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The Influence of Methionine and Vitamin E on the Morphological Picture of Pancreas of Albino Rats Intoxicated With Sodium Fluoride

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Abstract: Background: Sodium Fluoridehas protective effects on dental health. Excessive ingestion of fluoride may cause toxic and harmful effects. Chronic toxicity with fluoride is more common than acute toxicity**.** Methionine is a potential natural antioxidant**.** Vitamin E is a fat-soluble vitamin which acts as an antioxidant. It protects cells from free radical damage. It also enhances the immune function**. Aim of the work:** This work aims at evaluation of the protective role of Methionine, and Methionine in combination with vitamin E, on the pancreas of Albino rats intoxicated with Sodium Fluoride. **Materials and Methods:** This study included 24 adult male Albino rats which were divided into four equal groups: **Group I** (Control group): Each rat received 1 ml distilled water orally. ***Group II*** (Fluoride- treated group): Each rat received 10 mg NaF/kg b.w., once daily for 35 days. The prepared dose was given orally by a gastric tube**. Group III** (Fluoride and Methionine - treated group): Each rat received 10 mg NaF/kg b.w. orally by gastric tube once daily in addition to 2 mg methionine/rat/day using a syringe connected to gastric tube for 35 days**. Group IV**: Each rat received 10 mg NaF/kg b.w. orally by a gastric tube once daily in addition to 2 mg Methionine/rat/day orally by a gastric tube & vitamin E in a dose of 3mg/rat/day using a syringe connected to gastric tube for 35 days. Pancreatic samples were prepared for histopathological and Immunohistochemical study. **Results:** Heamatoxilen and Eosin study of sections of the pancreas of Albino rats from Sodium Fluoride-treated rats (group II) showed disturbance of the normal architecture of the pancreas in the form of variable degrees of cellular degeneration. There was an increase of interlobular connective tissue with dilated congested blood vessels and massive infiltration of inflammatory cells. Areas of highly vacuolated acini were also noticed. There was an obvious increase in the connective tissue by Masson's Trichrome stain. These group showed strong positive immunoreactions to PCNA in acinar cell and islets of Langrhans. Sections of the pancreas of Albino rat from group III that received sodium fluoride and Methionine showed an improvement of islets of Langerhans but with congested blood vessels and were surrounded by normal exocrine acinar cells of pancreatic tissues. Degeneration of few acini were also noted. Mild to moderate amount of connective tissue in between the pancreatic acini was seen by Masson's Trichrome. moderate positive immunoreaction to PCNA in acinar cell and islets of Langerhans was also noticed. As regards rats of group IV, most pancreatic specimens showed preserved normal pancreatic architecture, with most acinar cells; their nuclei appeared more or less similar to those in the control group. Normal appearance of islets of Langerhans with very mild infiltration with inflammatory cells was also observed. By Masson's Trichrome minimal amount of connective tissue was seen. Specimens also showed mild positive immunoreaction to PCNA in acinar and in islets of Langerhans. **Morphometric results:** Group III showed a significant ($P<0.01$) decrease in collagen fibers deposition when compared with group II and a significant ($P<0.01$) increase when compared with group I. Group IV showed a significant ($P<0.01$) decrease in collagen fibers deposition compared with group II and group III while it showed an insignificant ($P<0.01$) increase when compared with group I. Group III showed a significant ($P<0.01$) decreased PCNA immunoreaction compared with group II but a significant ($P<0.01$) increase when compared with group I. Group IV showed a significant ($P<0.01$) decreased PCNA immunoreaction compared with group II and group III but an insignificant ($P<0.01$) increase when compared with group I. **Conclusion:** These results indicate that Sodium Fluoride may cause pancreatic histological and immunohistochemical changes which may lead to a series of pathological abnormalities. Concurrent administration of NaF and vit. E in combination with Methionine alleviate the adverse effects of Fluoride.

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Keywords: Influence; Methionine; Vitamin E; Morphological; Picture; Pancreas; Rat; Intoxicate; Sodium Fluoride

1. Introduction:

Fluoride belongs to the halogen group of elements and is found naturally in soil, animals, water,

and plants [1]. Fluoride has protective effects on dental health. Less tooth decay was observed in communities consuming naturally fluoridated water compared to non-fluoridated areas [2]. Fluoride exists in water either naturally or added during water fluoridation. It also enters the body through burning coal, fluoride dust and fumes from industries using fluoride containing salt and hydrofluoric acid [3].

