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ORIGINAL ARTICLE

Allocation of Stem Cell Alterations in Spermatogenesis of Male Rats Affected by Zymafluor and Detection of Reverse Effect of Vitamin E on Testis Histology Using Immunohistochemical Techniques

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Abstract

Background/Aim: Zymafluor is a proven protective agent against dental caries and it is a naturally occurring agent present in water and soil. Aim of this study was to analyse the detrimental effect of sodium fluoride and opposing effect of vitamin E on spermatogenesis on testicular tissue of male rats using stem cell markers (aldehydes dehydrogenase 1A1 - ALDH1A1 and NANOG).

Methods: Four groups of male rats (10 rats in each) were used in this study as follow: group C regarded as a control group received normal saline, group F received sodium fluoride (*Zymafluor*) once daily, group FE received vitamin E followed by sodium fluoride and group E received vitamin E. The experiment was carried out for 28 days. After this period, the rats were sacrificed and the tissue of testis was processed for immunohistochemistry to stain with ALDH1A1 and NANOG stem cell biomarkers.

Results: A decrease in the expression of both ALDH1A1 and NANOG stem cell markers in group F was highly observed and statistically significant. The expression was improved in spermatic cells of group FE for both markers. Normally strong intensity of markers was observed in group E. The spermatogenesis was diminished in F group according to Jonhson score and improved in group FE, while group C and E displayed normal Johnson score for spermatogenesis.

Conclusion: Sodium fluoride had a toxic effect on testis affecting spermatogenesis and decreasing quality and quantity of mature sperm, but the administration of vitamin E reversed the toxic effect of sodium fluoride, acting as an antidot. ALDH1A1 and NANOG appeared as markers of stem cell expressed in spermatogenic niche and spermatocytes and affected by many chemicals destroying these cells such as sodium fluoride.

Key words: Zymafluor; Spermatogenesis; Testis, ALDH1A1; NANOG.

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Introduction

Fluoride anions are widely utilised in industry, agriculture and medicine and they are naturally occurring chemicals observed in soil and water.[1] In addition to being used as a disinfectant and for fluoridating water, sodium fluoride (*Zymafluor*) is a proven and effective preventative measure against tooth cavities. Additionally, it is used in combination with calcium and vitamin D to treat osteoporosis.[2] Fluoride can have both positive and negative effects. Several research have

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shown that low concentrations of sodium fluoride are safe and necessary for both people and many animals, especially for the growth and development of teeth and bones.[3] The World Health Organisation recommends the maximum level of 1.5 mg/L for fluoride in drinking water. High fluoride ingestion may lead to fluorosis and a weakened antioxidant defence system.[4] Chronic fluoride toxicity has been documented in recent years after extended exposure to industrial emissions, toothpaste, mouthwash and drinking water.[5] Because fluoride can pass through cell membranes, it can reach a variety of organs, including the liver, kidneys, skin and reproductive system. As a result, long-term exposure to fluoride may cause noticeable gastrointestinal problems, neurological illnesses and reproductive system disorders.[6] Long-term exposure to sodium fluoride has been linked to poor reproductive function and altered gonad histology in mice, rabbits and even chicken embryonic gonads, according to several studies.[7] According to earlier research on genotoxicity, high sodium fluoride consumption is linked to immunotoxicity, cytotoxicity and oxidative damage with consequent apoptosis.[8]

Aldehyde dehydrogenase 1A1 (ALDH1A1), is commonly used as a marker for progenitor and stem cells. It is frequently linked to Sertoli cells and specific populations of spermatogonial stem cells (SSCs) in the testis.[9] It contributes to the production of retinoic acid, which is essential for the start of meiosis.[10, 11] NANOG is a crucial transcription factor that supports self-renewal and pluripotency. Its expression is typically limited to particular undifferentiated spermatogonia in the adult testis.[12] Antioxidant medications can lessen the negative effects of sodium fluoride. The use of nutritional supplements to alter the toxicity of environmental toxins has received intriguing attention lately. By alleviating the molecular makeup of cell membranes, stopping lipid peroxidation and preventing the negative outcomes of reactive oxygen species, vitamin E (α-tocopherol), a naturally occurring antioxidant, deactivates the harmful roles of free radicals produced by stressful oxidative impairment.[13]

The current study sought to clarify potential histological and immunohistochemical alterations in rat testis tissue following experimental exposure to a lethal dosage of sodium fluoride and to explore the probable effectiveness of vitamin E in mitigating sodium fluoride toxicity.

