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Remedial Potential of *Olea europea* and *Punica granatum* Fruit Extracts on Testicular Histopathology in Fluoride Exposed Mice

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ABSTRACT

Purpose: This study was designed to study testicular-histopathology and lipid profile against 50ppm of fluoride (*ad labitum*) exposure in mice. Moreover, the curative potentials of vitamin E, *Punica granatum* and *Olea europea* fruit extracts were also studied.

Methods: Five groups (10 animals/group) were treated as follow (a) Control (C) (F free water-15 days); (b) NaF {50ppm F-ions in drinking water (10days) and F free water (5days)}; (c) NaF + vitamin E (NaFE) {50ppm F-ions (10days) and F free water+60µg vitamin E in corn oil (5days)}; (d) NaF+ *Punica granatum* fruit extract (NaFP) {50ppm F ions (10days) and F free water+0.2mL *P. granatum* fruit extract (5days)}; (v) NaF+ *Olea europea* fruit extract (NaFO) {50ppm F-ions (10days) and F free water +0.2mL *O. europea* extract (5days)}; All animals were sacrificed on day 16 to obtain blood for lipid profile and testes for histopathological studies.

Results: Results shows peculiar histopathological changes in the testis, including the loss of interstitial tissues, spermatogonia, spermatocytes, spermatids and maturing sperms. These signs were convincingly recovered in NaFO, followed by other two post treatment groups NaFP and NaFE. Similarly the micrometric data shows significant increase in the number of spermatogonia, spermatocytes and developing spermatozoa, in NaFO (CSA of seminiferous tubules, number of spermatogonia, spermatocytes:, sperms embedded in sertoli cells & number of dislodged sperms). The rise of High Density Lipoproteins (HDL) with a simultaneous decline in plasma cholesterol, Low Density Lipoprotein (LDL) and Very Low Density Lipoprotein (VLDL) in NaFO indicate the importance of these post treatments, against altered lipid profile of the F toxicology. Excellent testicular rehabilitation found in NaFO group that indicate the curative potential of *O.europea* against altered histopatology against F exposure in testes.

Conclusions: The severity of the mentioned pathology of NaF group indicates that *Olea europea* and *Punica granatum* post treatment can effectively eradicate the toxic changes of F exposure in lipid profile, while *Olea europea* fruit extract showed more ameliorationative capability against F-exposure in testes.

Key-words: *Fluoride; Olea europea; Punica granatum, Vitamin E, Histopathological changes in testes*

INTRODUCTION

Fluoride (F) toxicity is a significant health concern, particularly in regions with high natural fluoride levels in groundwater. Chronic exposure to fluoride can lead to dental and skeletal fluorosis, characterized by changes in tooth enamel and bone structure.[1] The major routes of fluoride exposure are drinking water,

smoke, pesticides, food stuffs and some F containing dental products. Acute fluoride toxicity, although rare, can result from excessive ingestion of fluoride-containing products, leading to symptoms such as nausea, vomiting, abdominal pain, and diarrhea.[2] When fluoride enters into the blood stream via intestine, it readily distributes throughout the body.[3] The World Health Organization recommends a fluoride level of 0.5 to 1.0 mg/L in drinking water to balance dental health benefits and the risk of fluorosis.[1]

NaF exposure causes significant elevation in F concentration in mice testes. It also enhances sperm deformity to great extent.[4] It also cause serious implications in male reproductive system such as loss of sperm count, spermatogenesis defect, sperm maturation and impairment differentiation.[5] A wide range of abnormalities related to F exposure include decline in the weight of testes, prostate, seminal vesicle, and dilation in the seminiferous tubules.[6] A study on rats showed that the cholesterol level was reduced as a result of fluoride administration.[7] The elevated levels of F leads to loss of sperm motility, lipid peroxidation, and decreased fertility in spermatozoa.[8]

