Role of Vitamin E in Combination with Methionine and L- carnosine Against Sodium Fluoride-Induced Hematological, Biochemical, DNA Damage, Histological and Immunohistochemical Changes in Pancreas of Albino Rats.

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Abstract
Excessive fluoride ingestion has been identified as a risk factor for fluorosis and oxidative stress. The present study was aimed to evaluate vitamin E in combination with methionine and L- carnosine as a potential natural antioxidant to mitigate the effects of sodium fluoride on hematological indices, DNA damage, pancreatic digestive enzyme activities and histological structure of pancreas through light, electron microscopic and immunohistochemical studies. Thirty-six of adult male albino rats were divided into six groups (6 rats in each group). Oral administration of sodium fluoride caused a statistical significant decrease in RBC, HCT, MCV, RDW, MCH, MCHC and PLT and increase in WBC, lymphocytes and granulocytes The levels of the these parameters were significantly reversed in the groups pretreated with vitamin E in combination with methionine and L- carnosine prior to NaF. Animals treated with NaF showed significant decrease in pancreatic digestive enzyme activities and protein levels as compared to the control group, while significant increase in animals treated with vitamin E in combination with methionine and L- carnosine prior to NaF. Also, NaF resulted in a significant decrease in serum total protein, albumin and blood glucose levels, while pretreated with vitamin E in combination with methionine and L- carnosine prior to NaF resulted in a significant increase in these parameters. Plasma malondialdehyde levels were significantly increased and the activities of erythrocyte superoxide dismutase were significantly decreased in the NaF treated group. However, vitamin E in combination with methionine and L- carnosine prior to NaF reduced the process of lipid peroxidation and increased the activity of SOD. NaF reduced DNA, RNA contents of the liver and significant increase DNA damage in liver and the frequencies of micro nucleated polychromatic erythrocytes (MN-PCE) in bone marrow. But, concurrent administration of NaF and vit. E in combination with methionine and L- carnosine for 35 days caused significant amelioration in all parameters was studied. Histologically, multiple vacuoles of variable size were observed in the cytoplasm of pancreatic acinar cells together with inflammatory cells infiltration in the stroma of pancreas of Na F treated group. Pancreas of animals treated with vit. E in combination with methionine and L- carnosine prior to NaF displayed amelioration in toxic effects of NaF. Intensive positive immunoreactivity for caspase- 3 was observed in the cytoplasm of most pancreatic acinar cells of NaF treated group which was of significant value. On the other hand the cytoplasmic acinar cells of vit. E in combination with methionine prior to NaF treated group and L-carnosine prior to NaF treated groups showed apparent reduction of caspase-3 immunoreactivity which were also of significant values. Dilatation and globular- shaped rER, intra-cisternal granules, few or even absence of zymogen granules and irregular shaped, pyknotic and heterochromatic nuclei were observed ultrastructurally in the cytoplasm of pancreatic acinar cells of NaF treated group. Ultrathin sections of serous cells of vit. E in combination with methionine prior to NaF treated group and L-carnosine prior to NaF treated group showed preservation of acinar cytoplasmic contents. These results indicate that sodium fluoride can inhibit pancreatic digestive enzyme activities and cause histological and immunohistochemical changes, which may lead to a series of biochemical and pathological abnormalities and concurrent administration of NaF and vit.E in combination with methionine and L-carnosine for 35 days to these animals alleviated the adverse effects of fluoride. [Fatma E. Agha[1], Mohamed O. El-Badry[2], Dina A. A. Hassan[3], Amira Abd Elraouf .**Role of Vitamin E in Combination with Methionine and L- carnosine Against Sodium Fluoride-Induced Hematological, Biochemical, DNA Damage, Histological and Immunohistochemical Changes in Pancreas of Albino Rats]**. *Life sci J 2012; 9(2):1260-1275].(ISSN:1097-8135*). http://www.sciencesite.com. **187**

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1.Introduction

Fluoride is widely distributed in nature in many forms and its compounds are being used extensively. Fluoride in small doses has remarkable prophylactic influence by inhibiting dental caries while in higher doses it causes dental and skeletal fluorosis (Shanthakumari et al., 2004). However, detrimental effects of high-fluoride intake are also observed in soft tissues (Monsour and Kruger, 1985). Fluoride enters the body through drinking water, food, toothpaste, mouth rinses, and other dental products; drugs and fluoride dust and fumes from industries using fluoride containing salt and hydrofluoric acid (Shulman and Wells, 1997). The fluorosis of human beings is mainly caused by drinking water; burning coal and drinking tea while that of animals is mainly by drinking water and supplementing feed additives such as calcium monohydrogen phosphate containing high levels of fluoride (Liu et al., 2003). Intake of high levels of fluoride is known to cause structural cha biological activities of some nges, altered activities of enzymes, metabolic lesions in the brain and influence the metabolism of lipids (Shivarajashankara et al., 2002). Acute poisoning can terminate in death due to blocking cell metabolism since fluorides inhibit enzymatic processes, particularly metalloenzymes responsible for important vital processes (Birkner et al., 2000). Recent studies revealed that fluoride induces excessive production of oxygen free radicals, and might cause the depletion in biological activities of some antioxidant enzymes like super oxide dismutase (SOD), antioxidant enzymes like

super oxide dismutase (SOD), catalase and glutathione peroxidase (GP*X*) (Chlubek, 2003; Shanthakumari et al.,2004). Toxic effects of fluoride on various biochemical parameters are known (Singh, 1984; Chlubek, 2003). Increased free radical generation and lipid peroxidation (LPO) are proposed to mediate the toxic effects of fluoride on soft tissues (Rzeuski et al., 1998) ;Shivarajashankara et al., (2001a,b) reported increased lipid peroxidation and disturbed antioxidant defense systems in brain, erythrocytes and liver of rats exposed to fluoride.

Although fluorosis has been investigated for many years, there are relatively few studies about its effect on the digestive system such as the pancreas. Enzyme secretions of the exocrine pancreas are required for hydrolysis of nutrients present in food and feed (Rinderknecht, 1993). Studies have shown that excess fluoride can cause DNA damage, trigger apoptosis and change cell cycle (Wang et al., 2004; Ha et al., 2004). The effects of fluoride on hematological parameters have been studied well in experimental models (Khandare et al., 2000; Cetin et al., 2004; Eren et al., 2005; Karadeniz and Altintas, 2008; Kant et al., 2009). However, there are limited studies about effects of chronic fluorosis on hematological parameters in human subjects living in endemic fluorotic areas (Uslu, 1981; Choubisa, 1996).

Excessive exposure to fluorides can evoke several oxidative reactions as induction of inflammation (Stawiarska-Pieta et al., 2007; 2008 & 2009; Shashi et al., 2010; Gutowska et al., 2011), cell cycle arrest and apoptosis in different experimental system (Thrane et al., 2001). Apoptosis is a complex process that involves a variety of different signaling pathways and results in multitude of changes in the dying cells. Many of events that occur during apoptosis are mediated by a family of cysteine proteases called caspases (Kumar et al., 2004). Sequential activation of caspase 3 plays a central role in the execution-phase of apoptosis (Gu et al., 2011). The antioxidative vitamins such as A, E and C and selenium or methionine (Met) and coenzyme Q have been shown to protect the body against many diseases which characterized by disruptive activity of free radicals (Littarru and Tiano, 2007).

Among the non-enzymatic antioxidants, vitamin E is listed; its activity has been studied to a reasonable extent. The anti-oxidative ability of methionine has been recognized to a lesser degree. Methionine may play the role of endogenous scavenger of free radicals (Stadtman and Levine, 2003). Cyclic oxygenation of Met and the reduction of methionine sulfoxide (MetO) may be an important antioxidative mechanism; perhaps, it influences the enzyme activity control (Stadtman et al., 2003). It is presumed that because of those processes, the methionine residues of proteins perform the function of reproducible scavengers of reactive oxygen and nitrogen species (Levine et al., 2000; Stadtman et al., 2002).

Methionine reduces the ototoxic, hepatotoxic, and nephrotoxic activity of some drugs (Abdel-Wahhab et al., 1999; Reser et al., 1999). It also demonstrates a protective influence upon the organism in the course of exposure to sodium fluoride (Błaszczyk et al., 2009&2010). It has been found that joint administration of vitamin E and methionine to rabbits is more efficient in protecting cells against disadvantageous influence of oxidative stress than administration of vitamin E only. This may suggest that methionine takes part in regeneration of the tocopherol radical (Birkner, 2002).

