sera, which were stored at −20ºC until analysis could be completed. The serum was used for the assay of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP). After blood collection, all animals were rapidly sacrificed and the liver of each animal was dissected,weighted and portion of it was preserved in 10% formalin (pH7.2) and subjected to histopathological examination. The remaining part then divided into two parts, the first part for the determination of hepatic antioxidants and malondialdehyde (MDA) as marker for lipid peroxidation. The second part for the determination of nucleic acids and total proteins. The first part of liver of each animal immediately homogenized in 50mM ice –cold phosphate buffer (pH 7.4) to give 20% homogenate (W/V) [20]. The homogenate was centrifuged at 1700 rpm and 4ºC for 10 min. and the supernatant was stored at -70ºC to the next day until analysis. This supernatant (20%) was used for the determination of hepatic MDA and it was further diluted with phosphate buffer solution to give 2% and 0.5% dilutions for the determination of hepatic catalase (CAT), superoxide dismutase (SOD) activities and reduced glutathione (GSH) level. The second parts of the samples fresh or frozen was taken, blotted using filter paper, weighted and homogenized in 0.9% sodium chloride solution for 5 minutes at 0ºC for the determination of nucleic acids and total proteins.

Biochemical assays

Determination of serum AST, ALT and ALP

Serum AST and ALT were assessed spectrophotometerically by the dinitrophenylhydrazene method [21], using spectrophotometer **(Shimadzu – Model UV 2401).** ALP was assayed by spectrophotometric method described by [22]. Commercially available reagent kits for assays AST, ALT and ALP were purchased from Roche Diagnostic kits (Germany).

Determination of nucleic acids and proteins:

Nucleic acids were extracted from liver by using trichloracetic acid (TCA) and ethanol according to the methods of [23,24]. Hepatic tissue were homogenized (10%) in Tris-EDTA; 0.01 M using a Potter-Elvehjem homogenizer with a Teflon pestle. 5.0 ml of 10% TCA was then added to the homogenate (1 ml) and kept in ice for 30 minutes, to allow complete precipitation of proteins and nucleic acids. The mixture was centrifuged and the precipitate obtained was washed thrice with ice cold 10% TCA. It was then treated with 95% ethanol to remove lipids. The final precipitate was suspended in 5.0 ml of 5% TCA and kept in a water bath, maintained at 90ºC for 15 minutes with occasional shaking which facilitated the quantitative separation of nucleic acids from proteins. The supernatant after centrifugation was used for the estimation of DNA and RNA. Tissue DNA content was measured by the method described by [25]. The blue colour developed was read spectrophotometrically at 640 nm. The value was expressed as mg/g tissue. Tissue RNA content was measured by the method of [26]. The reading was taken at 655 nm and was expressed as mg/g tissue.

Protein measurement:

The total protein content in liver samples were assayed according to [27], and the level was expressed as mg protein /g tissue.

Determination of hepatic antioxidants and lipid peroxidation

Malondialdehyde was estimated as index for lipid peroxidation by spectrophotometric method [28] using Oxis Research™ Co. Kit (USA) and was expressed as nmol/g tissue. The assay for hepatic CAT activity was

carried out spectrophotometerically by the modified method of [29] using 50 μL diluted liver homogenate using kit purchased from Oxis Research™ Co., USA. Hepatic SOD activity was determined spectrophotometerically by red formazan dye reduction procedure [30] using 50 μL diluted liver homogenate using Ransod kit from Randox Laboratories Co., UK. Hepatic GSH concentration was assayed spectrophotometerically by the modified method of [31], using Ransel kit obtained from Randox Laboratories CO., UK. The specific activity of hepatic CAT and SOD was expressed as units/g tissue, where the concentration of GSH was expressed as mmol/g tissue.

Histopathological examination was evaluated according to the method described by [32].

Statistical analysis:

Results were expressed as the mean values ± the standard error, using Microcal Excel™ for windows (Microcal Software, 2000), and statistical differences between groups were assessed by Student's t –test. Values of $P<0.05$ were considered significantly different.

