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The Impact of Sodium Fluoride on Redox the Impact of Sodium Fluoride on Redox Homeostasis an Antioxidant Defense Systems in Sperm Cells: an in Vitro Study

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ABSTRACT

Purpose: Fluoride is a ubiquitous element in nature that can be beneficial at low doses but toxic at high levels. The Holstein bulls used in this study are both economically significant and represent a species susceptible to environmental fluoride exposure through contaminated water and feed. The research seeks to evaluate the impact of sodium fluoride (NaF) on sperm function in farm animals, mimicking field conditions, and thereby elucidate potential effects on fertility. This is also of significant importance for food safety. This study aimed to investigate the effects of NaF on redox homeostasis and antioxidant defense mechanisms in bovine sperm cells.

Methods: Sodium fluoride was applied to bovine sperm samples at concentrations of 30, 60, 120, and 240 mM and incubation periods ranging from 5 to 30 minutes. Oxidative stress parameters were assessed by measuring malondialdehyde (MDA) levels and the activities of antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx), and reduced glutathione (GSH).

Results: NaF exposure led to a significant dose- and time-dependent increase in MDA levels, indicating oxidative stress. This was accompanied by a decrease in CAT and GPx activities and a reduction in GSH levels. The oxidative damage induced by fluoride in sperm cells was further supported by observed declines in sperm motility and viability.

Conclusions: NaF exposure disrupts the redox status of bovine sperm cells, leading to oxidative damage and a consequent reduction in fertilization potential. This study highlights the need for further research on chronic fluoride exposure and protective dietary strategies using antioxidants.

Keywords: *Fluoride toxicity; Redox homeostasis; Seminal plasma; Oxidative stress; Antioxidant defense systems; Bulls*

INTRODUCTION

Fluoride contamination in livestock is a widespread and well-documented issue, especially in areas with high fluoride levels in groundwater and feedstuffs. Holstein bulls have been considered particularly vulnerable to the harmful effects of fluoride due to their probable long-term exposure to the chemical through contaminated drinking water and feed consumption, as well as environmental sources. In regions where this breed is raised for milk and meat production, Holstein bulls have the potential for prolonged and continuous exposure to fluoride. Previous studies[1,2] have demonstrated that fluoride accumulates in biological tissues over time, which can have toxic effects on the reproductive system. Accordingly, to minimize systemic influences, our study aims to elucidate the specific effects of fluoride on bovine sperm function by directly exposing the sperm cells to sodium fluoride (NaF) under in vitro conditions.

Fluoride toxicity is largely mediated through oxidative stress, a process that specifically targets the cellular integrity of sensitive tissues such as the reproductive system. Excessive fluoride exposure

disrupts redox homeostasis, leading to increased production of reactive oxygen species (ROS) and a weakened antioxidant defense mechanism, thereby inducing cellular damage. Various epidemiological studies[3] have established significant associations between fluoride exposure and markers of oxidative stress, as well as infertility. These findings suggest that oxidative stress mechanisms, including lipid peroxidation (LPO), protein oxidation, and deoxyribonucleic acid (DNA) damage, play a crucial role in fluoride toxicity, ultimately leading to cellular dysfunction and pathological cell death[4,5].

Emerging evidence[6,7] indicates that fluoride can disrupt mitochondrial function by inhibiting oxidative phosphorylation, compromising mitochondrial membrane integrity, and consequently reducing adenosine triphosphate (ATP) production. Furthermore, it has been demonstrate[8,9] that fluoride can alter gene expression through epigenetic mechanisms, and these effects may cause lasting genetic changes in reproductive cells, particularly with chronic exposure. These findings underscore the critical implication that fluoride-induced oxidative stress may impact not only individual reproductive health but also have transgenerational effects.

Fluoride can traverse the blood-testis barrier and accumulate within testicular tissues, thereby inducing toxic effects at the testicular level. Elevated oxidative stress impairs sperm function by overwhelming antioxidant defense mechanisms, consequently diminishing overall reproductive capacity[10,11]. Fundamentally, oxidative stress diminishes sperm motility and functional competence by damaging sperm membranes with ROS[12,13]. Key antioxidant enzymes, including glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT), play a crucial role in protecting cells against oxidative damage[14,15]; however, their activities are significantly inhibited in response to fluoride toxicity[16]. Furthermore, certain investigations have revealed that fluoride can induce epigenetic modifications, leading to persistent alterations in gene expression, a phenomenon critical for elucidating the broader impacts of fluoride on the reproductive system[16,17].