Methods

Zymafluor® 1 mg tablets of sodium fluoride were acquired from *Mylan Company*, Paris, France. Vitamin E, 400 IU, in the form of soft gel capsules, was acquired from *Adrien Gagnon*, Brossard, Quebec, Canada. Forty healthy-looking, energetic male Wistar albino rats weighing between 200 and 250 g were acquired from the Animal House at the University of Mosul's Veterinary College. The rats' age ranged from 2.5 to 3.5 months. Prior to the experiment, the rats were acclimated for a week in appropriate cages under strict supervision, hygienic conditions and aeration with a light-dark 12:12 cycle, an average temperature of 24 ± 26 ºC, standard rodent food pellets and unlimited fresh water. A digital weighing scale digital balance was used to measure and record the animal weights at the start and finish of the experiment. The experiment lasted for 28 days.
Lethal dose (LD_{50}): Pilot experiments were validated and inclusive doses of vitamin E and sodium fluoride were determined based on prior research that is comparable to the therapeutic doses for humans.[14] The project was performed between January and August of 2025.

Study design

The present study was a randomised controlled experiment.[15] The rats were evenly divided into 4 groups (10 rats in each):

- Group C, regarded as a control group, consisted of 10 animals that received normal saline 1 mL/kg/day by oral canulation as a single dose daily for 28 days.
- Group F, the sodium fluoride receiving group, took 20 mg/kg/day sodium fluoride once daily for 28 days period by oral gavage using a metal oropharyngeal cannula and syringe.
- Group FE (vitamin E + sodium fluoride) received vitamin E in a dose of 3 mg/kg/day, which equals to 0.6 mL, by oral canulation daily followed by oral gavage of sodium fluoride after 24 h using the same dose of sodium fluoride that received group F, for 28 days.
- Group E included animals that received vitamin E alone for 28 days (3 mg/kg/day) orally by gavage.

After 28 days of the experiment, the rats were sacrificed by ether inhalation and testis specimens were removed, cleaned in a sodium chloride solution (0.9 %), dried by filter paper and fixed in

Table 1: Johnsen score classification for spermatogenesis[18]

Score	Histological observation
10	Full spermatogenesis: Many spermatozoa (mature sperm) present; organised epithelium.
9	Many spermatozoa present but the germinal epithelium is disorganised or sloughing.
8	Only a few spermatozoa (typically < 5-10) present in the tubule.
7	No spermatozoa, but many spermatids are present.
6	No spermatozoa and only a few spermatids are present.
5	No spermatozoa or spermatids, but many spermatocytes are present.
4	Only a few spermatocytes are present.
3	Only spermatogonia (stem cells) are present; no further maturation.
2	No germ cells at all; Sertoli cells only (Sertoli cell-only syndrome).
1	Tubular fibrosis: No cells (germ cells or Sertoli cells) in the tubular section.

Bouin's solution for a full day before being processed into paraffin blocks. Reichert's Rotatory Microtome was used to cut paraffin blocks into 5-µm-thick slices which were placed on positively charged slides, to prepare the specimens for histology and immunostaining.

Immunohistochemistry

For immunohistochemical staining analysis, anti-NANOG (*ABNOVA*, Cat No: MAB-12279) and anti-ALDH1A1 (*ABNOVA*, Cat No: MAB12300) primary antibodies were used. followed by employment of a secondary antibody detection kit (*Elabscience*, Cat No: E-IR-R213, rabbit/mouse specific HRP/DAB). The slides were dewaxed using xylene and progressively hydrated. The antigen was completely recovered after 20 minutes of pressure cooking with citrate buffer. The main anti-NANOG and anti-ALDH1A1 antibodies were allowed to warm up at room temperature for 30 minutes after being diluted to a 1:200 ratio using background lowering dilution buffer (*Abcam*, code ab64211). Chromogen staining and DAB were utilised after detection using labelled streptavidin-biotin from an *Elabscience* secondary antibody detection kit. Haematoxylin was used as a counterstain after the slides were quickly hydrated and mounted with DPX.[16]

Every testis tissue slide was subjected to a blind evaluation. Estimates were made for the staining intensity and proportion of ALDH1A1 and NANOG:

- Four staining intensity levels were available: 0 for no staining, 1+ for faint staining, 2+ for moderate staining and 3+ for significantly strong staining. The following percentage indicated the degree of staining: Less than 10 % of the cell stained favourably was represented by 0, 10–50 % by 2, 51–80 % by 3 and more than 80 % by 4.[17]
- Spermatogenesis was evaluated from the histological slides based on the Johnsen score (Table 1),[18] which is determined by the maturity level of spermatozoa present in the testicular tissue.