3. Results:

Biochemical assessments

As shown in **table (1);** rats intoxicated with D-GalN/LPS (G3) developed a state of hepatic damage as evident from a significant ($P < 0.001$) elevation in the serum marker activities of AST and ALP when compared with control group. Pretreatment with PJ (G4) afforded a significant protection against D-GalN/LPS – induced hepatitis, where the activities of AST, ALT and ALP were significantly ($P< 0.05$) decreased as compared to G3.

As shown in **table (2);** the toxicity of D-GalN/LPS was accompanied with significant ($P< 0.001$) decrease in the levels of DNA, RNA and protein contents as compared with G1. Also, there was a significant ($P<0.05$) increase in tissues DNA, RNA and protein contents as compared with G3.

With regard to MDA content , CAT & SOD activities and GSH concentration , the results in **Table (3)** showed that treatment of rats with D-GalN/LPS caused a significant ($P < 0.001$) increase in hepatic MDA content in comparison to G1, whereas pre-treatment with PJ (G4) resulted in a significant reduction in MDA content as compared with G3. Also, the treatment with D-GaIN/LPS resulted in a significant ($P<0.001$) decrease in CAT, SOD and GSH values in comparison to G1. Pre-treatment with PJ resulted in a significant ($P<0.05$) increase in the activities of CAT, SOD and GSH content in comparison with G3.

Table1. Effect of PJ on liver enzymes activities in D-GalN/LPS -induced hepatitis in rats.

Groups	AST U/ml	ALT U/ml	ALP IU/L
G1	38.6 ±1.3	37.3±1.4	124.5±3.83
G2	40.3±0.83	37.1±1.12	126.5±2.73
G3	50.4±2.64 b**	59.4±5.38 b**	301.6±14.5 b**
G4	46.9±1.3 a*	45.4±1.4 a*	274±7.1 a*

a: indicates significantly from G3 ; b: indicates significantly from G1 ; *:P< 0.05 and **: P<0.001 .

Table 2. Effect of PJ on Tissue DNA, RNA and Protein contents in D-GalN/LPS -induced hepatitis in rats.

Groups	DNA	RNA	Protein
	mg / whole liver		
G1	7.45 ± 0.16	8.96 ± 0.23	201.41 ± 4.22
G2	7.95 ± 0.27	8.39 ± 0.30	199.77 ± 5.25
G3	5.64 ± 0.22b**	6.34 ± 0.35b**	171.05 ± 6.14b**
G4	6.06 ± 0.14a*	7.45 ± 0.17a*	188.12 ± 5.06a*

a : indicates significantly from G3 ; b: indicates significantly from G1 ; *:P< 0.05 and **: P<0.001 .

Table 3. . Effect of PJ on Tissue MDA and antioxidants in D-GalN/LPS -induced hepatitis in rats.

Groups	MDA (nmol/g liver)	CAT (U/g liver)	SOD (U/g liver)	GSH (mmol/g t liver)
G1	4.4± 0.37	1.72±0.04	7.06±0.17	0.18±0.02
G2	4.32±0.35	1.7±0.05	7.14±0.06	0.19±0.01
G3	15±1.20b**	0.51±0.04b**	6.4±0.13b**	0.07±0.01b**
G4	9.5±0.46a**	0.81±0.03 a*	6.67±0.09a*	0.12±0.01a*

a : indicates significantly from G3 ; b: indicates significantly from G1 ; *:P< 0.05 and **: P<0.001 .