This study specifically evaluated the in vitro effects of fluoride directly on sperm cells, rather than systemic fluoride exposures. This approach aimed to isolate the effects of fluoride at the testicular level and exclude confounding variables commonly encountered in in vivo animal models. Holstein bulls were chosen for this study due to their genetic homogeneity, economic value, and previous exposure to fluoride-contaminated water and feed.

MATERIAL AND METHODS

Chemicals

Sodium fluoride used in the experiments was procured from Sigma-Aldrich (St. Louis, MO, USA). Unless otherwise specified, all chemicals were of analytical grade.

Experimental Design and Exposure Conditions

The acute and subacute toxicity of fluoride on reproductive cells was assessed using a method adapted from Tanyıldızı and Bozkurt[18]. For acute exposure, 30-60 mM NaF concentrations were used, while 120-240 mM NaF concentrations were employed for subacute exposure, based on prior literature[8,9]. These concentrations were considered appropriate for inducing physiological levels of oxidative stress in mammalian sperm and were therefore utilized as the optimal dose range in this study.

Animals and Semen Sample Collection

Twenty healthy and sexually mature Holstein bulls (aged 4-5 years), a breed commonly utilized for milk and meat production with well-defined reproductive characteristics, were included in this study. The sample size was determined based on previous studies to ensure adequate statistical power and minimize variability in sperm quality. All bulls were maintained under standard commercial breeding conditions with a balanced diet consisting of dry hay, alfalfa hay, and corn silage, and had ad libitum access to fluoride-free water. Experimental conditions were maintained at a constant room temperature of 22°C and 60% humidity. Following a two-week acclimatization period, the animals were included in the study, and efforts were made to minimize stress and variability during semen collection.

Semen samples were collected from all bulls (n=20), and concurrent dermatological examinations were performed. An average ejaculate volume of 6 ± 0.2 ml with a sperm concentration of 1.5 ± 0.3 x 10^9 sperm/ml was obtained from each bull. Semen was collected using the non-invasive artificial vagina method, a technique widely accepted in reproductive studies[19,20]. All samples were divided into five groups, ensuring equal volumes were taken from each bull, and sampling was conducted every morning for five consecutive days. One group served as the control, while the remaining four groups were treated with NaF solutions prepared at concentrations of 30, 60, 120, and 240 mM, respectively.

Sperm Incubation and Treatment Procedure

Fresh semen samples were diluted 1:1 with T-cell factor (TCF) buffer and subsequently mixed with the NaF solutions before incubation. Control samples underwent the same conditions, with distilled water

used in place of NaF. All samples were incubated at 37°C to simulate physiological conditions conducive to sperm metabolism and enzymatic activity.

Following incubation, samples were collected at 5, 10, 15, 20, and 30 minutes, and stored at -20°C for subsequent evaluation of acute oxidative stress responses. Given the limitations of the current laboratory setup, which lacked the capacity for -80°C or liquid nitrogen storage, a -20°C deep freezer was utilized for sample preservation. Cell viability assays, such as 3-(4,5-dimetiltiyazol 2-yl)-2,5-difeniltetrazolyum-bromür (MTT), were not performed in this study; future research is recommended to incorporate such analyses. As this study was conducted entirely in vitro, ethical committee approval was not required.

Biochemical Analyses

Malondialdehyde (MDA), an indicator of LPO, was quantified using the thiobarbituric acid reactive substances (TBARS) assay[21]. The reaction mixture consisted of 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid adjusted to pH 3.5, 1.5 ml of 0.8% thiobarbituric acid, and 0.2 ml of 20% tissue homogenate containing 1.15% KCl. The total volume was brought to 4 ml with distilled water, and the mixture was incubated in a boiling water bath for 1 hour. Following incubation, the mixture was cooled to room temperature and centrifuged at 3500 rpm for 10 minutes. The resulting pink supernatant was measured spectrophotometrically at a wavelength of 532 nm. TBARS levels were expressed as nmol TBARS/mg protein using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}. Protein concentration was determined using the method described by Lowry et al. [22], with bovine serum albumin employed as the standard. Malondialdehyde values were expressed as nmol MDA/mg protein, based on a 1,1,3,3-tetraethoxypropane standard curve.