Plants contain number of antioxidants such as vitamin E and C anthocyanin, carotenoids, flavonoids and poly phenols. All antioxidants play important role to reverse the damage caused by free radicals.[9] Exposure of vitamin E in recovery of testes against NaF exposure has been observed.[10] According to another study, the co-administration of calcium and vitamin-E with F resulted in a significant recovery from testicular disorders and oxidative stress in the testis and male accessory sex organs.[11]

Phytochemicals of Pomegranate (Punica granatum)

Pomegranate (*Punica granatum*) has been used in the traditional medicine of many cultures particularly in the Middle East.[12] Freshly prepared juice is rich in vitamin C and polyphenolic compounds,[13] and also a good source of vitamin B5, potassium and polyphenols, such as ellagitannins and flavonoids.[14] Lipid peroxidation is inhibited to greater degree by the application of *Punica granatum* peel extract.[15] *P. granatum* juice, decreased abnormal sperm rate when compared to the control group.[16] Fruit extract of pomegranate contain phenolic compounds like punicalagins, ellagitannins and gallotannins; variety of flavonoids e.g anthocyanins, flavonols; many organic acids like ellagic acid, gallic acid and polyphenols & tannins.[17]

Phytochemicals of Olive (Olea europea)

Olea europea contain different phytochemicals such as riboflavin, phenols, terpenoids, flavonoids, saponins, tannins, alkaloids, steroids, carbohydrates and proteins.[18] Phenolic compounds in olive oil show powerful antioxidant activities against LDL oxidation and decrease LDL/HDL cholesterol ratio.[19] *Olea europea* juice consumption impart positive effects on sperm quality and cure male infertility reproductive issues.[20] Fruit extract of *Olea europea* has phenolic compounds like hydroxytyrosol, oleuropein; flavonoids like flavonols, flavones, terpenoids, squalene; vitamin E; saponins and tannins.[21]

The study was designed to check the curative potential of vitamin E and fruit extracts of *Punica granatum* and *Olea europea* against Fluoride exposure in mice testes.

MATERIAL AND METHODS

Animal Rearing, Maintaince and Feeding

The present study was conducted on 50 male swiss webster albino mice, age 3-4 months animals reared in animal house of The Department of Zoology, University of Sargodha. They were kept under standard protocol of light and dark, humidity and temperature, water and food were provided *ad libitum*.

Dose Groups

Total 50 male animals were divided into five groups (each containing 10 animals) randomly.

(i) **Control (C) group:** This group was given regular drinking water (F free water) for 15 days.

(ii) **NaF group:** 50ppm F-ions in drinking water was given to animals for initial 10 days followed by F free water for next 5 days.

(iii) **NaF+Vitamin E (NaFE) group:** 50ppm NaF solution was given to animals for initial 10 days followed by F free water and 60µg vitamin E in corn oil for next 5 days.

(iv) **NaF+*Olea europea* (NaFO) group:** 50ppm NaF solution was given to animals for initial 10 days followed by F free water and 0.2mL *Olea europea*

pulp extract on regular basis of 24hrs for next 5 days.

(v) **NaF+*Punica granatum*(NaFP) group:** 50ppm NaF solution was given to animals for initial 10 days followed by F free water and 0.2mL *Punica granatum* pulp extract on regular basis of 24hrs for next 5 days.

Dose Preparation

Fluoride (NaF) Dose Prepartion

Analytical grade sodium fluoride (NaF) was used to prepare the required aqueous NaF solutions. A 1000-ppm NaF stock solution was prepared by dissolving 2.21g of NaF in 1L of water. The dose (50ppm) was then prepared by adding 95mL of water to 5mL stock solution as per requirement .

Fruit Extracts Prepartion

Olea europea and *Punica granatum* fruits were purchased from market and pulp was separated from seeds. The fruit was washed with tap water and ground by juicer blender after removing its seeds. Finally juice was centrifuged to obtain the supernatant from pulp.[22]

Organ's Recovery

On the day 16th of experiment, the animals were dissected for surgical removal of the testis. The excised organs were fixed in Bouin's fixative for histopathological study. Blood samples were collected with the help of syringes by heart puncture in vacuum tube vacutainers with gel and clot activator.