Statistical analysis

The SPSS (version 26) software was used for statistical analysis. Data was examined for normal distribution using Shapiro-Wilk lot testing. Categorical data were expressed as frequency (%) and compared to each other using Chi-square test. Numerical data were expressed as mean ± standard deviation (SD) and were first examined using one-way ANOVA and then group to group comparisons were performed using Tukey test, p-value less than 0.05 was regarded as significant.

Results

Sections of control group showed a normal structure of testis with strong expression of ALDH1A1 in the cytoplasm of primary spermatocytes, secondary spermatocytes, spermatozoa and Sertoli cells as shown in Figure 1A. In histological sections of F group, the expression of ALDH1A1 was negative or low intensity and the histology of the cells was sloughed displaying overt vacuolation of spermatocytes and decrease in number of mature spermatozoa as shown in Figure 1B. Testis specimens from group FE showed a mild to moderate improvement in the histology of testicular cells with moderately intense expression pattern of ALDH1A1 (Figure 1C). The slides from group E

showed a histological pattern largely similar to C group and mainly strong intensity expression of ALDH1A1, as shown in Figure 1D.

Expression of NANOG immunohistochemical marker was mainly confined to spermatogonia which are regarded as the stem cells of sperm development. In group C, the spermatogonia showed a strong intensity of NANOG marker expression with histologically normal cells as shown in Figure 1E. In sections obtained from F group, the spermatogonia were diminished and vacuolated and expression of NANOG was mainly negative, as represented in Figure 1F. The sections examined from group FE revealed an improvement in the spermatogonia cells both histologically and immunohistochemically where the expression of NANOG biomarker ranged from mild to moderate, represented in Figure 1G. The E group also showed a histological structure of spermatogonia similar to C group and similarly strong intensity of NANOG stem cell biomarker expression, as shown in Figure 1H.

Staining intensity showed significant difference for both markers ($p < 0.001$). For ALDH1A1, the highest intensity of “3” was found in the control group (70 %) followed by the E group (60 %) while it was absent in the other two groups. The “2” intensity was highest in the FE group (80 %) and was rule the percentage found in the E and C groups but absent in the F group. The “1” intensity was seen only in the F group (60 %) and FE group (20 %). The “0” intensity was seen in the sodium fluoride (F) group only, as shown in Figure 2.

Regarding NANOG marker, the highest “3” intensity was recorded in the control and vitamin E groups, the “2” intensity was equally distributed between the vitamin E group and the vitamin E + sodium fluoride group, the “1” intensity was most frequent in the vitamin E + sodium fluoride group and observed as well in sodium fluoride group while the “0” intensity was again only seen in the sodium fluoride group, as shown in Figure 3.