Selenium-dependent glutathione peroxidase (Se-GPx; E.C. 1.11.1.9) activity was measured spectrophotometrically at 37°C and 412 nm using cumene hydroperoxide and glutathione (GSH) as substrates, with Ellman's reagent[23]. Catalase (CAT; E.C. 1.11.1.6) activity was assessed according to the method described by Aebi[24]. In this method, one unit of enzyme activity was defined as the decomposition of 1 µmol of hydrogen peroxide per minute at 25°C. The change in absorbance at 240 nm was measured as an indicator of CAT activity. Reduced GSH levels in the tissues were also determined spectrophotometrically using Ellman's reagent[25]. Protein concentrations were quantified using the Lowry method[22], as described previously.

Statistical Analyses

The data were analyzed using IBM SPSS version 19.0. Results are presented as mean ± standard error (SE). The distribution of variables was assessed using the Shapiro-Wilk test. For data that did not exhibit normality, the Mann-Whitney U test was employed, while Student's t-test was used for pairwise comparisons of normally distributed data. For comparisons involving multiple groups, a one-way analysis of variance (ANOVA) followed by the Tukey HSD post-hoc test was performed. Correlations between variables were determined using Spearman's rank correlation coefficient (r). Statistical significance was set at $p \leq 0.05$.

Ethical Approval Statement

This entire research was conducted in vitro using isolated bovine sperm cells. As live animals were not directly involved in the study, ethical committee approval was not required under the ARRIVE 2.0 guidelines, European Union Directive 2010/63/EU, and relevant institutional ethical regulations. No pain, suffering, stress, or physiological impairment was inflicted upon any animals during the course of this study.

This study examined the effect of NaF on bovine sperm quality under in vitro conditions. Although MTT or similar assays were not included in this study, future research incorporating such tests is recommended to better understand the additional mechanisms underlying fluoride toxicity.

RESULTS

What was investigated in vitro in this study are the sperm quality under the action of sodium fluoride. Other possible mechanisms of fluoride toxicity should be better explained by future studies implementing the MTT assay or any other plausible assay.

Data Presentation and Figures

Figures 1–4 were consolidated into a single table to enhance clarity and facilitate review of standard deviations. Data previously depicted in these figures are presented visually in Table 1 for improved interpretability. Vertical bars now represent the percentage of activity in enzyme-related graphs, ensuring easier comparison and clearer understanding (see Table 1).

Effect of Sodium Fluoride on MDA Levels in Seminal Plasma

Sodium fluoride exposure at concentrations of 30, 60, 120, and 240 mM led to a significant, dose- and time-dependent increase in MDA levels in seminal plasma. The increase began at 10 minutes post-treatment and continued progressively, reaching the highest level at 30 minutes.

At 10 minutes, MDA levels were significantly elevated in the 60, 120, and 240 mM groups compared

to the control (p = 0.05). At 15 minutes, the 30 mM group also showed a significant increase versus control (p = 0.0327), with a further significant difference observed between the 30 and 120 mM groups (p = 0.0016), confirming a dose-response relationship.

After 20 minutes, the distinctions among treatment groups became more pronounced. The 240 mM NaF group showed a significantly higher MDA level compared to the control (p = 0.0010) and to the 60 mM group (p = 0.0027).

At 30 minutes, MDA levels peaked across all groups:

- 30 mM: 2-fold increase (p = 0.0327)
- 60 mM: 3-fold increase (p = 0.0100)
- 120 mM: 5-fold increase (p = 0.0015)
- 240 mM: 6-fold increase (p = 0.00013)

Additionally, a significant difference between the 120 mM and 240 mM groups (p = 0.0042) supported a dose-dependent progression of oxidative stress.