Histological Preparations and Observations

After fixation, testis were washed in 50%, 70%, 90% and 100% alcohol for 3-5 hours for dehydration. After that, testes were placed in molten histological paraffin wax (at 56-58 ^{0}C) for 3-5 hours for proper embedding. Thin sections of 2-3µ were obtained by microtome (ERMA TOKYO 422) and stained by H & E. Prepared sections were finally observed under stereoscopic compound microscope (Labomed CXM2), using digital camera (Sony DSC-W35).

Micrometric Studies

The diameter of seminiferous tubules was measured from two right angle positions to calculate the circumference of tubules with the help of pre-calibrated ocular micrometer on 40X, under research binocular microscope (Labomed CSM2).

CSA of the seminiferous tubules was calculated individually for each animal. To calculate the cross-sectional area (CSA), length and width were obtained with the help of Bezier tool drawn upon each seminiferous tubules in corelDRAW11.

CSA of a semicircle were calculated by using the following formula:

CSA = (Length x Width/4)π

The total number of spermatogenic cells (separately for spermatogonia, spermatocytes, attached sperms and dislodged sperms) were counted from the photographs of slides displayed in Coral Draw11 in all the groups and standardized against the 2916µm²area. All micrometric readings were taken from random sections in Coral Draw11 and were processed for statistical analysis.

RESULTS

Histological Results

Histological results of Control group showed well placed seminiferous tubules and healthy interstitial tissues. The rounded sections of seminiferous tubules indicate many concentric rings of spermatogonia and spermatocytes arranged in a centripetal fashion. The innermost region of the tubules shows spermatids and spermatozoa properly imbedded in the conical sertoli cells. However a few spermatozoa were found located in the central lumen of the seminiferous tubules (Fig A).

The histopathological signs of NaF exposed groups include selective obliteration of the interstitial tissue. The spermatogonial cells were not as closely packed as they appeared in control group sections. Moreover, there were fewer spermatocytes as compared to control group. The spermatids and developing spermatozoa were also scanty in numbers, whereas cellular debris of the spermatocytes and developing spermatozoa was found to appear in the center of the seminiferous tubules. However some maturing free spermatozoa were found in the lumen of tubules (Fig B).

In NaFE group sections slight improvement in terms of number of spermatogonia, spermatocytes and developing spermatozoa was observed, nevertheless debris of dead spermatozoa in the center of tubules was clearly seen (Fig C).

Similar histological disposition as seen in the NaFE group was also evident in the NaFP group with huge amounts of debris from the dead spermatogenic cells being frequently seen and which was almost choking the entire central lumen of the tubules (Fig D).

In NaFO group there was lesser number of spermatogonia and spermatocytes as compared to NaFP and NaFE group sections, the spermatogonia and spermatocytes were clearly differentiable. The CSA of

the seminiferous tubules was seemingly decreased. The center of seminiferous tubules was free of any debris, all the developing spermatozoa appeared embedded and clear signs of regeneration of the interstitial tissue were seen. The histological landmarks of healthy testes were most prominent in the NaFO group. Also it has clearly differentiable spermatogonia and spermatocytes and a lack of debris in the center of the tubules. Additionally, there were clear signs of regeneration of the interstitial tissue (Fig E).

Micrometric Results

Highest CSA of seminiferous tubules was found in C (276.10±24.4µm²) followed by NaFP (260.41±58.3µm²), NaF (252.49±44.3µm²), NaFE (242.51.±57.0µm²) and NaFO (221.34±64.9µm²) group. Similarly, maximum number of spermatogonia was found in C (16.7±0.51) followed by NaFO (15.2±0.92), NaFE (11.6±0.4), NaFP (11.5±0.37) and NaF (7.6±0.42) groups. While large number of spermatocytes was found in C (17.5±0.24) followed by NaFP (16.5±0.44), NaFO (13.6±0.66), NaFE (12±0.61) and NaF (6.9±0.40) groups. Whereas, obvious number of imbedded sperms was found in C (15.1±0.37) followed by NaFE (15±0.33), NaFP (13.4±0.37), NaFO (13.2±0.53) and NaF (7.5±0.34) groups. Additionally, maximum disloged sperms was found in NaFP (10.8±0.72) followed by NaFE (8.7±0.3), NaFO (7.1±0.31), NaF (4.9±0.23) and C (3.6±0.30) groups (Table. 1).