Carnosine is a well known antioxidant acting as a scavenger of active oxygen radicals and peroxynitrite radical implicated in cell injury (Fontana et al., 2002). Carnosine also has SOD-like activity (Guney et al., 2006), and acts indirectly by preserving GSH which is an antioxidant itself playing a pivotal role in reducing lipid peroxides. In addition, the cytosolic buffering activity of carnosine prevents proton and lactate accumulation which is involved in the pathogenesis of oxidative tissue injury (Gariballa and Sinclair, 2000). Improved microcirculation in the injured tissue by carnosine is another factor responsible for reduced lactate accumulation (Stvolinsky and Dobrota, 2000). Recently, it was found that carnosine could also exhibit antioxidant activity by acting at the molecular level causing a dose-dependent up-regulation of hepatic catalase mRNA expression (Liu et al., 2008). The present study was undertaken to assess whether vitamin E plus methionine or L- carnosine may prevent or alleviate the effects of sodium fluoride on hematological indices, pancreatic digestive enzyme activities, DNA damage and histological structure of pancreas through histological and immuno-histochemical studies.

2.Materials and Methods

2.1.Materials:

2.1.1. Chemicals:

Sodium fluoride, methionine and L-carnosine powder (Fluka, Switzerland) were procured from Sigma Chemical (USA). All other chemicals were analytical reagent grade and chemicals required for all biochemical assays were obtained from Sigma–Aldrich Chemicals Co (St. Louis, MO, USA), and Merck (Darmstadt, Germany).

2.1.2. Experimental animals

Thirty-six of adult male Wister albino rats weighing 120–130 g were obtained from animal house of Helwan farm, Egypt. The animals were housed under standard laboratory conditions (12 h light and 12 h dark) in a room with controlled temperature (24.3°C) during the experimental period. The rats were provided ad libitum with tap water and fed with standard commercial rat chow. Animal procedures were performed in-accordance with Guidelines for Ethical Conduct in the Care and Use of Animals

Experimental design

After one week of acclimation, animals were divided into six groups (6 rats in each group).

Group (1) served as control, received distilled water orally by gavages once daily for 35 days.

****Group (2)*** received vit.E (3 mg /rat/day) in combination with methionine (2 mg /rat/day) orally by gavages for 35 days (Stawiarska-Pieta et al., 2009 & 2007).

****Group (3)*** received L-carnosine in a dose of 5 mg/kg bw orally by gavages once daily for 35 days (Soliman et al., 2001).

Group (4) received NaF in a dose of 10 ml/kg bw orally by gavages once daily for 35 days **(**Blaszczyk et al., 2008).

Group (5) received vit. E (3 mg /rat/day) in combination with methionine (2 mg /rat/day) followed by NaF in a dose of 10 mg/kg bw orally by gavages once daily for 35 days.

Group (6) received L-carnosine in a dose of 5 mg/kg bw followed by NaF in a dose of 10 ml/kg bw orally by gavages once daily for 35 days.

2.2.Methods:

2.2.1. Blood collection and tissue homogenate

At the end of the treatment, blood samples were collected from the anaesthetized rats, by direct puncture of the right ventricle and from the retro-orbital vein plexus. Whole blood was used to assay hematological variables, while the serum was used to assay glucose, total protein, and albumin level. Malondialdehyde (MDA) was determined in plasma and superoxide dismutase (SOD) activities in erythrocyte. In addition, pancreas was collected for the estimation of pancreatic digestive enzyme activities and protein concentration.

2.2.2. Biochemical analysis

A-Clinical Hematological Variables

White blood cells (WBC), red blood cells (RBC), haematocrit (Hct), haemoglobin (Hb), mean cell volume (MCV), red cell distribution width (RDW), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and platelet (PLT) were measured on a Sysmex Hematology Analyzer (model K4500).

B-Pancreatic digestive enzyme activities

The pancreas from rats was homogenized and centrifuged. The supernatant was saved for determining the activities of lipase (EC 3.1.1.3), protease, and amylase (EC 3.2.1.1). Lipase was determined at 37°C by a pH-stat titration using tributyrin as substrate according to the method of Erlanson-Albertsson et al., (1987). Protease activity was analyzed with the modified method of Lynn and Clevette-Radford, (1984) using azocasein as substrate. One lipase or protease unit is defined as the amount of enzyme that hydrolyses 1 μmol of substrate per minute. Amylase was determined by the iodometric method (Harms and Camfield, 1966). One amylase unit is the amount of enzyme that hydrolyses 10 mg of starch in 30 min.In pancreatic homogenates; protein concentration was determined using Lowry's method (Lowry et al., 1951)

C-Other serum biochemical analyses

Glucose, total protein and albumin levels were determined using Johnson & Johnson label kits and a Vitros750 model autoanalyser

D-Determination of plasma malondialdehyde (MDA) levels and erythrocyte superoxide dismutase (SOD) activities

Plasma MDA levels, and erythrocyte SOD activities were determined as described by Yoshioka et al., (1979); Sun et al., (1988) respectively.

2.2.2. Determination of nucleic acid (DNA and RNA) contents

Total DNA and RNA contents in the liver were determined according to pears, (1985).

DNA fragmentation was quantified by Di-phenyl Amine l (DPA) method according to Gibb et al., (1997).

2.2.3. Micronucleus assay

Rats were sacrificed 24 h after treatment. Rat's femora were removed through the pelvic bone. The epiphyses were cut and the bone marrow was flushed out by gentle flushing and aspiration with fetal calf serum (Valette et al., 2002). The cell suspension was centrifuged and a small drop of the re-suspended cell pellet was spread onto slides and air-dried. The bone marrow smears were made in five replicates and fixed in absolute methanol and stained with May-Grünwald/Giemsa (D'Souza et al., 2002). Scoring the nucleated BMCs and the percentage of micronucleated BMCs (polynucleated MN-BMCs) was determined by analyzing their number in 3000 BMCs per rat.

2.2.4.Histological,immunohistochemical,and morphometric studies:

- For ***routine histological examination***, the pancreatic specimens were fixed in 10% neutral buffered formaldehyde and processed for paraffin sections of 5μm thickness. Sections were stained with Hematoxylin and Eosin **(**Bancroft and Stevens, 1996).

- *Additional sections were prepared :*

-**For *caspase- 3 immunohistochemical staining*** for detection of apoptosis in pancreatic acinar cells**.** A standard avidin-biotin complex method with alkaline phosphatase detection was carried out. Formalin-fixed paraffin-embedded sections were dewaxed in xylene and rehydrated through graded alcohol to distilled water. The sections were subjected to antigen retrieval by boiling in a microwave for 20 min in 0.01 M sodium citrate buffer (pH 6.0). The primary antibody to caspase-3 (Transduction Laboratories, Lexington, KY) was applied at a dilution of 1:1000 and incubated overnight at 4°C. After incubation, the slides were treated with biotinylated rabbit antimouse immunoglobulin (1:600 for 30 min; Dako Ltd., Ely, UK) washed as before, and then treated with streptavidin and biotinylated alkaline phosphatase according to the manufacturer's instructions (Dako). The slides were then washed, and the signal was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. A negative control reaction with no primary antibody is always carried out alongside the reaction containing sample. The specificity of the caspase-3 antibody was confirmed by comparison with control antibodies (Ansari et al., 1993).

- For ***electron microscopic examination*** pancreatic tissue specimens were immediately fixed in 2.5 % phosphate buffered gluteraldehyde (ph 7.4) at 4ºC for 24 hours and post fixed in 1% osmium tetraoxide for one hour, then dehydrated in ascending grades of ethanol. After immersion in propylene oxide, the specimens were embedded in epoxy resin mixture. Semithin sections (1μm thickness) were cut, stained with toluidine blue and examined by light microscopy. Ultrathin sections (80-90nm thickness) were stained with uranyl acetate and lead citrate **(**Bozzola and Russell, 1998) and were examined and photographed with JEOL 1010 transmission electron microscope.

- For ***quantitative morphometric measurement*** the number of caspase3 positive pancreatic acinar cells were counted in five non overlapping fields of vision from each slide of all animals of each group at X 400 magnification using Leica Qwin 500 C image analyzer computer system and were expressed as cell number per μm^2.