Excessive ingestion of fluoride may cause toxic and harmful effects. Chronic toxicity of fluoride is more common than acute toxicity. The effects of chronic ingestion of fluoride depend not only on the duration and dose but also on several other factors such as nutritional status, renal function, and interactions with other trace elements [4]. Fluoride is one of the most reactive elements. With toxic amount of fluoride in the body, fluoride attacks oxygen and disrupt the metabolism resulting in hydrogen peroxide production [5]. In addition, fluoride results in excessive production of free radicles that disrupt the antioxidant formation [6] Chronic application of a superoptimum NaF dose induces ultrastructural changes in the cells of the submandibular gland, the pancreas and the liver of young rats. The mitochondria are the most damaged organelles in the cells of examined organs [7].

Methionine is an aliphatic, sulfur-containing, essential amino acid, and a precursor of succinyl-CoA, homocysteine, cysteine, creatine, and carnitine [8]. Methionine is a potential natural antioxidant. It demonstrated a protective role in the course of exposure to the sodium fluoride. Administration of methionine reduces the oxidative stress status [9].

Vitamin E is a fat-soluble vitamin with several forms, but alpha-tocopherol is the only one used by the human body. It acts as an antioxidant as it protects cells from free radical damage. It also enhances the immune function. Vitamin E plays an important role in the production of prostaglandins, which are responsible for regulating body processes, such as blood pressure and muscle contraction [10]. Multiple antioxidants that are active against different oxidants are required to cope with oxidative stress in vivo.

The aim of this study Is to evaluate the toxic effects of Sodium Fluoride on the pancreas of Albino rats. Also, it demonstrates and compares the protective effects of administration of Methionine, and Methionine in combination with vitamin E, on the pancreas of Albino rats treated with Sodium Fluoride.

2. Materials

A- Animals:

The present study was carried out on 24 adult Albino rats aged 8 weeks old and weighing 185-225 g each. The rats were divided into four groups. The rats were obtained from the Animal house, Faculty of Veterinary Medicine, Benha University, Egypt. Each group was kept in separate clean cages under good hygienic conditions, approved by the Animal Use and Care **Committee, under controlled light cycle (12 h light/12 h dark), The rats were housed in uniform** husbandry conditions at a temperature of 25±1ºC, with a relative humidity of 50±10%. The rats were freely supplied with sterilized food and water during the experiment. Ethical clearance for the use of animals was got from the Institutional Animal Ethics Committee prior to the beginning of the work.

B- Drugs:

1-Sodium F luoride:

Sodium Fluoride was obtained from El-Gomhoria Chemicals Company in the form of white powder soluble in water; l gm of NaF was dissolved in 100 ml distilled water, so l ml of NaF solution contained 10 mg of NaF. The dose of NaF was given as 10 mg NaF/kg b.w., and was administered orally via a gastric tube for 35 days [11].

2- Methionine

Methionine was obtained from Sigma Chemicals Company, Mumbai, India in the form of powder soluble in water, l gm of Methionine was dissolved in 100 m l distilled water, and so l ml of Methionine solution contained 10 mg of Methionine [11].

3-Vitamin E

Vitamin E was obtained from Sigma Chemical Co., Mumbai, India. The dose of vitamin E is 3mg/rat/day using a syringe connected to a gastric tube for 35 days [12].

Experimental design:

This study icludes24 adult male Albino rats which were divided into 4 equal groups as follows:

Group I (Control group): Rats had received 1 ml distilled water orally by a gastric tube once daily for 35 days.

Group II *(Fluoride - treated group): The rats had received 10 mg NaF/kg b.w., once daily for 35 days. The prepared dose was given orally by a gastric tube* [11].

Group III *(Fluoride*+Methionine - treated group): The rats had received 10 mg NaF/kg b.w. orally by gastric tube once daily in addition to 2 mg Methionine/rat/day using a syringe connected to a gastric tube for 35 days [11].

Group IV: The rats were received 10 mg NaF/kg b.w. orally by a gastric tube once daily in addition to 2 mg Methionine/rat/day orally by a gastric tube & vitamin E in a dose of 3mg/rat/day using a syringe connected to a gastric tube for 35 days [12].

Pancreas Extraction:

At the end of experiment, the rats of all groups were anesthetized by ether. The pancreas was extracted through opening the anterior abdominal wall, locating the stomach on the left side of the rat and then

gently (so as to avoid tearing) separating the pancreas from the stomach and duodenum by using two forceps.