Histopathological investigation of liver

The histological evidence authenticated the injury caused by D-GalN/LPS and the protection offered by PJ to hepatocytes. **Fig. 1** Illustrated a section of control rat liver showing normal architecture. In **Fig. 2,** a section of liver of PJ treated rat showing normal liver parenchyma with central vein and cords of hepatocytes. Microscopical examination revealed loss of architecture and cell necrosis with inflammatory collections in the central zone in D-GalN/ LPS -induced rats (G3, **Fig.3**). Pre-treatment with PJ(G4) prevented the histopathological changes in liver induced by D-GalN/LPS **(Fig .4).**

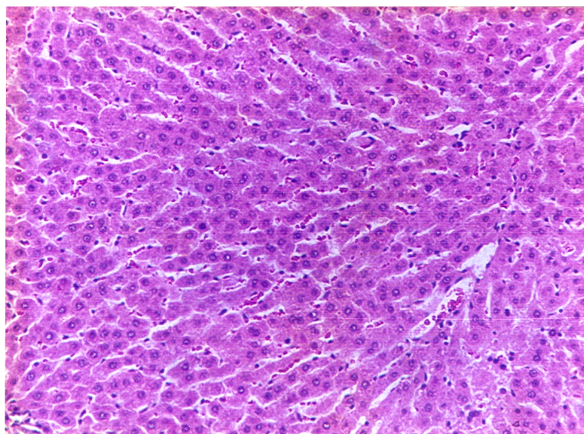

Fig. 1

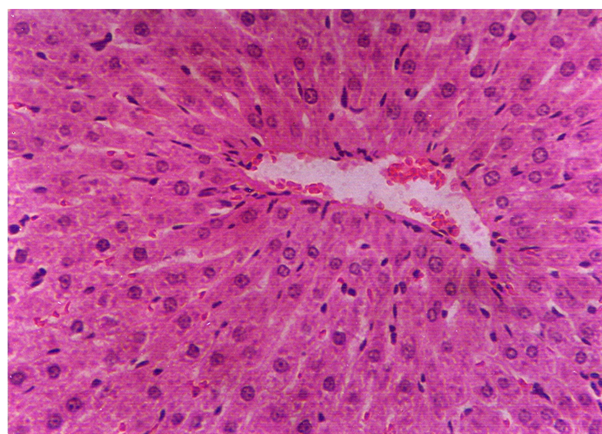

Fig. 2

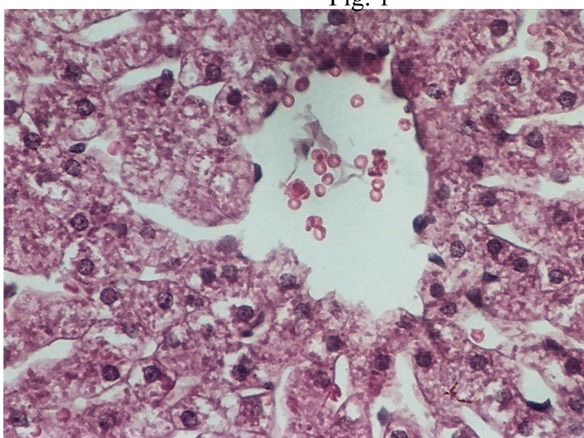

Fig.3

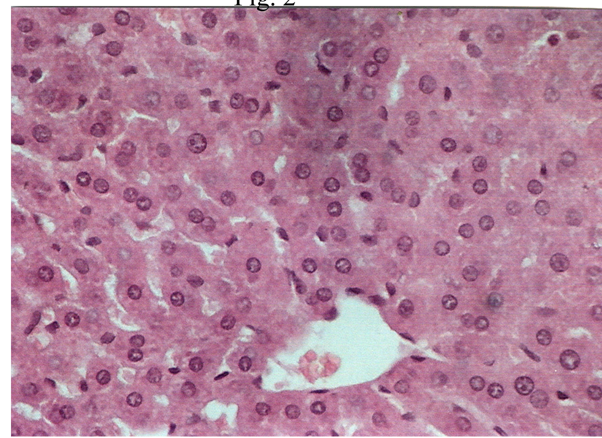

Fig.4

(H & E X 400)

4. Discussion

In the current study, D-Ga1N / LPS intoxication resulted in a significant increase in the serum level of diagnostic marker enzymes (AST, ALT and ALP) as compared to the control group. This is an indicative of cellular leakages and loss of functional integrity of cell membrane in liver. This observation is in agreement with [19] and [33]. The elevation of transaminases could be taken as an index of liver damage and this may be due to the fact that D-GalN / LPS administration disrupts plasma membrane permeability causing leakage of the enzymes from the cells. These findings is confirmed with the results of the histopathological investigation which revealed loss of architecture and cell necrosis with inflammatory collections in the central zone. In this study, oral pretreatment with PJ attenuated the D-GalN / LPS-induced elevation in the level of these diagnostic marker enzymes demonstrating the cytoprotectve activity of PJ which preserved the structural integrity to the hepatocellular membrane and liver cells architecture damage caused by D-GalN / LPS.