These findings demonstrate that NaF-induced oxidative stress intensifies over time and with increased dosage, resulting in greater MDA production and potential sperm dysfunction.

Antioxidant Enzyme Activities and GSH Levels in Seminal Plasma

Se-GPx Activity:

NaF exposure caused a dose-dependent reduction in Se-GPx activity:

- 30 mM: 75% of control (p < 0.05)
- 60 mM: 66% (p < 0.01)
- 120 mM: 60% (p < 0.001)
- 240 mM: 49% (p < 0.001)

The greatest inhibition was observed at 240 mM, indicating a pronounced suppression of antioxidant defense.

Catalase Activity:

CAT activity also declined with increasing NaF concentrations:

- 30 mM: 73% of control (p < 0.05)
- 60 mM: 65% (p < 0.001)
- 120 mM: 62% (p < 0.001)
- 240 mM: 50% (p < 0.001)

This indicates a substantial impairment of enzymatic antioxidant capacity due to fluoride exposure.

Reduced GSH Levels:

GSH content decreased in all treatment groups after 30 minutes:

- 30 mM: 82% of control (p < 0.05)
- 60 mM: 77% (p < 0.01)
- 120 mM: 64% (p < 0.001)
- 240 mM: 50% (p < 0.001)

This suggests that NaF disrupts redox homeostasis via depletion of non-enzymatic antioxidants.

Correlation Between MDA Levels and Antioxidant Parameters

MDA and CAT Activity:

Strong inverse correlations were observed at higher NaF doses:

- 60 mM: r = -0.810 (p < 0.01)
- 120 mM: r = -0.928 (p < 0.001)
- 240 mM: r = -0.875 (p < 0.001)

MDA and Se-GPx Activity:

Severe inverse correlations were also found between MDA and Se-GPx activity:

- 60 mM: r = -0.560 (p < 0.01)
- 120 mM: r = -0.759 (p < 0.001)
- 240 mM: r = -0.976 (p < 0.001)

MDA and Reduced GSH Levels:

Similar patterns were observed with GSH:

- 60 mM: r = -0.451 (p < 0.05)
- 120 mM: r = -0.622 (p < 0.01)
- 240 mM: r = -0.693 (p < 0.01)

Table 1. Figures 1-4 have been combined into a single table

NaF (mM)	MDA (nmol/ml)	Se-GPx (%control)	CAT (%control)	GSH (%control)
30	2.0*	75*	73*	82*
60	3.0**	66**	65***	77**
120	5.0***	60***	62***	64***
240	6.0***	49***	50***	50***

Note: * p < 0.05, **p < 0.01, ***p < 0.001 vs. control group
MDA: Malondialdehyde; Se-GPx: Selenium-dependent glutathione peroxidase; CAT: Catalase; GSH: Reduced glutathione.