2.2.5. Statistical analysis:

Data were computerized and expressed as mean ± standard deviation using Microsoft office excel 2007 soft ware, where the differences between the four groups were analyzed using student 's t-test . The results were considered statistically significant if $p<0.05$.

3.Results:

3.1 Biochemical Results:

There was a statistical significant decrease in RBC, HCT, MCV, RDW, MCH, MCHC and PLT and a statistical significant increase in WBC, lymphocytes and

granulocytes in sodium fluoride (NaF) treated group as a compared to control group. The levels of the above parameters were significantly reversed in the groups pretreated with vitamin E in combination with methionine and L- carnosine prior to NaF (Table1)

As shown in Table (2): Animals treated with NaF showed significant decrease ($P < 0.001$) in pancreatic digestive enzyme activities and protein levels as compared to the control group, while animals treated with vitamin E in combination with methionine and L- carnosine prior to NaF showed statistical significant increase in pancreatic digestive enzyme activities and protein levels as compared to NaF treated group. The results also indicated that, treatment with NaF resulted in a significant decrease in serum total protein, albumin and blood glucose levels as compared to the control group. On the other hand, pretreated with vitamin E in combination with methionine and L- carnosine prior to NaF resulted in a significant increase in serum total protein, albumin and blood glucose levels (Table 3). Plasma malondialdehyde levels were significantly increased and the activities of erythrocyte superoxide dismutase were significantly decreased in the NaF group compared to the control group. The administration of vitamin E in combination with methionine and L- carnosine prior to NaF reduced the process of lipid peroxidation and increased the activity of SOD (Table 4)

3.2. Determination of nucleic acid (DNA and RNA) contents:

Oral administration of sodium fluoride for 35 days resulted in a significant reduction in the DNA, RNA contents of the liver a and significant increase DNA damage in liver and the frequencies of micro nucleated polychromatic erythrocytes (MN-PCE) in bone marrow. However, concurrent administration of NaF and vitamin E in combination with methionine and L- carnosine for 35 days caused significant amelioration in all parameters was studied (Table 5).

3.3.Histological Results:-

3.3.1.Light microscopic results:

Light microscopic examination of pancreas of control group showed rounded to oval serous acini, the exocrine portion of the pancreas, and pancreatic islets of Langerhans, the endocrine portion of the pancreas, that were packed in a connective tissue stroma (fig.1A). Each acinus was lined by pyramidal cells. Each had a spherical, vesicular and basal located nucleus containing a prominent nucleolus and surrounded by a basophilic cytoplasm. However the apical part was occupied by acidophilic zymogen granules (fig.1B). The pancreatic acini of vit.E in combination with methionine and L-carnosine treated groups were more or less similar in histological structure to those of control group (figs.1C&1D). Histological alternations were observed only in pancreas of NaF treated group. These changes occurred in the form of multiple vacuoles of variable size in the cytoplasm of pancreatic acini (figs.2A&2B). The serous cells had deeply stained pyknotic and peripheral situated nuclei (figs.2B&2C). Furthermore inflammatory cells infiltrations were commonly observed in the stroma of pancreas (fig.2A) especially in area around blood vessels (fig. 2C). Pancreas of vit. E in combination of Na fluoride as evidenced histologically by complete absence of vacuoles and normal appearance of nuclei of pancreatic acinar cells and absence of inflammatory cells infilterations in the stroma of pancreas.. Thus serous cells of pancreatic acini and stroma of pancreas in the last two groups regained its normal structure to be more or less similar to those of control group (figs.2D, 2E&2F)

3.3.2-Immunohistochemical Results:

Pancreatic acinar cells of control group, vit.E in combination with methionine treated group and L-carnosine treated group revealed negative caspase- 3 immunohistochemical reactivity. However few scattered cells exhibited faint light brown granules in their cytoplasm (figs.3A, 3B&3C). Intensive positive immunoreactivity for caspase- 3 was observed in the cytoplasm of most pancreatic acinar cells of NaF treated group (fig.3D). On the other hand the cytoplasmic acinar cells of vit. E in combination with methionine prior to NaF and L-carnosine prior to NaF treated groups showed apparent reduction of caspase-3 immunoreactivity to be more or less similar to the control group (figs.3E&3F) **.**

3.3.3-Morphometric and statistical Results:

As regards the statistical study concerning caspase- 3 immunoreactivity, there was a statistically significant increase in the mean number of caspase- 3 positive pancreatic acinar cells in NaF treated group, when compared with the control group. There was also significant decrease in the mean number of caspase- 3 positive pancreatic acinar cells in vit. E in combination with methionine prior to NaFand L-carnosine prior to NaF treated groups when compared with NaF treated group. On the other hand, vit.E in combination with methionine and L-carnosine treated groups showed no significant differences in the mean number of caspase- 3 positive pancreatic acinar cells in comparison to the control group the mean number of caspase- 3 positive pancreatic acinar cells in comparison to the control group.

3.3.4-Electron microscopic Results:

In control group, electron microscopic examination of exocrine pancreatic cells revealed well developed rough endoplasmic reticulum (rER) in their basal regions and great amount of zymogen granules in the apical parts. Each cell had a large basal, spherical and euchromatic nucleus containing a prominent nucleolus. Multiple mitochondria were also obviously scattered in the pancreatic acinar cell cytoplasm (fig.4A). The cytoplasm of pancreatic acinar cells of vit.E in combination with methionine and L-carnosine treated groups showed normal ultrastructure, that were more or less similar to those of control group (figs.4B&C). In NaF treated group, few or even absence of zymogen granules were observed in the cytoplasm of pancreatic acinar cells. The rER saccules showed dilatation and exhibited a globular- shape in some parts. Intra-cisternal zymogen granules were contained in multiple globular- shaped rough endoplasmic reticulum. Most acinar cells had irregular shaped, pyknotic and heterochromatic nuclei (figs4D&E).

Ultrathin sections of serous cells of vit. E in combination with methionine prior to NaF treated group.showed preservation of acinar cytoplasmic contents to be more or less similar to those of control group. The cytoplasm of pancreatic acinar cells of vit.E in combination with methionine prior to NaF treated group regained its normal structure. Rough endoplasmic reticulum showed neither dilatation nor globular shape and

Table (1): Effect of vitamin E in combination with methionine and L- carnosine on sodium fluoride-induced changes in blood parameters.

Parameters	Control group	Vit.E + Meth. treated group	L-Carnosine treated group	NaF treated group	Vit.E and Meth. +NaF treated group	L-Carnosine + NaF treated group
WBC (10^3/ul)	7.55±0.035[a]	8.05±0.336[a]	8.0±0.353[a]	10.85±0.318**[b]	9.15±0.388*[c]	8.5±0.212**[c]
Lymph (10^3/ul)	5.36±0.091[a]	5.76±0.243[a]	6.1±0.604[a]	8.6±0.049**[b]	6.16±0.113**[c]	5.48±0.011***[c]
GRA (10^3/ul)	1.18±0.07[a]	1.28±0.195[a]	1.27±0.215[a]	2.58±0.02**[b]	1.20±0.11**[c]	1.16±0.15**[c]
Lymph%	73.6±0.282[a]	73.95±0.247[a]	74.6±0.565[a]	81.25±0.318**[b]	74.45±0.388**[c]	73.5±0.494**[c]
GRA%	17.1±0.63[a]	17.35±2.43[a]	17.25±2.72[a]	26.45±1.52*[b]	17.65±0.318*[c]	17.4±0.282*[c]
RBC (10^6/ul)	6.59±0.049[a]	6.4±0.082[a]	6.5±0.127[a]	5.23±0.049***[b]	6.49±0.12*[c]	6.47±0.102**[c]
HCT (%)	43.4±0.282[a]	42.85±0.53[a]	42.95±0.318[a]	41.05±0.03**[b]	43.0±0.353*[c]	43.7±0.353*[c]
MCV(fL)	67±0.707[a]	64.5±0.35[a]	65±0.707[a]	63.5±0.212*[b]	66.5±0.282*[c]	67.0±0.707*[c]
RDW (%)	27.95±0.459[a]	27.05±0.035[a]	26.7±0.565[a]	25.8±0.707*[b]	27.15±0.176*[c]	26.5±0.141*[c]
MCH(pg)	23.7±0.212[a]	22.5±0.848[a]	23.25±0.388[a]	20.8±0.212**[b]	23.3±0.282**[c]	22.35±0.03**[c]
MCHC (g/dl)	33.85±0.813[a]	32.55±0.247[a]	32.7±1.34[a]	30.05±0.035*[b]	32.5±0.353**[c]	32.4±0.212**[c]
HGB (g/dl)	13.65±0.106[a]	13.25±0.167[a]	13.55±0.247[a]	12.95±0.035*[b]	13.65±0.106*[c]	13.7±0.071*[c]
PLT (10^3/ul)	544±0.707[a]	539±1.41[a]	540.5±1.06[a]	537±0.707*[b]	543.5±0.212*[c]	542.5±0.494*[c]

Data are presented as mean ± SE of the six animals *P<0.05, **P<0.01and*P<0.001**
(b) Significantly different from control group. (c) Significantly different from NaF treated group.
Within each row, means superscript with the same letter are not significantly different.