Histopathological analyses:

Pancreatic specimens were fixed in 10% neutral buffered formaldehyde in order to be processed for light microscopic study. Fixed materials were dehydrated in ascending grades of ethanol and embedded in paraffin wax and of 5-micrometer thick sections were prepared and subjected to staining with Hematoxylin-Eosin (Hx & E), and Masson's Trichrome (MT), [13]

Immunohistochemical staining of the proliferating cell nuclear antigen (PCNA):

Paraffin-embedded rat liver sections were deparaffinized and hydrated. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 5 minutes. Sections were incubated over night with PCNA monoclonal antibody and rinsed with phosphate buffer saline (PBS) for 5 minutes. The monoclonal antibody was then linked with biotinylated goat anti-mouse IgG antibody for 30 minutes. After being washed with PBS for 3-5 minutes, the sections were incubated with streptavidin-conjugated peroxidase for 35 minutes. A brown colored reaction was developed by *exposing sections to* 3, 3-diaminobenzidine tetrahydrochloride solution (DAB) for 5 minutes and washed in distilled water. Sections were countered-stained with hematoxylin [14]. PCNA positive cells were counted in 10 randomly selected, non overlapping fields and expressed as the numberof PCNA positive cells/mm2.

Morphometric study:

The mean area percentage of collagen fibers deposition and PCNA immunoreaction was quantified in five images from five non-overlapping fields of each rat using Image-Pro Plus program version 6.0 (Media Cybernetics Inc., Bethesda, Maryland, USA).

Statistical analysis

All the data collected from the experiment was recorded and analyzed using IBM SPSS Statistics software for Windows, Version 23 (IBM Corp., Armonk, NY, USA). One-way analysis of variance (ANOVA) with Post Hoc LSD test was used to compare differences among the groups. In each test, the data was expressed as the mean (M) value, standard deviation (SD) and differences were considered to be significant at $P< 0.01$.

3. Results:

1- Light microscopic results:

Heamatoxilen and Eosin study of sections in the pancreas of Albino rats from the control group showed islets of Langerhans appearing pale pink oval or rounded areas inside the pancreatic lobules and were being formed of circular groups of cells rich in capillaries and surrounded by normal pancreatic acini which appeared rounded or oval in shape. They were lined with pyramidal cells arranged around a narrow acinar lumen and containing zymogen granules in their cytoplasm and they were separated from each other by very little connective tissue septa **(Fig.1)**. Compared with the control specimens, examination of pancreatic sections obtained from Sodium Fluoride-treated rats (group II) showed disturbance of the normal architecture of the pancreas in the form of variable degrees of cellular degeneration. There was an increase of interlobular connective tissue with dilated congested blood vessels and massive infiltration of inflammatory cells. Areas of highly vacuolated acini were also noticed. Focal areas of acinar degeneration that were completely destroyed leaving empty spaces were also detected **(Fig.2)**. Sections in the pancreas of Albino rat from group III that received sodium fluoride and Methionine showed an improvement of islets of Langerhans but with congested blood vessels and were surrounded by normal exocrine acinar cells of pancreatic tissues. Degeneration of few acini were also noted **(Fig.3)**. As regards rats of group IV, most pancreatic specimens showed preserved normal pancreatic architecture, with most acinar cells; their nuclei appearing more or less similar as in the control group. Normal appearance of islets of Langerhans with very mild infiltration with inflammatory cells was also observed **(Fig.4)**.

By Mason's Trichrome stain in the control group, minimal connective tissue between pancreatic acini and islets of Langerhans was observed **(Fig.5)**. In Sodium Floride -treated rats (Group II), there was an obvious increase in the connective tissue between the pancreatic acini, islets of Langerhans and around blood vessels **(Fig.6)**. Group III which received Sodium Fluoride and Methionine showed mild to moderate amount of connective tissue in between the pancreatic acini, minimal amount of connective tissue separating islets of Langerhans **(Fig.7)**. Group IV appeared more or less similar to the control group **(Fig.8)**.