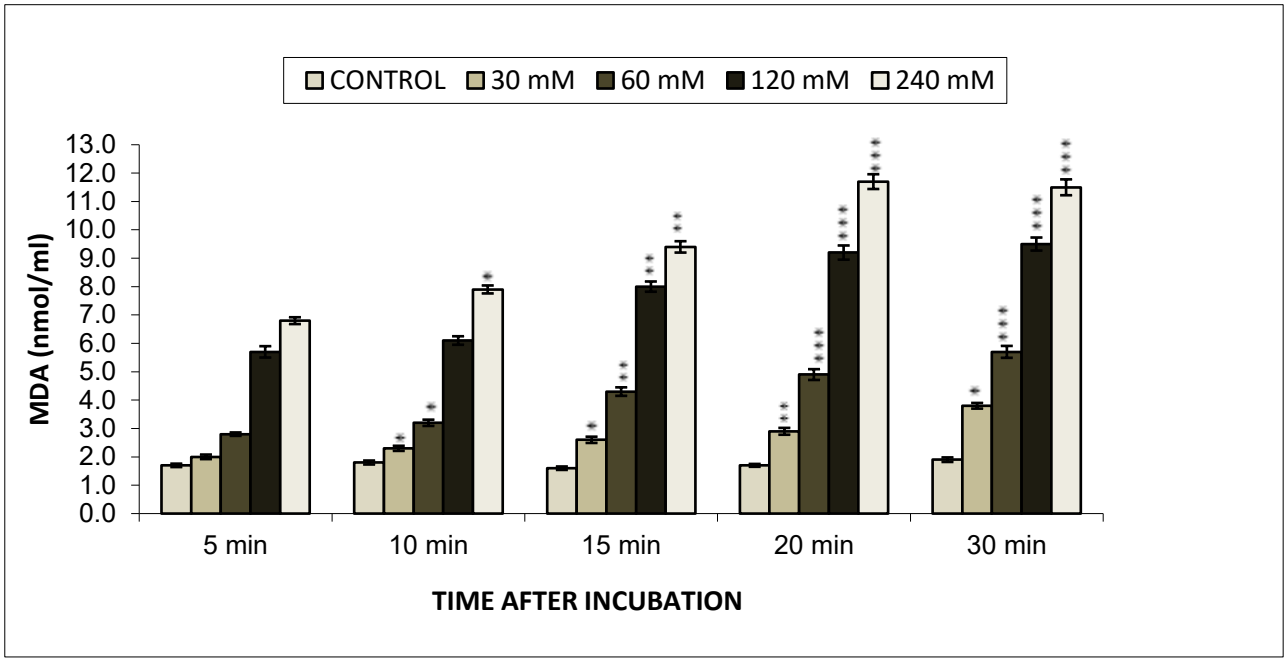

Figure 1. Doses of NaF and MDA levels in the seminal plasma at different time points

Note: Values are expressed as the mean at each time point
*, **, *** Statistically significant according to the control group (*p<0.05; **p<0.01;*** p<0.001)

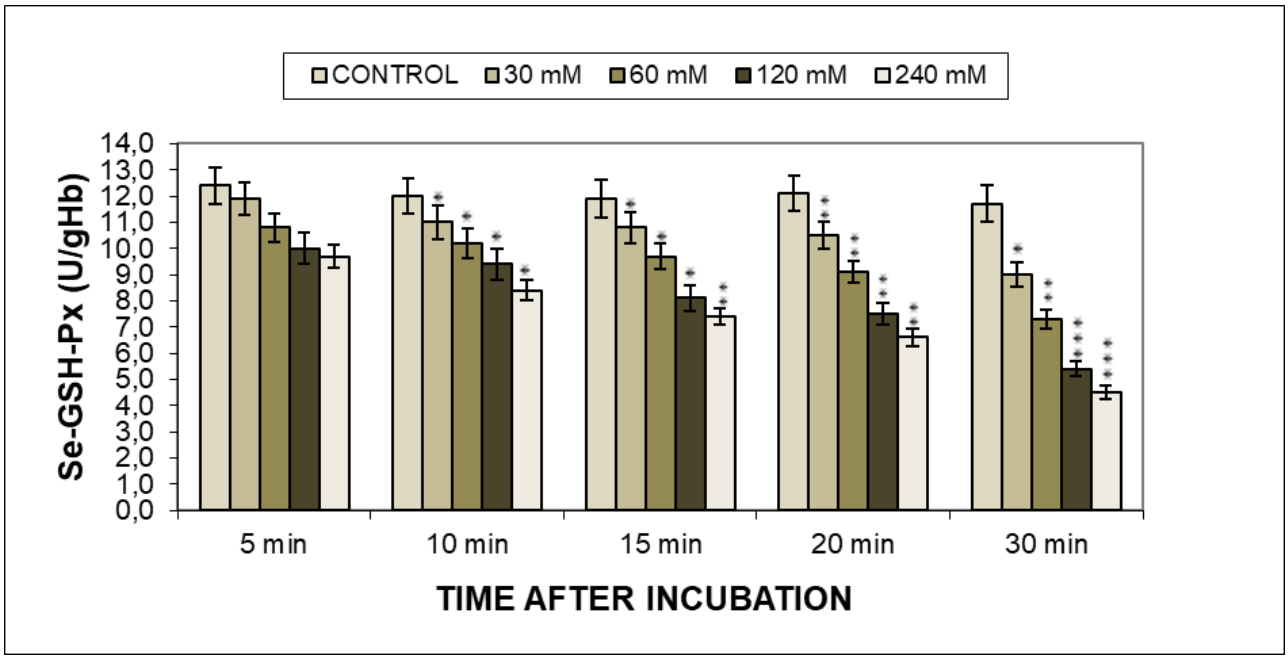

Figure 2. Doses of NaF and Se-GPx activity in the seminal plasma at different time points