Table (2): Pancreatic digestive enzyme activities (U/g tissue) and Protein levels (mg/g tissue) in control and experimental groups.

Parameters	Control group	Vit.E +Meth. treated group	L-Carnosine treated group	NaF treated group	Vit.E and Meth. + NaF treated group	L-Carnosine + NaF treated group
Pancreatic lipase (U/g tissue)	342.5 ± 2.25[a]	341.75±2.41[a]	340.25±0.65[a]	319.5±2.93***[b]	345.25±1.24***[c]	336.0±2.35**[c]
Pancreatic amylase (U/g tissue)	470±2.96[a]	469±3.47[a]	463.5±1.29[a]	423±3.95***[b]	489±2.03***[c]	455.5±1.6***[c]
Pancreatic Protease (U/g tissue)	50.33±1.97[a]	47.53±0.67[a]	48.35±0.78[a]	39.4±0.272**[b]	50.25±0.754***[c]	48.05±1.89**[c]
Pancreatic Protein levels (mg/g tissue)	4.683±0.242[a]	4.478±0.158[a]	4.833±0.05[a]	3.633±0.147**[b]	4.448±0.221**[c]	4.768±232**[c]

Data are presented as mean ± SE of the six animals. **P <0.01 and ***P<0.001.
(b) Significantly different from control group. (c) Significantly different from NaF treated group.
Within each row, means superscript with the same letter are not significantly different.

Table (3): Serum total protein, albumin and blood glucose levels (g/dl) in control and experimental groups

Parameters	Control group	Vit.E +Meth. treated group	L-Carnosine treated group	NaF treated group	Vit.E and Meth. + NaF treated group	L-Carnosine + NaF treated group
Total protein(g/dl)	7.32±0.08[a]	7.34±0.052[a]	7.35±0.04[a]	7.04±0.03*[b]	7.323±0.023***[c]	298±0.02***[c]
Albumin(g/dl)	4.79±0.01[a]	4.81±0.16[a]	4.72±0.17[a]	3.99±0.07***[b]	4.32±0.01**[c]	294±0.031**[c]
Glucose(g/dl)	123±0.35[a]	123.3±0.96[a]	124.5±1.43[a]	97±0.35***[b]	123.8±0.4***[c]	117±0.62***[c]

Data are presented as mean ± SE of the six animals. *P<0.05, **P<0.01and*P<0.001**
(b) Significantly different from control group. (c) Significantly different from NaF treated group.
Within each row, means superscript with the same letter are not significantly different.

Table (4): Plasma MDA (nmol/mL) and erythrocyte SOD (U/mL) in control and experimental groups.

Parameters	Control group	Vit.E +Meth. treated group	L -Carnosine treated group	NaF treated group	Vit.E an Meth. + NaF treated group	L-Carnosine + NaF treated group
MDA (nmol/mL)	3.443±0.154a	3.085±o.129a	3.035±0.13a	6.232±0.177***b	4.222±0.164***c	3.845±0.11***c
SOD (U/mL)	6.693±0.174a	6.958±0.093a	7.012±0.062a	3.592±0.176***b	5.9±0.149***c	6.267±0.319***c

Data are presented as mean ± SE of the six animals. ***P<0.001
(b) Significantly different from control group. (c) Significantly different from NaF treated group.
Within each row, means superscript with the same letter are not significantly different.

Table (5): Hepatic DNA, RNA contents (mg/g tissue weight), % of hepatic DNA fragmentation and frequency of micro nucleated polychromatic erythrocytes in bone marrow of control and experimental groups.

Parameters	Control group	Vit.E +Meth. treated group	L –Carnosine treated group	NaF treated group	Vit.E and Meth. + NaF treated group	L-Carnosine + NaF treated group
DNA(mg/g tissue weight)	0.499±0.012a	0.536±0.016a	0.51±0.01a	0.345±0.01***b	0.517±0.01***c	0.578±0.008***c
RNA(mg/g tissue weight)	0.281±0.007a	0.290±0.006a	0.293±0.005a	0.193±0.004***b	0.251±0.009***c	0.267±0.009***c
% of hepatic DNA fragmentation	0.779±0.013a	0.746±0.01a	0.743±0.019a	1.65±0.17***b	0.721±0.013***c	0.709±0.012***c
MN-PCE/3000 in bone marrow	19.5±0.969a	18.167±0.523a	17.833±0.658a	78.166±1.707***b	23.167±0.867***c	21.667±0.658***c

Data are presented as mean ± SE of the six animals. ***P<0.001
(b) Significantly different from control group. (c) Significantly different from NaF treated group.
Within each row, means superscript with the same letter are not significantly different.

and complete absence of intra-cisternal granules. Increases in number of zymogen granules were also observed. The nucleus regained its normal spherical and euchromatic contents with a prominent nucleolus (fig.4F).

Most acinar cells of L-carnosine prior to NaF treated group also regained their normal ultrastructre. However, few pancreatic acinar cells showed mild dilatation and globular shape of rER (fig.4G) .

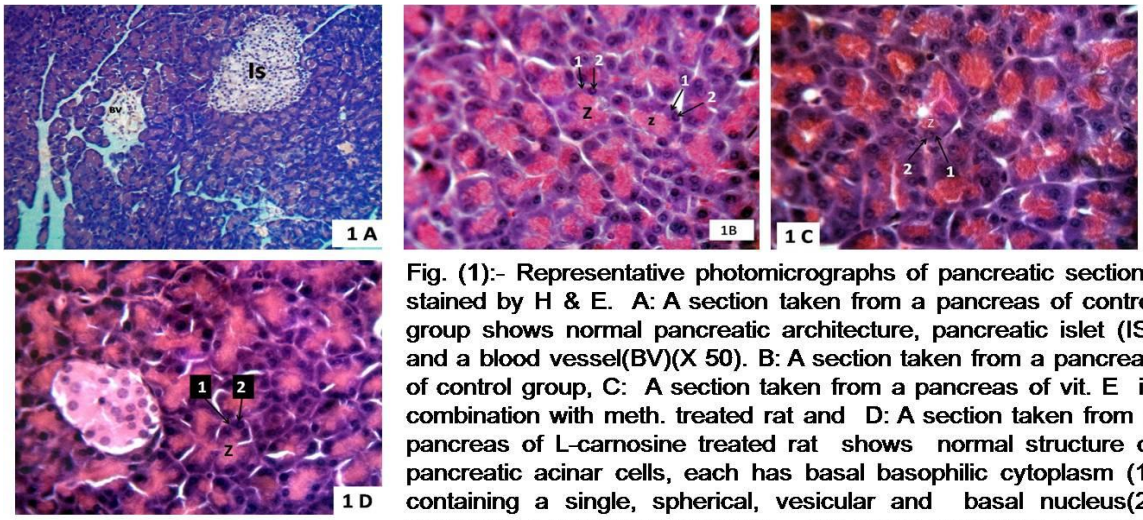


Fig. (1):- Representative photomicrographs of pancreatic sections stained by H & E. A: A section taken from a pancreas of control group shows normal pancreatic architecture, pancreatic islet (IS) and a blood vessel(BV)(X 50). B: A section taken from a pancreas of control group, C: A section taken from a pancreas of vit. E in combination with meth. treated rat and D: A section taken from a pancreas of L-carnosine treated rat shows normal structure of pancreatic acinar cells, each has basal basophilic cytoplasm (1) containing a single, spherical, vesicular and basal nucleus(2) containing a prominent nucleolus and apical acidophilic zymogen granules(Z)(X400).