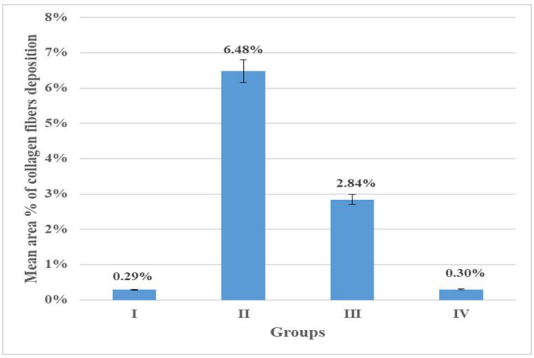
2- Immunohistochemical study of the proliferating cell nuclear antigen (PCNA):

Sections of pancreas of rats from the control group showed a negative immunoreaction for PCNA in acinar cell and islets of Langerhans **(Fig.9)**. Pancreas of rats from group II (treated with Sodium Fluoride) showed strong positive immunoreactions for PCNA in acinar cell and islets of Langrhans **(Fig.10)**. The reaction was moderately positive in the sections of pancreas from group III which rats were treated with Sodium Fluoride and Methionine **(Fig.11)**. The immune study of specimens obtained from group IV that was given Sodium fluoride, Methionine and Vit E showed a mild positive immunoreaction to PCNA in acinar cell and islets of Langerhans. **(Fig.12)**.

3- Morphometric results

The mean area percentage of collagen fibers deposition and PCNA immunoreaction is presented in Tables (1) and (2) and in Histograms (1) and (2). Group III showed a significant (P<0.01) decrease in collagen fibers deposition when compared with group II and a significant (P<0.01) increase when compared with group I. Group IV showed a significant (P<0.01) decrease in collagen fibers deposition compared with group II and group III while it showed an insignificant (P<0.01) increase when compared with group I.

Group III showed a significant (P<0.01) decreased PCNA immunoreaction compared with group II but a significant (P<0.01) increase when compared with group I. Group IV showed a significant (P<0.01) decreased PCNA immunoreaction compared with group II and group III but an insignificant (P<0.01) increase when compared with group I.



Histogram (1): Showing the mean area % of collagen fibers deposition in all groups

Table (1): Showing the mean area % and SD of collagen fibers deposition in groups I, II, III and IV with comparison between all groups by Post Hoc LSD test.

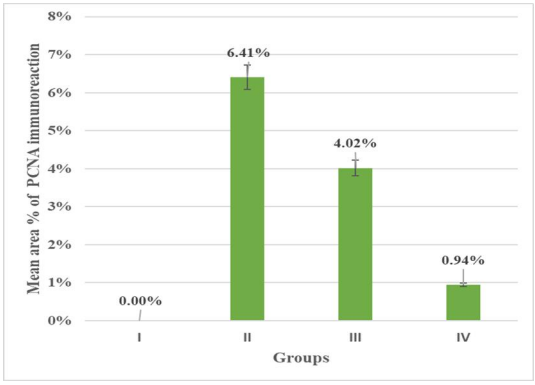
	Group I	Group II	Group III	Group IV
Mean	0.29%	6.48%	2.84%	0.3%
SD	0.0525	0.5776	0.2677	0.08373
Significance at P < 0.01	2,3	1,3,4	1,2,4	2,3

1=sig. with group I 2=sig. with group II 3=sig. with group III 4=sig. with group IV

Table (2): Showing the mean area % and SD of PCNA immunoreaction in groups I, II, III and IV with comparison between all groups by Post Hoc LSD test.

	Group I	Group II	Group III	Group IV
Mean area %	0.0%	6.41%	4.02%	0.94%
SD	0	0.4501	0.1673	0.2079
Significance at P < 0.01	2,3,4	1,3,4	1,2,4	1,2,3

1= sig. with group I 2=sig. with group II 3=sig. with group III 4=sig. with group IV



Histogram (2): Showing the mean area % of PCNA immunoreaction in all groups.

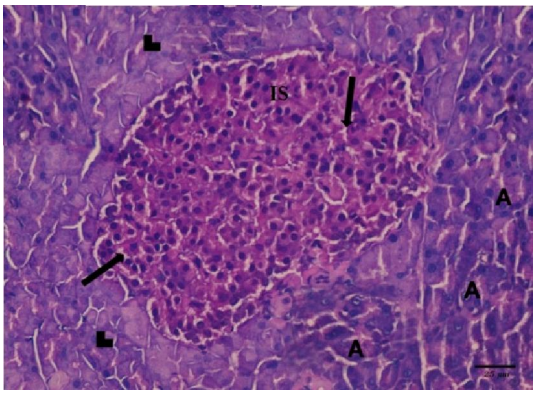


Fig. (1): A photomicrograph of section in the pancreas of Albino rats from the control group showing islets of Langerhans (IS) rich in capillaries (arrow) and surrounded by more or less normal pancreatic acini (A) containing zymogen granules in their cytoplasm (arrow head). **(H & E x 400)**