Biochemical Results

Biochemical results of lipid profile indicate highest level of LDL was found in NaF (96.6±8.04) followed by C(67.6±2.27), NaFO (45±6.503), NaFE(41±3.114) and NaFP(38.4±5.334) groups. While, large level of VLDL was found in NaF(66±2.07) followed by NaFO (39.8±3.67), C(32.2±0.86), NaFP(26±2.98) and NaFE(16.2±0.37) groups. Whereas, maximum cholesterol level was found in NaF (174.2±10.77) followed by C(158.6±6.46), NaFO (110.8±5.81), NaFP (99.2±5.75) and NaFE (88.2±2.59) groups. Moreover, enhanced level of triglycerides was found in NaF (334±4.70) followed by NaFO (215±9.74), C (151.6±5.35), NaFP (92±5.14) and NaFE (77±2.12) groups. (Table. 1)

Table 1. Show lipid profile parameters related to testes in male mice (CSA= cross sectional area)

Micrometric Parameters	**Mean±SEM**				
	C	**NaF**	**NaFP**	**NaFO**	**NaFE**
***Mean CSA seminiferous tubules ¥(p<0.0001) (µm^2)	276.10.43±24.4^a	252.49±44.3^b	260.41±58.3^e	221.34±64.9^d	242.51.±57.0^c
***Mean number of spermatogonia/seminiferous tubules (µm^2) ¥ (p<0.001)	16.7±0.51^a	7.6±0.42^b	11.5±0.37^c	15.2±0.92^d	11.6±0.4^c
**Mean number of spermatocytes/seminiferous tubule (µm^2) ¥(p<0.001)	17.5±0.24^a	6.9±0.40^b	16.5±0.44^e	13.6±0.66^d	12±0.61^c
**Mean number of sperms embedded in sertoli cells ¥(p<0.001)	15.1±0.37^a	7.5±0.34^b	13.4±0.37^c	13.2±0.53^c	15±0.33^a
**Mean number of dislodged sperms/seminiferous tubules ¥(p<0.001)	3.6±0.30^a	4.9±0.23^b	10.8±0.72^e	7.1±0.31^d	8.7±0.3^c

¥: analyzed by ANOVA, *** highly significant, **: very significant, *: significant, no star: no significant difference. abc the mean values in a row not sharing a common superscript differ significantly (p≤0.05) with each other.

Table 2. Shows lipid profile in NaF and *Olea europea* and *Punica granatum* treated male albino mice

Biochemical Parameters	Mean±SEM				
	C	NaF	NaFP	NaFO	NaFE
**Plasma level of LDL (mg/dL) ¥(p<0.001)	67.6±2.27[a]	96.6±8.04[b]	38.4±5.334[c]	45±6.503[c]	41±3.114[c]
***Plasma level of VLDL (mg/dL) ¥ (p<0.0001)	32.2±0.86[a]	66±2.07[b]	26±2.98[d]	39.8±3.67[e]	16.2±0.37[c]
***Plasma level of cholesterol (mg/dL) ¥(p<0.0001)	158.6±6.46[a]	174.2±10.77[b]	99.2±5.75[d]	110.8±5.81[e]	88.2±2.59[c]
**Plasma level of triglycerides (mg/dL) ¥(p<0.0001)	151.6±5.35[a]	334±4.70[b]	92±5.14[d]	215±9.74[e]	77±2.12[c]

¥: analyzed by ANOVA, *: (p≤0.05); **: (p≤0.001); *** (p≤0.0001). Any two groups not sharing a common lower case superscript differ significantly (p≤0.05) with each other.