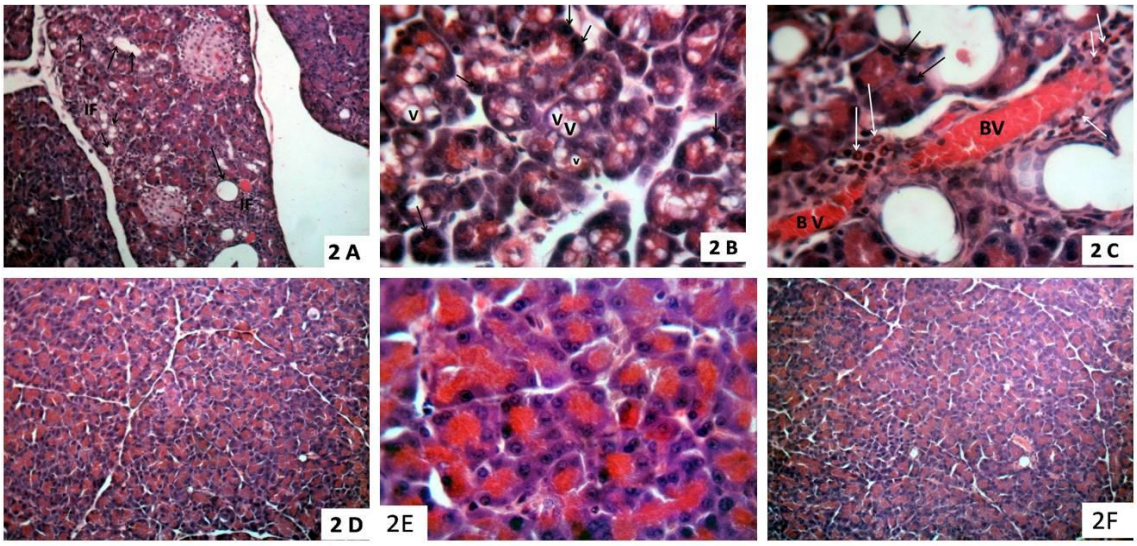


Fig.(2):- Representative photomicrographs of pancreatic sections stained by H & E : A , B and C: Sections taken from pancreas of NaF treated rats. A: Shows multiple vacuoles (arrows) of variable size in the cytoplasm of pancreatic acinar cells and inflammatory cells infiltrations (IF) in the stroma of pancreas (X 50). B: Shows multiple vacuoles (V) in the cytoplasm of pancreatic acinar cells and the peripheral pyknotic and darkly stained nuclei (arrows) of pancreatic acinar cells (X400). C: Shows inflammatory cells infiltrations (white arrows) in the stroma of pancreas around blood vessels (BV) and the peripheral, pyknotic and darkly stained nuclei (black arrows) of pancreatic acinar cells (X400). D and E: Sections taken from pancreas of vit.E in combination with meth. prior to NaF treated rats (D: X 50, E: X 400) and F: A section taken from a pancreas of L- carnosine followed by Na F treated rat show the apparent normal looking of pancreatic acini and stroma (X 400).

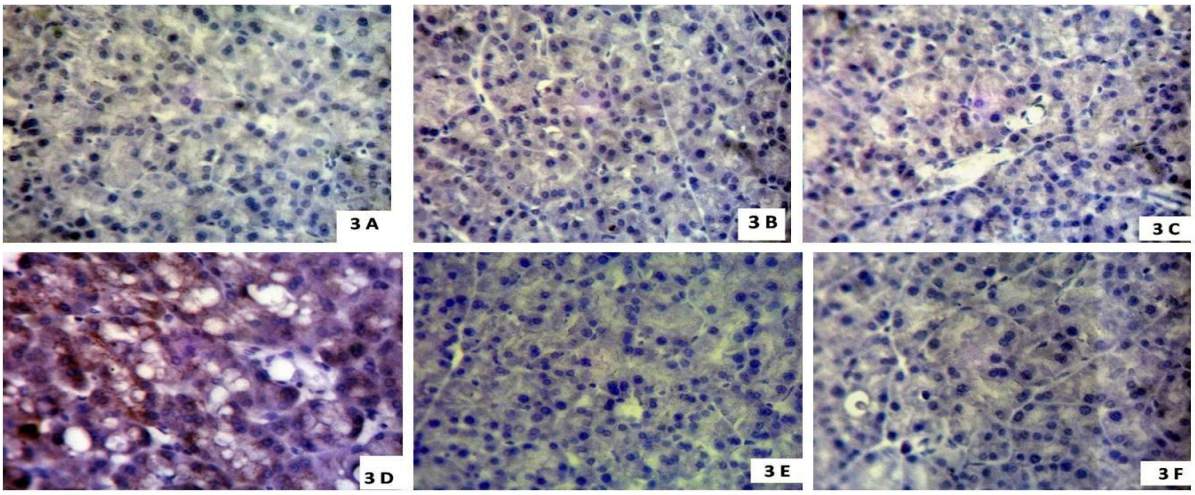
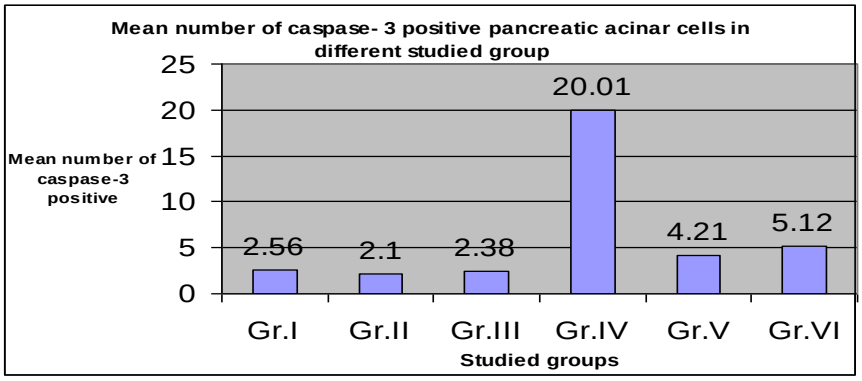


Fig. (3):- Expression of caspase- 3 immunohistochemical staining (X 400). A: A section obtained from a pancreas of control rat, B: A section obtained from a pancreas of vit. E in combination with meth. treated rat and C: A section obtained from a pancreas of L-carnosine treated rat show faint cytoplasmic reactions for caspase-3 in few pancreatic acinar cells. D: A section obtained from a pancreas of Na F treated rat shows strong cytoplasmic reactions for caspase-3 immunoreactivity in the cytoplasm of most pancreatic acinar cells. E: A section obtained from a pancreas of vit. E in combination with meth. prior to Na F treated rat and F: A section obtained from a pancreas of L-carnosine prior to Na F treated rat shows week cytoplasmic immuno- reactions for caspase-3 in pancreatic acinar cells.

Table (6): The mean number of caspase 3 positive pancreatic acinar cells in the different studied group

Groups	Control group (I)	Vit.E +meth. treated group (II)	L –Carnosine treated group (III)	NaF treated group (IV)	Vit.E and meth.+ NaF treated group (V)	L-Carnosine + NaF treated group (VI)
Mean± SD	2.56±0.32	2.10±0.42	2.38±0.66	20.01±0.5	4.21±0.53	5.12±0.14
P. value		P1>0.05 NS	P2>0.05 NS	P3 < 0.05 *	P4<0.05 *	P5<0.05 *

P1, P2, P3 = P. value of each group versus control group P4, P5= P. value in group V and VI versus group IV
NS= Non significant * = Significant



Histogram (1): The mean number of caspase 3 positive pancreatic acinar cells in the different studied groups.