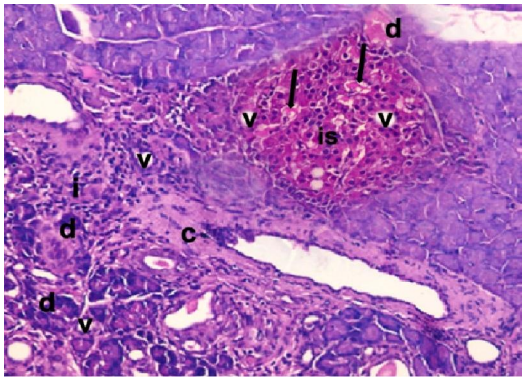


Fig. (2): A photomicrograph of section in the pancreas of Albino rats from group II (treated with sodium fluoride) showing: Many degenerated acini (d) were surrounded by massive infiltration of inflammatory cells (i), with collagen fibers (C) beside the islets of Langerhans (is). Notes: Destruction and distortion of endocrine cells with the presence of vacuolations (V) in the islets of Langerhans & congested blood vessel (arrow)**. (H & E x 400)**

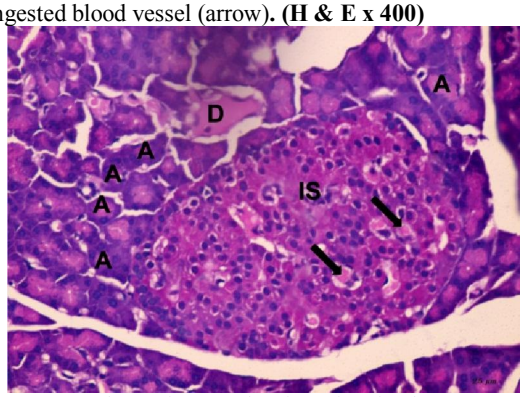


Fig. (3): A photomicrograph of section in the pancreas of Albino rat from group III (received Sodium Fluoridean Methionine) showing: An improvement of islets of Langerhans (IS) with congested blood vessels (Arrows). They were surrounded by normal exocrine acinar cells (A) of pancreatic tissues with degeneration of few acini (D). **(H & E x 400).**

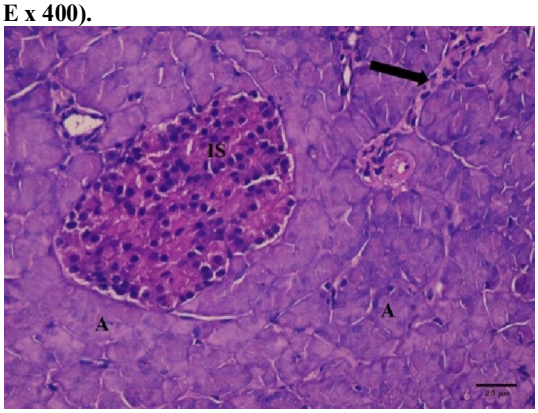


Fig. (4): A photomicrograph of section in the pancreas of Albino rat from group IV (received Sodium Fluoride, Methionine and Vit E) showing islets of Langerhans (IS) surrounded by most probably normal exocrine acinar cells (A) of pancreatic tissues. Notes: mild infiltration with inflammatory cells (arrow). **(H & E x 400)**

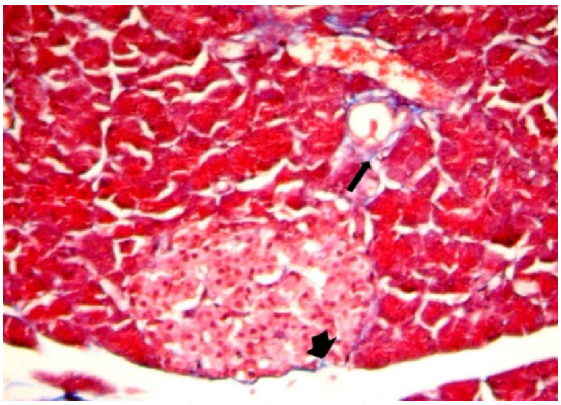


Fig. (5): A photomicrograph of section of rat's pancreas from the control group showing minimal connective tissue between pancreatic acini (Arrow), and islets of Langerhans (Arrow head). **(Mason's Trichrome ×400)**