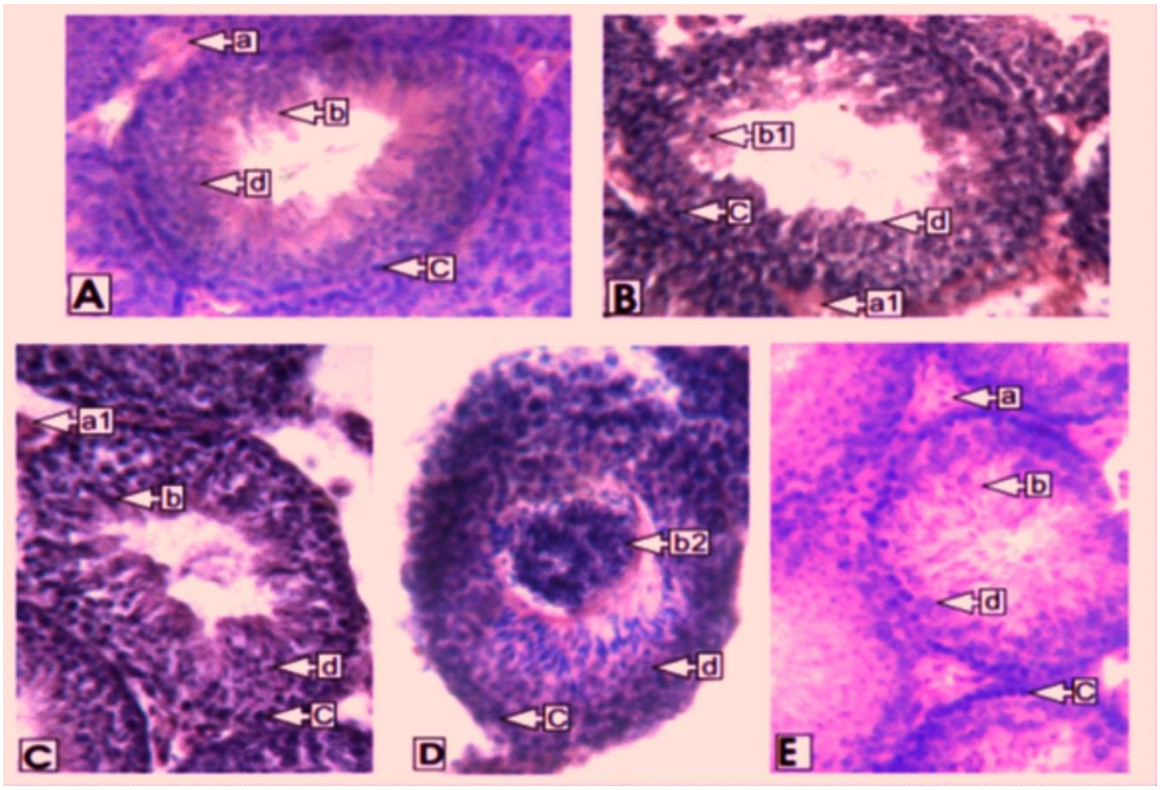

Figure 1. Histological sections of testis (400X). A: control; **B:** NaF; **C:** NaFE; **D:** NaFP; **E:** NaFO. a: interstitial cells, a1: fibrosis of interstitial cells, b: maturing sperms, b1:area of necrosis of spermatocytes, b2: discarded spermatogonial cells, c: spermatogonia, d: spermatocytes

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DISCUSSION

There are various repeated indications in the available literature that clearly indicate NaF toxicity upon testicular structure along with various parameters of sperm.[2] On the whole our findings of testicular histopathology that includes obliteration of interstitial tissues, loss of spermatogonia, spermatocytes and the

presence of debris in the center of the seminiferous tubules are well-aligned with previous studies on fluoride-induced testicular toxicity.[23] For instance, significant histopathological changes in the testes of rats exposed to fluoride leads to degeneration of seminiferous tubules and reduction in spermatogenesis. These findings are consistent with our observations (Fig 1B). Whereas the testicular histological architecture was found rescued in the post treatments groups (NaFE, NaFP and NaFO). However, out of the three post treatments olive (*Olea europea)* fruit pulp extract was found best in this regard. These findings clearly point out the importance of *Olea europea* fruit extract treatment to rescue from testicular toxicology of F exposure. Olive leaf extract had a protective effect against testicular damage induced by oxidative stress in rats, improving the histological architecture and increasing the number of spermatogonia and spermatocytes.[24] This supports our above findings that olive extract significantly rescues testicular histology post fluoride exposure.