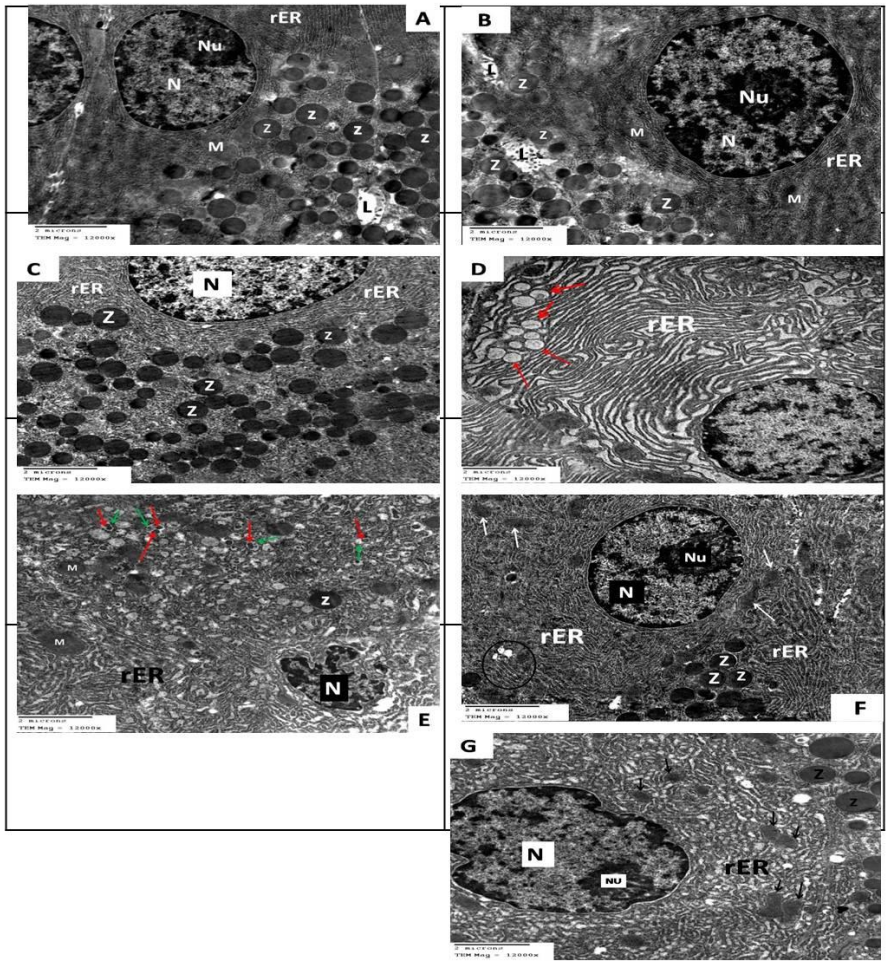


Fig. (4):- Representative electronmicrographic ultrathin sections of pancreatic acinar cells cytoplasm (O.M. X 12000). A: A section obtained from a pancreas of control rat, B: A section obtained from a pancreas of vit. E in combination with methionine treated rat and (C) A section obtained from a pancreas of L-carnosine treated rat. A and B: Show zymogen granules(Z) facing lumen(L), rough endoplasmic reticulum(rER), mitochondria(M) and an euchromatic nucleus(N) containing a prominent nucleolus(Nu) . C: Shows zymogen granules(Z), rough endoplasmic reticulum(rER) and a nucleus(N). D and E: Sections obtained from pancreas of Na F treated rats. D: Shows marked dilatation of rough endoplasmic reticulum(rER) , globular shape of rough endoplasmic reticulum(arrows) and complete absence of zymogen granules and E: shows dilatation of rough endoplasmic reticulum(rER), intra-cisternal zymogen granules (red arrows) in the globular endoplasmic reticulum (green arrows), few zymogen granules(Z), mitochondria(M) and an irregular shape, pyknotic and heterochromatic nucleus(N). F: A section obtained from a pancreas of vit. E in combination with meth. prior to fNa F treated rat and G: A section obtained from a pancreas of L-carnosine prior to Na F treated rat show preservation of cytoplasmic contents in pancreatic acinar cells to be more or less similar to the control group. F: Shows normal rough endoplasmic reticulum(rER), multiple apical zymogen granules(z), multiple mitochondria(arrows), an euchromatic nucleus(N) containing a prominent nucleolus(Nu) and Golgi apparatus(circle). G: Shows multiple zymogen granules (Z), mitochondria (arrows), an euchromatic nucleus (N) containing a prominent nucleolus(Nu), mild dilatation and globular shape in the rough endoplasmic reticulum(rER).

4.Discussion:-

In the present study**,** we evaluated the protective effects of vitamin E in combination with methionine and L- carnosine against the pancreatic toxicity induced by NaF in rats. Treatment with NaF resulted in a significant decrease in RBC, HCT, MCV, RDW, MCH, MCHC and PLT and a statistical significant increase in WBC, lymphocytes and granulocytes as compared to control group. The levels of the above parameters were significantly reversed in the groups pretreated with vitamin E in combination with methionine and L-carnosine prior to NaF. These results were in-agreement with Cetin et al., (2004); Karadeniz and Altintas, (2008); Tiwari & Pande (2009); Kant et al., (2009) who reported significant increase in WBC and decrease in RBC, packed cell volume, WBC count, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration and neutrophil counts, as well as the hemoglobin and hematocrit values in mice, goats and rabbits with chronic fluorosis. Rao and Vidyunmala, (2009) observed that a significant decrease in RBC, hemoglobin, PCV, MCH relative to the respective controls and with no change in WBC and MCHC. Changes of these values in mice have been attributed to rate of bioaccumulation of fluoride in blood. Banu Priya et al., (1997) reported that fluoride intoxication depressed bone marrow activity in cattle. Machalinska et al., (2002) revealed that fluoride induced disorders in hematopoietic organs in mice and in human hematopoietic progenitor cells. Machalinski et al., (2000); Mittal and Flora, (2007) reported that sodium

fluoride at 50 mg/L, in drinking water caused significant depletion in blood δ--aminolevulinic acid dehydratase (ALAD) activity and platelet counts (PLT). Vitamin E supplementation during sodium fluoride exposure had limited beneficial effects in restoring altered biochemical variables. The fluoride-induced anemia may result from inhibition of globulin synthesis, depression of erythropoiesis, or a decrease in the level of blood folic acid (Cetin et al., 2004). Aydogan et al., (2008) suggested that L-carnosine supplemention can be used to improve the RBC quality or to protect them from oxidative damage in survival of RBC in the circulation. Caylak et al., (2008) reported that, Hb levels in lead–methionine were significantly higher than the lead group ($p<0.01$). In the present study, animals treated with NaF showed significant decrease in pancreatic digestive enzyme activities and protein levels as compared to the control group, while animals treated with in the vitamin E in combination with methionine and L- carnosine prior to NaF showed statistical significant increase in pancreatic digestive enzyme activities and protein levels as compared to NaF treated group. These results were in-accordance with Zhan et al., (2005b) who reported the activities of pancreatic lipase, protease and amylase were significantly decreased when exposed to 100, 250, and 400 mg NaF/ kg in their diets for 50 days. These effects might be an important reason for growth depression induced by fluorosis. Excessive production of free radicals induced by fluoride may damage the structures of digestive enzymes and reduce their activities (Liu et al., 2003). Hara and Yu, (1995) indicated that salivary amylase was inhibited by NaF. Also, Mulimani and Gopal, (1989) mentioned that pancreatic amylase activity has been found to be inhibited by sodium fluoride. Some enzymes, such as peptidases, alpha amylases, phosphatases, and ATPases, are activated by calcium ions and are inhibited by fluoride due to calcium binding to fluoride in the catalytic centre (Machoy, 1987).

In the present study, treatment with NaF resulted in a significant decrease in serum total protein, albumin and blood glucose levels as compared to the control group. On the other hand, pretreated with vitamin E in combination with methionine and L- carnosine prior to NaF resulted in significant increase in serum total protein, albumin and blood glucose levels. These results were in-agreement with Kanbur et al., (2009) who observed a decrease in total protein and albumin levels in the group that was administered fluoride. Stawiarska-Pieta et al ., (2009) showed that an increase in protein level in the pancreas of rats receiving antioxidants with NaF an increase was statistically significant in comparison with the controls and amounted to 92.31%. Bouaziz et al., (2006) who showed a significant decrease in serum levels of total protein and albumin and marked hypoglycemia in fluoride-treated mice. Some researcher has reported fluoride to change blood glucose (Eraslan et al., 2007). Cenesiz, et al., (2005) reported that, total protein levels were decreased ($p<0.01$ in the NaF group compared to the control group.