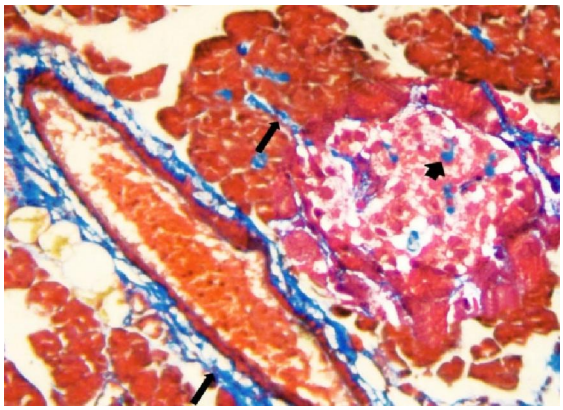


Fig. (6): A photomicrograph of section of rat's pancreas from group II (treated with Sodium Fluoride) showing massive infiltration with collagen fibers between the pancreatic acini & blood vessels (Arrow) and islets of Langerhans (Arrow head). **(Mason's Trichrome ×400)**

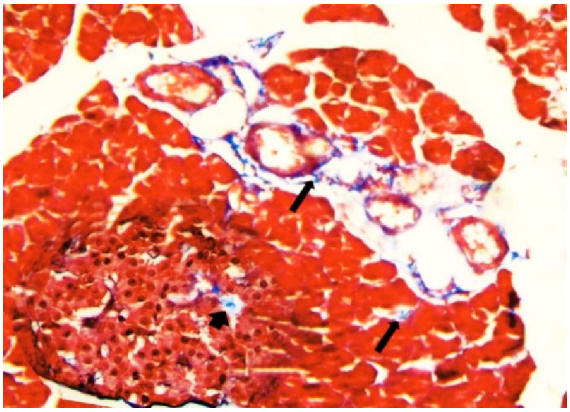


Fig. (7): A photomicrograph of section of rat's pancreas from group III (received Sodium Fluoride and Methionine) showing mild to moderate amount of connective tissue in between pancreatic acini (Arrow), with minimal amount of connective tissue (Arrow head) separating islets of Langerhans. **(Mason's Trichrome ×400)**

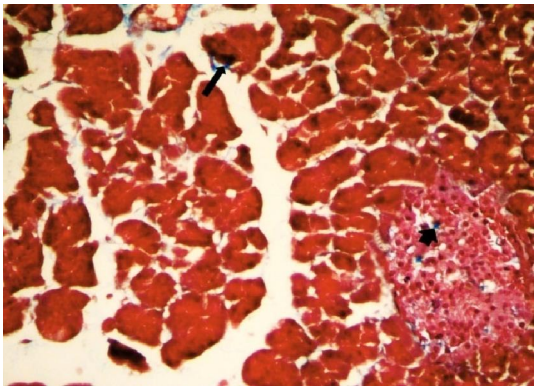


Fig. (8): A photomicrograph of section of rat's pancreas from group IV (received Sodium Fluoride, Methionine and Vit E) showing minimal connective tissue between pancreatic acini (Arrow), and islets of Langerhans (Arrow head). **(Mason's Trichrome ×400)**

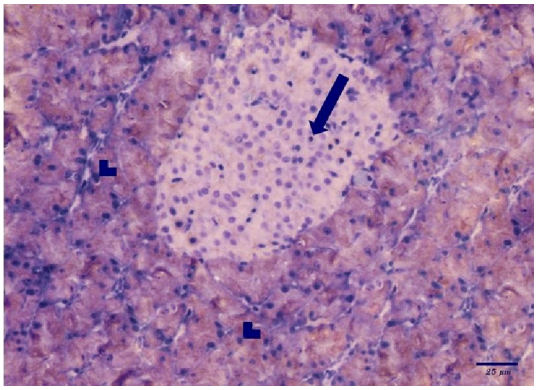


Fig. (9): A photomicrograph of pancreas sections of rats from the control group showing negative immunoreaction in acinar cells (arrow head) and islets of Langerhans (arrow). **(PCNA x400)**