In the current study, liver damage induced by D-GalN/LPS was accompanied by a significant inhibition of liver DNA, RNA and protein contents. The metabolism of D-GalN may deplete several uracil

nucleotides including UDP-glucose, UDP-galactose and UTP which are trapped in the formation of uridine diphosphogalactosamine and it has thought that D-GalN induces liver injury by inhibiting the synthesis of RNA and protein through a decrease in hepatic UTP concentration which finally evokes the necrosis of liver cells [34,35]. Furthermore, bacterial endotoxin such as LPS is among the agents that cause immunological stimulation of Kupffer cells [36]. Activation of Kupffer cells contributes to liver injuries by releasing cytotoxic agents, inflammatory cytokines and ROS, this may lead to severe oxidative damage of the liver cells [37] and the cellular components like cell membrane, lipids, proteins and DNA **[38].** In addition, [39] also reported that D -GalN/LPS intoxication increases the neutrophil infiltration into the liver cells with increased release of ROS species from the activated neutrophils.

In the present work, administration of GalN/LPS caused also a significant increase in the MDA level together with a decease in the activity of the antioxidant enzymes CAT and SOD) and the non-enzymatic antioxidant GSH level in hepatic tissue reflecting an oxidative stress state. Our results are in agreement with those observed by [19,40] who reported a significant increase in lipid peroxides accompanied with decreases in the antioxidant parameters CAT and SOD due to D-GalN/LPS intoxication. This selective inhibition of antioxidant enzymes activities might be justified by the suggestion of [41] that D-GalN can selectively block hepatic transcription and indirectly blocks hepatic protein synthesis.

In this study, oral pre-treatment with PJ effectively protected the liver from the toxicity of D-Ga1N / LPS by decreasing the oxidation process proved by decreasing hepatic MDA together with increasing the serum activities of CAT and SOD as well as GSH level. Concomitantly, it partly prevented liver enzymes from elevation indicating the protection of the cell membrane from free radicals attack. The histopathological examination confirmed these results showing the improvement in the signs of necrosis and cellular infiltration together with the marked prevention of protein ; DNA and RNA loss in the hepatic tissue. These results are in accordance with the findings of [42], which demonstrated in vitro that besides scavenging free radicals and ROS, pomegranate also prevents DNA damage and with the results of [43] who confirmed the ability of pomegranate to protect DNA and preventing chromosomal damage in mice. In addition, [44] demonstrated that pomegranate extract afforded up to 60% protection against hepatic lipid peroxidation due to the maintenance of the GSH levels and activities of CAT, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase. Furthermore, [45] found a direct correlation between antioxidant capacity and antimutagenic activity of pomegranate peels.

There is a possibility that orally administered PJ exerts a preventive effect on liver injury progression in D-Ga1N/LPS treated rats through its indirect antioxidant action to maintain antioxidant defense system in addition to its direct antioxidant action to scavenge ROS and to inhibit lipid peroxidation . The hepatoprotective property of PJ may be attributed to the presence of different bioactive components, mainly polyphenols, ellagitannins, condensed tannins, and anthocyanins which have antioxidant properties.

Thus, the present study confirms the hepatoprotective action of pomegranate against D-GalN/LPS induced hepatitis in rats which may accept a clinical importance because there is close resemblance between the multifocal necrosis produced by D-GalN/LPS and the lesion of viral hepatitis in humans. In coclusion; pomegranate could serve as a better remedy for liver disease and controlled clinical studies in viral hepatitis would be worthwhile.

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