Verma and Guna Sherlin, (2002) indicated that NaF treatment from day 6 of gestation throughout lactation caused a significant reduction in glucose and protein content in the serum of both P- and F1-generation rats than control. Compared with the vitamin E co-treated animals, amelioration in protein concentrations was significantly higher with vitamins C and C+D+E in both P- and F1-generation rats. Zang et al., (1996) reported a significant decrease in serum proteins in individuals with poor nutrition and living in high-fluoride areas. The significant recovery on co-treatment with vitamins C, E and C+D+E could be attributed to the action of these vitamins as free radical scavengers. Wilde and Yu, (1998) opined that the toxicity of free radicals is greater if fluoride can impair the production of free radical scavengers such as ascorbic acid and glutathione and this can be prevented by the additional supplementation with vitamins C and E. The antidotal effect of vitamin E is by preventing the oxidative damage caused by fluoride, which increases peroxides and free radicals of reactive oxygen species in tissues. The protective role of free radical scavenger is by the hydrogen donor ability of tocopherol. Vitamin E channels the conversion of oxidized glutathione (GSSG) to reduced glutathione (GSH), which in turn helps compression of mono- and dehydroascorbic acid to maintain ascorbic acid levels. It also has an inhibitory effect on the conversion of free or protein-bound -SH to –SS groups, thus maintaining -SH groups (Basu and Dickerson, 1996) .

Fluoride is well known to affect protein synthesis by causing impairment in polypeptide chain initiation (Rasmussen, 1982), weak incorporation of amino acids into proteins (Helgeland, 1976), abnormal accumulation or inhibition of RNA synthesis (Holland, 1979). Decreased protein synthesis during fluorosis has been attributed to a decrease in activity of a group of enzymes catalyzing the key process of cellular metabolism (Chinoy et al., 1993). The enzymes are glutamine synthetase catalyzing certain stages of amino acid biosynthesis and methionine activating enzymes of the liver (Zahvaronkov and Strochkova, 1981).

In the present study, plasma malondialdehyde levels were significantly increased and the activities of erythrocyte superoxide dismutase were significantly decreased in the NaF group compared to the control group. The administration of vitamin E in combination with methionine and L- carnosine prior to NaF reduced the process of lipid peroxidation and increased the activity of SOD. These results were in-accordance with Błaszczyk et al., (2008) who reported NaF caused increased concentration MDA and decreased activity of ant oxidative enzyme (SOD). The administration of vitamin E increased the activity of SOD; it also reduced the process of lipid peroxidation. It has been found that combined doses of vitamin E and methionine were most effective in inhibiting lipid peroxidation process. The results confirmed the antioxidative properties of methionine. He and Chen, (2006) reported that the contents of MDA in oral mucosa and liver tissue of fluoride group were significantly higher than those of control group ($P < 0.01$). Zhan et al., (2005a) found that Serum malondialdehyde and the activities of superoxide dismutase were significantly decreased in the NaF group compared to the control group. Vani and Reddy, (2000) showed that fluoride increases free radical production and at the same time inhibits the ant oxidative enzyme

SOD which probably make the tissue more susceptible to biochemical injury. Patel and Chinoy, (1998) concluded that fluoride impaired the functioning of the SOD enzyme in ovary of mice. Sun et al., (1998) also reported a decrease in SOD activity in the liver, kidney, and heart of fluoridated mice. Abdel-Kader et al., (2011) observed that significant reduction in MDA concentrations in lead- Methionine and lead –vit E groups when compared to lead group, Błaszczyk et al., (2010) suggested that fluoride reduces the efficiency of the enzymatic antioxidative system in the liver and kidney. The slight increase of the activity of superoxide dismutase after administration of methionine may indicate its protective influence upon that enzyme. Caylak et al., (2008) reported that MDA levels in lead–methionine were significantly reduced. Methionine influences the metabolism of elements participating in the biosynthesis of superoxide dismutase isoenzymes. The reduced availability of any of them would then cause an inhibition of the synthesis and a diminished activity of SOD (Bourdon et al., 2005). Corrales et al., (2002) observed that administration of methionine should protect the liver from oxidative stress caused by fluorine. However Patra et al., (2001) found that in rats methionine administration may lead to an increased activity of SOD in the liver. Superoxide dismutase catalyses a reaction, in which the superoxide anionic radical is rendered harmless and molecular oxygen as well as hydrogen peroxide, get formed. The total activity of SOD depends upon the activity of iso-enzymes MnSOD and CuZnSOD (Oyanagui, 1984).

Sharonov et al., (1990); Hipkiss and Chana, (1998) reported the advantage of carnosine as a universal antioxidant, relating its ability to give efficient protection against oxidative stress. Also, carnosine was able to diminish the level of the primary products of lipid peroxidation previously accumulated (Dupin et al., 1988). Therefore, it appears that carnosine exerts multipotent antioxidant ability by inhibiting the injurious action of free radicals and diminishing previously accumulated toxic products. Thus carnosine could be recommended as an antioxidant, beside the classical antioxidants, such as vitamins E and C, flavonoids and carotenoids (Babizhayev and Steven, 1996). In addition, the fact that carnosine is water-soluble and located at the site of primary free-radical generation makes it even more valuable.

In the present study, oral administration of sodium fluoride for 35 days resulted in significant reduction in the DNA, RNA contents of the liver and significant increase DNA damage in liver and the frequencies of cells with MN in bone marrow. However, concurrent administration of NaF and vitamin E in combination with methionine and L- carnosine for 35 days caused significant amelioration in all parameters studied. These results were in-agreement with Trivedi et al., (2008) who observed oral administration of sodium fluoride for 30 days resulted in significant reduction in the DNA, RNA, and protein contents of the liver and kidneys. However, concurrent administration of NaF and black tea extract for 30 days caused significant amelioration in all parameters studied. Shashi, (2003) revealed significant ($P<0.001$) decline in the DNA and RNA Content and total proteins in adrenal gland of fluoridated animals of both sexes compared to the control. The significant decline of DNA and RNA in the adrenal gland of fluoridated rabbits in this investigation may be due to alterations in DNA polymerase activity and changes in enzymes involved in nucleic acid synthesis. Shashi, (1993); Shashi et al., (1994); Patel and Chinoy, (1998) reported that sodium fluoride (5 mg/kg body weight) was effective from the 45th day of treatment in causing a significant decline in DNA and RNA levels of mice ovary and uterus, indicating alterations in nucleic acid and protein metabolism in these organs.

Jacinto-Aleman et al., (2010) suggested that excessive fluoride ingestion has been identified as a risk factor for fluorosis and oxidative stress. The oxidative stress results from the loss of equilibrium between oxidative and antioxidative mechanisms that can produce DNA fragmentation, resulting in apoptosis. Individual exposure to fluoride leads to DNA damage in brain may have occurred via two mechanisms (i) indirectly, in which free radicals generated by fluoride attack hydrogen bond of DNA molecule to give various DNA adducts and (ii) directly, fluoride attacks free -NH group. Supplementation of vitamin E with fluoride protected fluoride induced oxidative stress and DNA damage in brain. These protective values of vitamin E could be attributed to its ability to efficiently scavenge lipid peroxyl radicals before they attack membrane lipids leading to the removal of cell-damaging free radicals. Clarke et al.,(2008); He and Chen, (2006) revealed that DNA damage rate in fluoride group was 50.20% in oral mucosal cells and 44.80% in hepatocytes higher than those in the control group ($P < 0.01$). Zhang et al., (2006) found that fluoride can induce DNA damage in osteoblasts. Wang et al., (2004) observed that fluoride can cause lipid peroxidation, DNA damage, and apoptosis in human embryo hepatocytes. Oxidative stress, DNA damage and modifications of membrane lipids can be induced in hepatocytes by excess fluoride (Shanthakumari et al., (2004). Ha et al., (2004**)** reported that the rate of DNA damage of group treated with sodium fluoride was significantly higher than the control grouped.

Patel et al., (2009) observed that a significant increase in mean frequency of micronuclei induction in NaF treated groups when compared to control. Li et al., (2000) found that the micronucleus rate of adults from the high-fluoride area and mice drinking high-fluoride water was higher than that of the control group ($P<0.05$). Fluoride may be mutagenic, Meng and Zhang, (1997) showed the frequencies of cells with MN in peripheral blood lymphocytes of workers exposed mainly to fluoride were statistically significantly higher than controls. Wu and Wu, (1995) stated that in persons with fluorosis as well as those considered "healthy", the MN rate was significantly higher than in a neighboring control group drinking low-fluoride water. Suzuki et al., (1991) revealed that a significant increase in micronucleated polychromatic erythrocytes was observed 24 H after intraperitoneal injection of sodium fluoride at a dose of 30 mg/kg body weight. In the in vitro micronucleus test, the frequency of micronucleated polychromatic erythrocytes was increased significantly at concentrations of 2 and 4 MM. These results indicate

that the micronucleus test may be useful in evaluating the cancer risk of sodium fluoride.