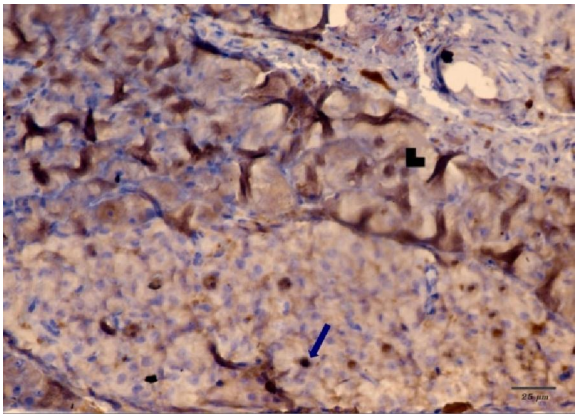


Fig. (10): A photomicrographs of pancreas of rats from group II (treated with Sodium Fluoride) showing a strong positive immunoreactions in acinar cells (arrow head) and islets of Langerhans (arrow). **(PCNA x400)**

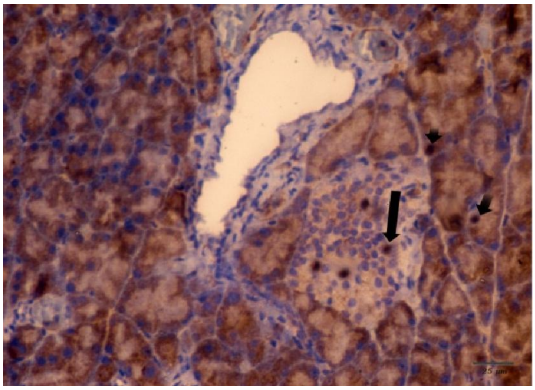


Fig. (11): A photomicrograph of pancreas of rats from group III (treated with Sodium Fluoride and Methionine) showing a moderate positive immunoreaction in acinar cells (arrow head) and mild to moderate positive immunoreactions islets of Langerhans (arrow). **(PCNA x400)**

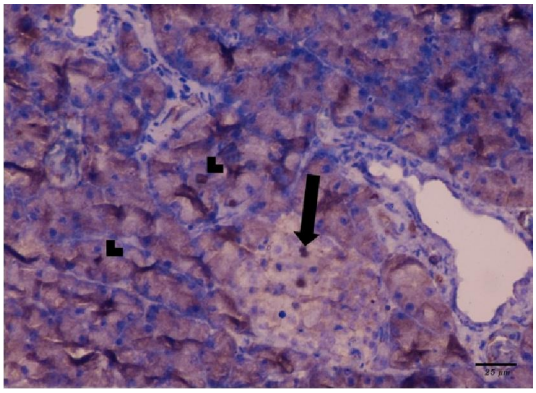


Fig. (12): A photomicrographs of pancreas of rats from group IV (treated with Sodium fluoride, Methionine and Vit E) showings a mild positive immunoreaction in acinar cells (arrow head) with mild positive immunoreaction in islets of Langerhans (arrow). **(PCNA x400)**

4. Discussion:

In this study each rat received 10 mg NaF/kg b.w., once daily for 35 days. The pancreas was extracted. Specimens examination by Heamatoxilen and Eosin showed disturbance of the normal architecture of the pancreas in the form of variable degrees of cellular degeneration. There was an increase of interlobular connective tissue with dilated congested blood vessels, and massive infiltration by inflammatory cells. Areas of highly vacuolated acini were also noticed. Focal areas of acinar degeneration that were completely destroyed leaving empty spaces were detected. This finding was similar to the histological findings of **Shashi et al (2010)** who found that the rats which received NaF for 36 days showed degenerative changes in the pancreatic acini. There was an increase in the intercellular spaces between acini; periductal fibrosis; and inflammation of acini.

Perilobular and periductal inflammation were more advanced. There was an extensive proliferation of connective tissue [15].

Barbara et al (2009) found focal vacuolar degeneration of cells and inflammatory infiltrations appeared in pancreas of rats which received sodium fluoride [16]. In a previous study, **Chlubek *et al* (2003)** elicited hyperglycemia in rats exposed to 50 or 100 ppm fluoride in drinking water over four months [17]. Studies on rats have suggested that fluoride toxicity may produce glucose intolerance and abnormalities in insulin secretion and pancreatic structure [18].

Fluoride toxicity is associated with reactive oxygen species (ROS) induction. Excessive ROS production can lead to lipid peroxidation, and Malondialdehyde (MDA) is the important indicator of lipid peroxidation [19].

Zhan et al (2005) revealed that excessive fluoride inhibits the activities of pancreatic lipase and protease and causes observable ultrastructural changes [20]. 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.