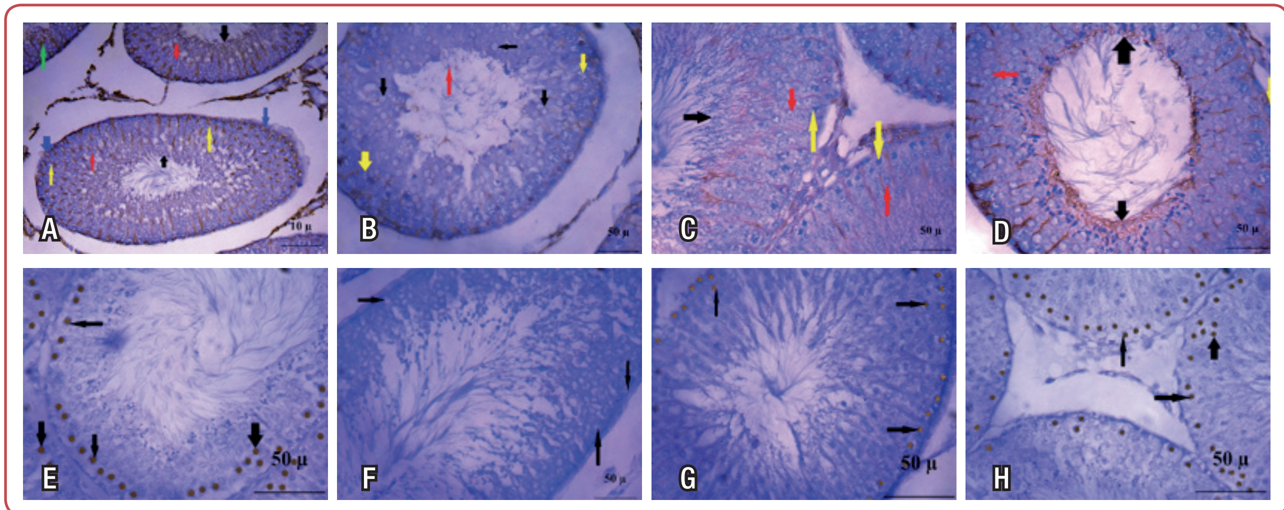

Figure 1: Photomicrograph of rat testis of study groups

A: Immunohistochemical strong intensity (3+) staining of aldehydes dehydrogenase 1A1 (ALDH1A1) of control (C) group showing normal testis structure, normal spermatozoa (black arrow), secondary spermatocytes (red arrow), primary spermatocytes (yellow arrow), spermatogonia (blue arrow), Sertoli cells (green arrow) 100X. B: Immunohistochemical mild intensity (1+) staining of ALDH1A1 of sodium fluoride (Zymalfluor) (F) group showing vacuolation of primary and secondary spermatocytes (black and yellow arrow, respectively) and diminished number of spermatozoa (red arrow) 400X. C: Immunohistochemical moderate intensity (2+) staining of ALDH1A1 of vitamin E + sodium fluoride (FE) group showing spermatozoa (black arrow), spermatocytes (red arrow), spermatogonia (yellow arrow) 400X. D: Immunohistochemical strong intensity (3+) staining of ALDH1A1 of vitamin E (E) group showing normal structure of testis, spermatozoa (black arrow), spermatogonia (red arrow) 400X. E: Immunohistochemical strong intensity (3+) staining of NANOG of control (C) group showing normal spermatogonia (black arrow) 400X. F: Immunohistochemical negative intensity (0) staining of NANOG of sodium fluoride (F) group showing vacuolation of spermatogonia (black arrow) 400X. G: Immunohistochemical moderate intensity (2+) staining of NANOG of vitamin E + sodium fluoride (FE) group showing spermatogonia (black arrow) 400X. H: Immunohistochemical strong intensity (3+) staining of NANOG of vitamin E (E) group showing normal spermatogonia (black arrow) 400X.

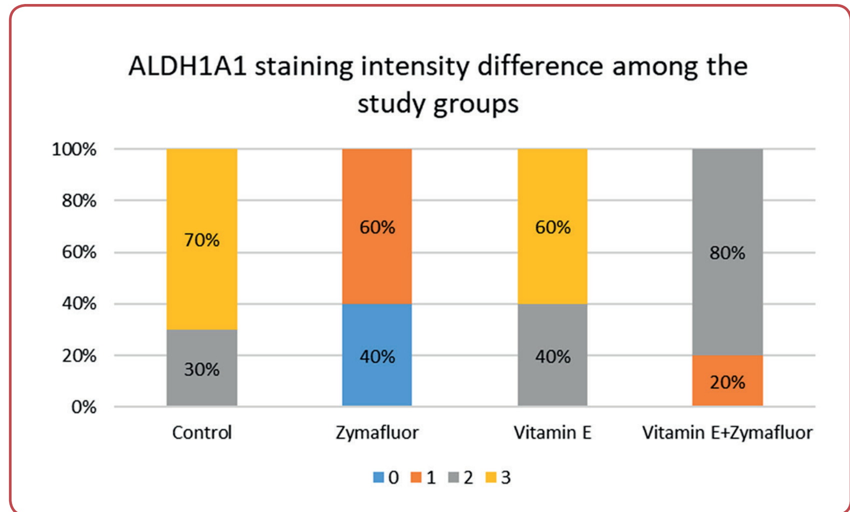

Figure 2: Stack graph showing aldehydes dehydrogenase 1A1 (ALDH1A1) staining intensity difference among the study groups