Jordao et al., (2009) demonstrated the increased DNA damage induced by acute ethanol administration and DNA protection when ethanol is administered together with methionine. Kang et al., (2002) revealed that L - Carnosine prevented both TPA and hydrogen peroxide induced DNA fragmentation Pretreatment with vitamin C (400 mg/kg/day) or vitamin E (100 mg/kg/day) for 5 days before acute ethanol administration (5 g/kg) inhibits the generation of the hydroxyl-ethyl radical by 30 and 50%, respectively, and prevents oxidative DNA damage, which is high in groups receiving no antioxidants Navasumrit et al., (2000). Bagchi et al., (1998) reported that pretreatment of animals with antioxidants such as vitamin C, vitamin E succinate and grape seed proanthocyanidin decreased O- tetradecanoylphorbol-13-acetate (TPA) induced DNA fragmentation in the liver and brain.

Histological examination of the exocrine portion of pancreas of fluoridated rats of the present study exhibited structural alternations. The most prominent alternation was the occurrence of multiple vacuoles in the cytoplasm of pancreatic acini. This finding was in consistence with the histological findings of many investigators who reported the occurrence of vacuolar degeneration in pancreas of sodium fluoride treated animals (Stawiarska-Pieta et al., 2007, 2008 &2009; Shashi et al., 2010). The same finding was attributed by Cicek et al., (2005); Shashi et al., (2010) who explained that fluoride toxicity leads to loss of selective permeability of the cell membrane, resulting in dilatation of cytoplasmic component secondary to intracellular fluid and electrolyte redistribution.

Light microscopic examination of pancreas of sodium fluoride treated group of the present work showed also inflammatory cells infiltrations in the stroma of pancreas especially around blood vessels. This coincided with the work of previuos studies, Stawiarska-Pieta et al., (2007, 2008 &2009); Shashi et al., (2010) who reported during their histological examination, that fluorosis induced inflammation in pancreas. Gutowska et al., (2011) also reported that excessive exposure to fluoride can result in inflammatory reactions involving macrophages and their differentiation. Fluoride has been considered to play an important role in pathogenesis of chronic fluoride toxicity as it increases the production of reactive oxygen species (ROS) and lipid peroxidation (Machoy, 1987; Chinoy&Patel,1998; Chlubek, 2003). Other studies reported reduction of anitioxidant defense system (enzymatic and non enzymatic) in different tissues of animals treated with fluoride (Shivarajashankara et al., 2001a; Zhan et al., 2005a).

In rats of vit. E in combination with methionine prior to NaF and L-carnosine prior to NaF treated groups, no histological changes were observed, concerning vacuolar degeneration and inflammation in the pancreas that were clearly observed in Na F treated group, during the present light microscopic study. The light microscopic results obtained in this study correlated with the previous studies concerning the influence of different antioxidants on pancreas (Stawiarska-Pieta et al., 2007, 2008, & 2009). Vitamin E prevents the organism from the activity of free oxidative radicals (Stawiarska-Pieta et al., 2009). Stawiarska-Pieta et al., (2007) during their experimental studies stated that simultaneous administration of vitamin E in animal intoxicated with fluoride increases the activity of antioxidative enzymes and added that vitamin E is usually administrated along with methionine or vitamin C. The functional role of L-carnosine is still not completely understood, however several studies indicate that the dipeptide content of L-carnosine exerts protective actions against metal- and or antibodies-mediated toxicity by acting as anti-oxidant and free-radical scavenger (Kohen et al., 1988; Preston et al., 1998*;* Horning et al., 2000; Trombley et al., 2000; Carlo et al., 2011). Littarru & Tiano, (2007) reported that antioxidants have a beneficial influence upon oxidation-reduction equilibrium. Different studies confirmed that administration of antioxidants to animals intoxicated with sodium fluoride inhibits the alterations observed in exposure to fluorides, which clearly shows their preventive action on brain (Chinoy and Patel, 2000) and testis (Krasowska et al., 2004).

The pyknotic and darkly stained nuclei that were observed during light microscopic examination, the irregular shaped and heterochromatic nuclei that were also seen during the electron microscopic study and the intensive expression of caspase- 3 that were demonstrated immunohistochemically in the cytoplasm of pancreatic acinar cells of Na F administrated group of the present work which was of a statistical significant value when compared to the control group, confirmed the induction of apoptosis by Na F administration, Gomez et al., (2001) revealed through their immunohistochemical study that inflammation of pancreas causes activation of apoptosis. Apoptosis is an active regulated cell death. The characteristics of apoptosis include; a series of biochemical and morphological changes, such as caspase family activation, nucleosomal DNA fragmentation, cell volume loss and chromatin condensation (Vaskivuo et al., 2000). Chronic fluorosis induces oxidative stress leading to generation of free radicals and alterations in antioxidants or reactive oxygen species (ROS) scavenging enzymes (Sharma and Chinoy, 1998; Shivarajashankara & Shivashankara, 2002). ROS have been implicated as potential modulators of apoptosis. It has been suggested that oxidative stress plays a role as a common mediator of apoptosis. Some studies revealed that LPO is one of the molecular mechanisms involved in chronic fluoride- induced toxicity. Fluoride ion, although a non oxidant ion, causes oxidative stress indirectly leading to increase in lipid peroxide levels. Lipid peroxidation impairs a variety of intra and extra mitochondrial membrane system that may contribute to apoptosis (Shivarajashankara et al., 2001a,b).

The present histological study revealed regaining of the normal appearance and contents of nuclei of pancreatic acinar cells in last two groups. The present immunohistochemical and statistical studies of the same groups showed also marked reduction in caspase 3 expressions in pancreatic acinar cells which indicated reduction of apoptosis. It has been reported that methionine has an important role in inhibition of apoptosis and methionine restriction induces apoptosis of prostate cancer cells (Lu et al., 2002). Vitamin E is

also the most important lipophilic antioxidant and resides mainly in the cell membrane, thus helping to maintain membrane stability (Stoyanovsky et al., 1995). As mentioned before, several studies indicate that the dipeptide content of carnosine exerts protective actions against metal- and or antibodies-mediated toxicity by acting as anti-oxidant and free-radical scavenger (Kohen et al., 1988; Preston et al., 1998*;* Horning et al., 2000; Trombley et al., 2000; Carlo et al., 2011). Consequently antioxidants could reduce cell death in different cellular structures caused by oxidants and free radicals effects of sodium fluoride , for example, blocking apoptosis induced by changes in mitochondrial membrane permeability and subsequent release of cytochrome c and caspase activation (Ramanathan et al., 2003). Reduction of apoptosis in last two groups could be also attributed by Patel & Chinoy, (1997); Chinoy & Patel, (1998); Sharma & Chinoy, (1998) who stated that antioxidant reduce LPO caused by fluoride in rat resulting in stability in mitochondrial membrane system and reduction in apoptosis.

The present electron microscopic examination of pancreatic acinar cells of fluoridated animals revealed dilatation and globular shape of rough endoplasmic reticulum. Some of these globular shape rough endoplasmic reticulum contained intra-cisternal zymogen granules. Zymogen granules were markedly decreased in number or even absent. These findings could be attributed by Quijeq et al., (2002) through their several studies which showed that fluoride inhibits protein synthesis. These findings were previously studied in sodium fluoride treated animals (Matsuo et al., 2000; Zhan et al., 2005b) and were attributed by Matsuo et al., (2000) to be due the toxic effect of fluoride that disrupts the export of zymogens from the rough endoplasmic reticulum. During the secretary process, incompletely assembled and aggregated products are selectively retained in the rough endoplasmic reticulum. Excessive protein accumulation in the rough endoplasmic reticulum results in formation of intra-cisternal granules and decreases in number of zymogen granules (Pfeffer and Rothman, 1987; Arias & Bendayan, 1993).

Stawiarska-Pieta et al., (2009) stated that application of antioxidants leads to an increase in the protein level, which may confirm our electron microscopic results in last two groups that showed apparent increase in the number of zymogen granules and no or mild dilatation in rough endoplasmic reticulum.

In conclusion, the combined administration of antioxidants showed beneficial effects upon hematological, biochemical, histological and immunohistochemical alterations which developed in pancreas following sodium fluoride intoxication. This proves that oxidation processes play a significant role in fluoride toxicity, and that the administration of antioxidants in response to an increased risk of exposure to fluoride compounds may protect the human body against their harmful effects.

5. **References:**

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