In the current study sections from the pancreas of Albino rats from group III which received Sodium Fluoride and Methionine showing moderate improvement of islets of Langerhans but yet with congested blood vessels surrounded by normal exocrine acinar cells of pancreatic tissues. Degeneration of few acini was noted. Mild to moderate amount of connective tissue in between pancreatic acini was also observed. This was in agreement with **Dorreia et al (20019)** who found that the light microscopic examination of the rat pancreas of group III which received Sodium fluoride and Methionine revealed a regression of the previously mentioned histological findings in group II which received Sodium Fluoride only apart from residual vacuoles. The lobular architecture was preserved and the acini were more regular [21]. Many papers have reported that Methionine plays a key role in antioxidant processes [22].

Because of the regulatory role of Methionine in endogenous antioxidant enzymes and other metabolic processes. This amino acid may play a leading role in reducing the prevalence of cancer. Methionine plays an essential role in the immune system through its metabolites. In this regard, **Maddocks et al. (2016)** found that this amino acid directly influences the function of the immune system because of Methionine catabolism that leads to an increase in the production of glutathione, taurine, and other metabolites [23]. Methionine is also readily used by the hepatocytes for the direct synthesis of glutathione, which is a low-molecular-weight antioxidant [24].

Campbell et al. (2016) observed an alteration of the oxidative activity in a branch of the pentose phosphate pathway (PPP) after increasing Methionine supplementation. They also found that pre-incubating cells with Methionine increased cellular tolerance to the thiol oxidizing agent diamide with relation to oxidative pentose phosphate [25]. Methionine supplementation can intervene in the natural antioxidant capacity of an organism by leading to the production of endogenous enzymes that reduce oxidative stress and, in turn, DNA damage [9].

As regards rats of group IV which received NaF, Methionine and vitamin E, most specimens showed preserved normal pancreatic architecture, and most acinar cells and their nuclei appeared more or less similar to those in the control group. Normal appearance of islets of Langerhans with very mild infiltration with inflammatory cells was also observed. This finding goes in agreement with **Iwona et al.; (2008)** who showed that simultaneous administration of Methionine and vitamin E is more efficient in protecting cells from oxidative stress than administration of vitamin E alone [26]. The mechanisms and dynamics of action of vitamin E as a radical-scavenging antioxidant have been investigated extensively and are well documented [27]. Vitamin E components are not only antioxidants protecting polyunsaturated fatty acids from free radical attack, but they also seem to have stabilizing role in cell membranes, where molecular organization plays a very important role [28]. It has been found that combined doses of vitamin E and Methionine were most effective in inhibiting lipid peroxidation processes. The results confirmed the antioxidative properties of Methionine [26]. On the other hand, advantageous effect of Methionine with vitamin E has been exercised upon the activity of catalase CAT, which increased by 7%, and upon the concentration of MDA (decrease by 75%), the reduction of which was more profound than after administration of vitamin E alone [22]. The results of the previous studies indicate that Methionine and vitamin E have opposite effects on accumulation of Fluorides in hard tissue in rats. By stimulating Fluoride accumulation, Methionine reduces the adverse effect of fluorides on soft tissue, while vitamin E; which prevents excessive accumulation of Fluorides in bones and teeth; protects these tissues from fluorosis. Therefore, it seems that combined application of both compounds would be optimal for the prevention of the adverse effects of chronic Fluoride intoxication [12].

In the current study sections of pancreas of rats from the control group showed a negative immunoreaction for PCNA in acinar cell and islets of

Langerhans. Pancreas of rats from group II (treated with Sodium Fluoride) showed strong positive immunoreactions for PCNA in acinar cell and islets of Langrhans. The reaction was moderately positive in the sections of pancreas from group III which rats were treated with Sodium Fluoride and Methionine. The immune study of specimens obtained from group IV that was given Sodium fluoride, Methionine and Vit E showed a mild positive immunoreaction to PCNA in acinar cell and islets of Langerhans. This in agreement with **Ehab et al (2014)** who found that the Strong positive reaction for PCNA in the most damaged hepatocytes in liver sections. PCNA has been used extensively in the identification of proliferating damage hepatocytes and its nuclei, which we observed as brownish black stained [29].

Conclusion:

These results indicate that sodium fluoride may cause pancreatic histological and immunohistochemical changes which may lead to a series of pathological abnormalities. Concurrent administration of NaF and vit. E in combination with Methionine alleviate the adverse effects of fluoride.

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