The micrometric findings also support the histological results, as we have found significant reduction in CSA of seminiferous tubules, number of spermatogonia and spermatocytes, number of embedded sperms, while there was a significant rise in the number of dislodged sperms, indicating F toxicity on the spermatogenic cells. Except in NaFP group, the trend of decline in CSA of seminiferous tubules continued in NaFE and NaFO groups. While there was a significant increase in the number of spermatogonia and spermatocytes in NaFE, NaFO and NaFP groups as compared to NaF group. The highest number of spermatocytes was counted in the NaFP group, indicating an enhancing capability towards the meiotic phase of sperm production. Pomegranate juice significantly improved sperm parameters and reduced oxidative damage in the testes,[25] which aligns with our findings.

The similar trend was noted for the number of embedded spermatozoa. However the number of dislodged spermatozoa was also increased in NaFE, NaFO, NaFP then NaF group (Fig:1). These findings clearly indicate a rescuing effect of Vit.E, *Olea europea* and *Punica granatum* fruit extracts post F exposure on the testicular health and spermatogenic capability. While olive extract brought highest increase in the number of spermatogonia indicating a rescuing affect on the mitotically dividing sperm mother cells. The highest number of spermatocytes was counted in NaFP group indicating the enhancing capability towards meiotic phase of sperm production.

However highest number of embedded spermatozoa were counted in NaFE group, while highest number of detached spermatozoa were counted in NaFP indicating delayed sperm detachment in NaFE group which is necessary for sperm maturation. Vitamin E supplementation mitigated the adverse effects of fluoride on reproductive parameters in male rats, improving sperm count, motility, and morphology.[25] This supports our findings that vitamin E (NaFE) had a rescuing effect on testicular health and spermatogenic capability.

Indications are present in the available literature showing significant alterations in lipid profile on F exposure.[26] We found that the HDL levels were significantly low in NaF then C, NaFE, NaFP and NaFO groups. The plasma LDL was highest in NaF followed by C, NaFO, NaFE and NaFP groups. The significant secondary increase in HDL and decline in LDL plasma concentration in NaFE, NaFP and NaFO groups shows health promoting effect of the two fruit extracts, which goes parallel to the rescuing capacity of Vit.E.

The highest VLDL mean value was detected in NaF followed by NaFO, C, NaFP and NaFE groups indicating that Vit.E and *Punica granatum* post treatment caused a significant decline in VLDL levels then both NaF and C groups. The results obtained for plasma triglycerides are similar to that of VLDL, where we have seen a significant affect towards decrease in plasma triglycerides in NaFE, NaFO and NaFP groups, where the same remained unusually high in NaF group as compared to control.

The F exposure also led to a significant increase in plasma cholesterol then C, while in all three post treatment groups (NaFE, NaFP, NaFO) there was a drastic decline indicating 'there' significance towards controlling the plasma cholesterol levels. These findings indicate that *Olea europea* and *Punica granatum* fruit extracts are even better than Vit.E for amelioration of lipid profile upsets of F exposure.

CONCLUSIONs

The findings suggest that *Olea europea* fruit extract was the best to bring about ameliorations in testicular parameters.The promising results of *Olea europea* and *Punica granatum* in mitigating fluoride toxicity suggest potential for broader clinical applications. Future research should focus on large-scale human trials, mechanistic studies, and developing standardized formulations to ensure safety, efficacy, and ease of use. Exploring synergistic effects with other treatments could further enhance therapeutic outcomes.

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CONFLICT OF INTERESTS

None

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