Control: group received normal saline 1 mL/kg /day for 28 days; Zymafluor: group received 20 mg/kg/day sodium fluoride once daily for 28 days; Vitamin E group received vitamin E alone for 28 days (3 mg/kg/day); Vitamin E+ Zymafluor: group received vitamin E followed by sodium fluoride for 28 days.

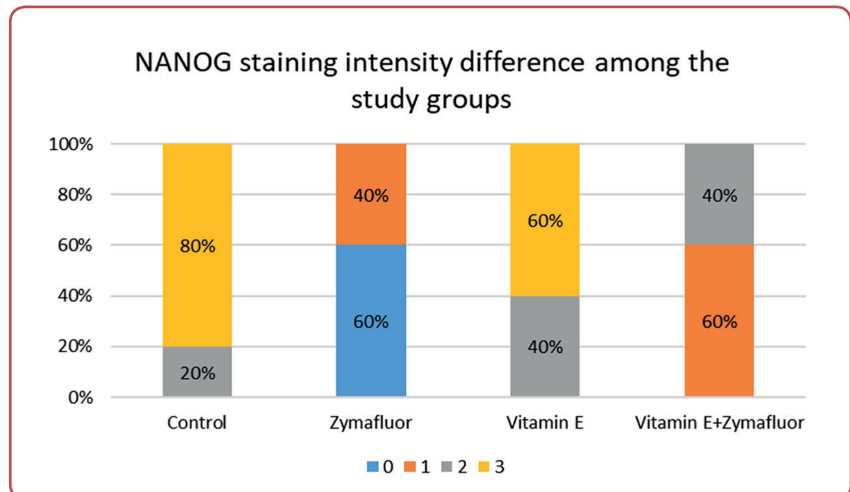

Figure 3: Stack graph shows NANOG staining intensity difference among the study groups

Control: group received normal saline 1 mL/kg /day for 28 days; Zymafluor: group received 20 mg/kg/day sodium fluoride once daily for 28 days; Vitamin E group received vitamin E alone for 28 days (3 mg/kg/day); Vitamin E+ Zymafluor: group received vitamin E followed by sodium fluoride for 28 days.

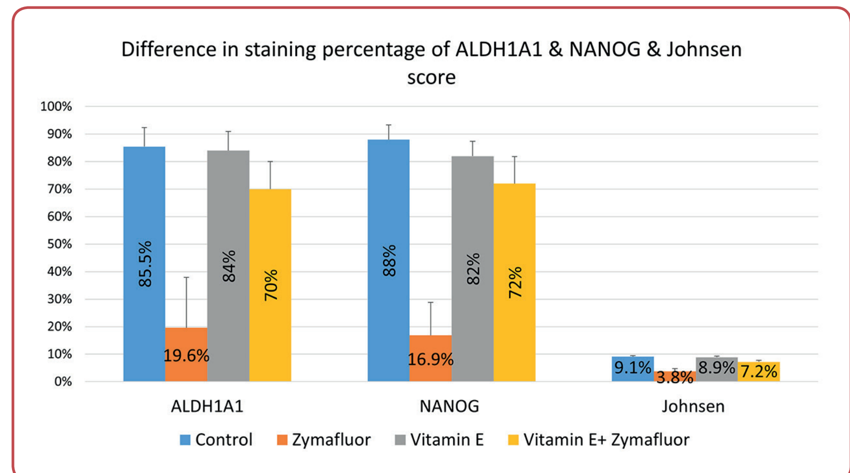

Figure 4: Difference in staining percentage of aldehydes dehydrogenase 1A1 (ALDH1A1) and NANOG and Johnsen score

Bars = mean; Error bars= standard deviation; Control: group received normal saline 1 mL/kg /day for 28 days; Zymafluor: group received 20 mg/kg/day sodium fluoride once daily for 28 days; Vitamin E group received vitamin E alone for 28 days (3 mg/kg/day); Vitamin E+ Zymafluor: group received vitamin E followed by sodium fluoride for 28 days.

The staining percentage and Johnsen score initially showed significant difference between all groups (ANOVA) as shown in Figure 4.

Table 2: Comparison between study groups in relation to difference in staining percentage of aldehydes dehydrogenase 1A1 (ALDH1A1) and NANOG and Johnsen score

Marker	**Group comparisons** (Tukey test, p-value)		
	Control (C)	Sodium fluoride (F)	Vitamin E (E)
ALDH1A1	0.000↔F 0.992↔E 0.032↔FE	0.000↔E 0.000↔*FE*	0.063↔*FE*
NANOG	0.000↔F 0.580↔E 0.008↔FE	0.000↔E 0.000↔*FE*	0.161↔*FE*
Johnsen score	0.000↔F 0.890↔E 0.000↔FE	0.000↔E 0.000↔*FE*	0.000↔*FE*

When comparing groups to each other (Post-hoc Tukey) the control group showed significant difference from both (F) and (FE) groups but not from the (E) group for all three parameters. The sodium fluoride (F) group showed significant difference from both the (E) and (FE) groups for all three parameters as well. The vitamin E group (E) showed significant difference from the (FE) group only for the Johnsen score but not for the immunohistochemical markers staining percentage. These results are summarised in Table 2.

Discussion

ALDH1A1 and NANOG are frequently employed as immunohistochemistry (IHC) markers in investigations of sodium fluoride toxicity in rat testis to assess the condition of germline stem cells and the extent of tissue regeneration or destruction.[19] A well-known reproductive toxin, sodium fluoride causes oxidative stress, which causes germ cell death and structural degeneration of the seminiferous tubules.[20]

ALDH1A1 usually acts as a progenitor cell and stem cell marker. It is frequently linked to Sertoli cells and specific SSC populations in the testis. It contributes to the formation of retinoic acid, which is essential for meiosis initiation.[21] However, NANOG is thought to be a crucial transcription factor that supports self-renewal and pluripotency. Its expression is typically limited to particular undifferentiated spermatogonia in the adult testis.[22]

As the “reserve” of the testis, NANOG is an important marker in testicular toxicity research. In essence, we are recording the loss of the testis’s capacity for self-healing when we see a decline in NANOG-positive cells following sodium fluoride treatment.[9] According to Sun et al, sodium fluoride causes a “domino effect” that specifically targets the undifferentiated spermatogonia (where NANOG is produced), the most vulnerable cells in the seminiferous tubules. Sodium fluoride reduces antioxidant enzymes like catalase (CAT) and raises the levels of reactive oxygen species (ROS). DNA fragmentation occurs in stem cell nuclei when ROS levels are high. The cell down-regulates NANOG as it enters an apoptotic (programmed cell death) state since it is a nuclear transcription factor that is in charge of DNA stability and self-renewal.[23] When mitochondria are damaged by sodium fluoride, cytochrome c is released and caspase-3 is activated. Kim et al has indicated that NANOG, a life/stemness marker, decreases when caspase-3, a death marker, increases. Signals from Sertoli cells, often known as the “nurse” cells, are necessary for NANOG expression. Sertoli cells atrophy and vacuolise as a result of sodium fluoride thereby “turning off” the environmental cues that maintain NANOG activity.[24] High concentrations of sodium fluoride (150 mg/L) caused considerable apoptosis in germ cells in mice and rats, according to research findings.[25] Reduction of NANOG-positive cells was found, which is directly supported by the observation of Heaney et al and Ali et al that the “undifferentiated” population declined first. Although there are not many studies that only look at sodium fluoride and NANOG, studies on other environmental toxins (such as phthalates or cadmium) reveal a pattern that is almost the same: the toxin inhibits the NANOG/OCT4 axis, which causes the tubules to appear “Sertoli-cell-only” (SCO), which is consistent with the low Johnsen Score it was probably seen.[26, 27] Studies conducted by Pushpalatha et al and others have demonstrated that sodium fluoride decreases the expression of androgen receptors and testosterone. The loss of NANOG-positive stem cells is indirectly caused by the decline of testosterone signalling, which is necessary to preserve the spermatogonial niche.[28]

ALDH1A1 is a functioning enzyme in testicular toxicity, not merely a marker. Its decrease in the presence of sodium fluoride acts as a “metabol-

ic red flag" for the sperm's support system and blood-testis barrier failure. There are three main ways whereby ALDH1A1 intensity and dispersion decrease. ALDH1A1 is a crucial enzyme in the synthesis of retinoic acid, the "master switch" that instructs a spermatogonium to cease being a stem cell and begin becoming a sperm cell (meiosis).[29] In the first mechanism, oxidative stress induced by sodium fluoride leads to the interruption of the retinoic acid (RA) pathway, which inhibits the enzymatic activity of ALDHs. As a result, RA levels decrease in the absence of ALDH1A1.[30] Accordingly, the germ cells lose their chemical "marching orders" and separate from the basement membrane, resulting in the "sloughing" that was observed.[31] The second mechanism is responsible for Sertoli cell dysfunction. Because Sertoli cells, which provide germ cells with both nutritional and physical support, have high levels of ALDH1A1, sodium fluoride causes the cytoskeleton of these cells to collapse, which is frequently observed as vacuolisation. Subsequently, the overall area of ALDH1A1 reactivity decreased as the Sertoli cells shrank or died. Toxins can enter the tubule more deeply as a result of the loss of the blood-testis barrier (BTB).[32] The third mechanism involves a failure of detoxification. Endogenous aldehyde generated during lipid peroxidation (a consequence of oxidative stress) are detoxified by ALDH1A1. A significant lipid peroxidation is triggered by sodium fluoride, leading to elevated quantities of hazardous malondialdehyde, which consequently "overwhelm" or downregulate the ALDH1A1 enzymes. The cell is unable to defend itself against sodium fluoride harmful effect, starting a vicious cycle.[33]

A significant disturbance of the testicular metabolic niche is indicated by the observed decrease in ALDH1A1 reactivity in rats treated with sodium fluoride. The downregulation of ALDH1A1 probably promotes the sloughing of the germinal epithelium since it is essential for retinoic acid signalling and the detoxification of lipid peroxidation products.[34] This finding, along with the reduction in NANOG expression, indicates that sodium fluoride affects both the SSC ability to regenerate and the Sertoli cells' ability to sustain function, which ultimately results in the Johnsen score decline that has been reported.[35, 36]

On the other hand, vitamin E supplementation successfully lessens these harmful impacts. Vitamin E stabilises the mitochondrial membrane and reduces reactive oxygen species produced by sodium fluoride by functioning as a lipid-soluble antioxidant.[37] By preserving ALDH1A1 and NANOG expression, this defence mechanism sustains the spermatogonial niche`s integrity. As a result, vitamin E supplementation preserves the germinal epithelium and produces a considerably higher Johnsen score, indicating that it may be a powerful cytoprotective treatment against fluoride-induced reproductive failure.[38] Vitamin E appears to have a protective impact at the signalling level by modulating the MAPK pathway. Exposure to sodium fluoride causes JNK and ERK, recognised mediators of oxidative stress-induced apoptosis in testicular germ cells, to become phosphorylated. Vitamin E suppresses these pathways, preventing the downregulation of the metabolic enzyme ALDH1A1 and the pluripotency marker NANOG. The improved Johnsen Scores in the vitamin E-supplemented group demonstrate how this molecular preservation successfully stops the apoptotic cascade, preserving the seminiferous tubules' structural integrity.[39]

Conclusion

This study concludes by confirming that exposure to sodium fluoride causes notable changes in the expression of important testicular stem cell markers, namely ALDH1A1 and NANOG. Both germ cell self-renewal and Sertoli cell support are failing, as evidenced by the observed decline in these markers and a dose-dependent decline in the Johnsen score. Nevertheless, these harmful effects were effectively reduced by using vitamin E as a cytoprotective agent. Vitamin E promoted the recovery of spermatogenesis by preserving the integrity of the SSC niche and increasing the immuno-reactivity of ALDH1A1 and NANOG. These findings demonstrate how vitamin E can prevent sodium fluoride toxicity by blocking pro-apoptotic signalling and maintaining the crucial chemical structure needed for sperm development.

Ethics

The study was approved by the Ethics Committee of the Faculty of Medicine, Mosul University, decision No UOM/COM/MREC/24-25(10), dated 26 December 2024.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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