Note: Values are expressed as mean ± SE
*, ** Statistically significant according to the control group (*p<0.05; **p<0.01)

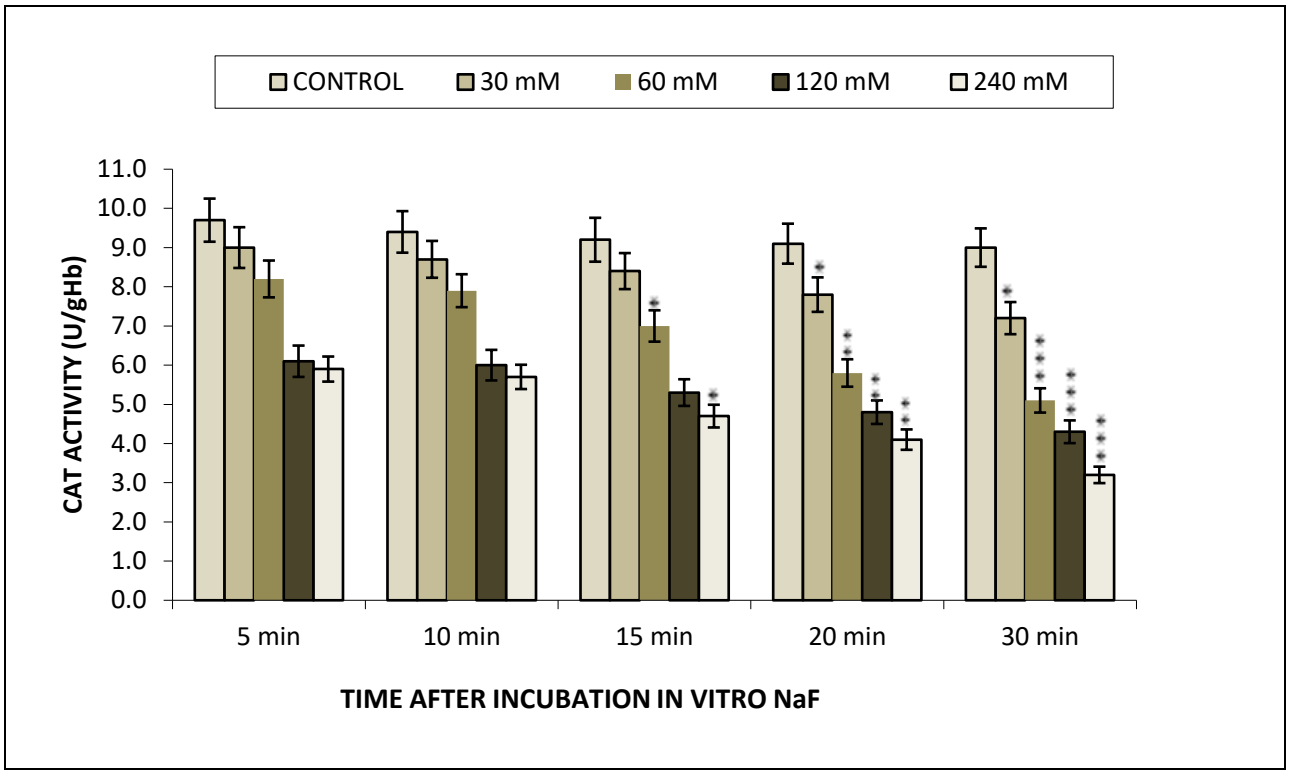

Figure 3. The Doses NaF and CAT activity in The seminal plasma at different time points

Note: Values are expressed as mean ± SE
*, **, *** Statistically significant according to the control group (* p<0.05; **p<0.01; p<0.001)

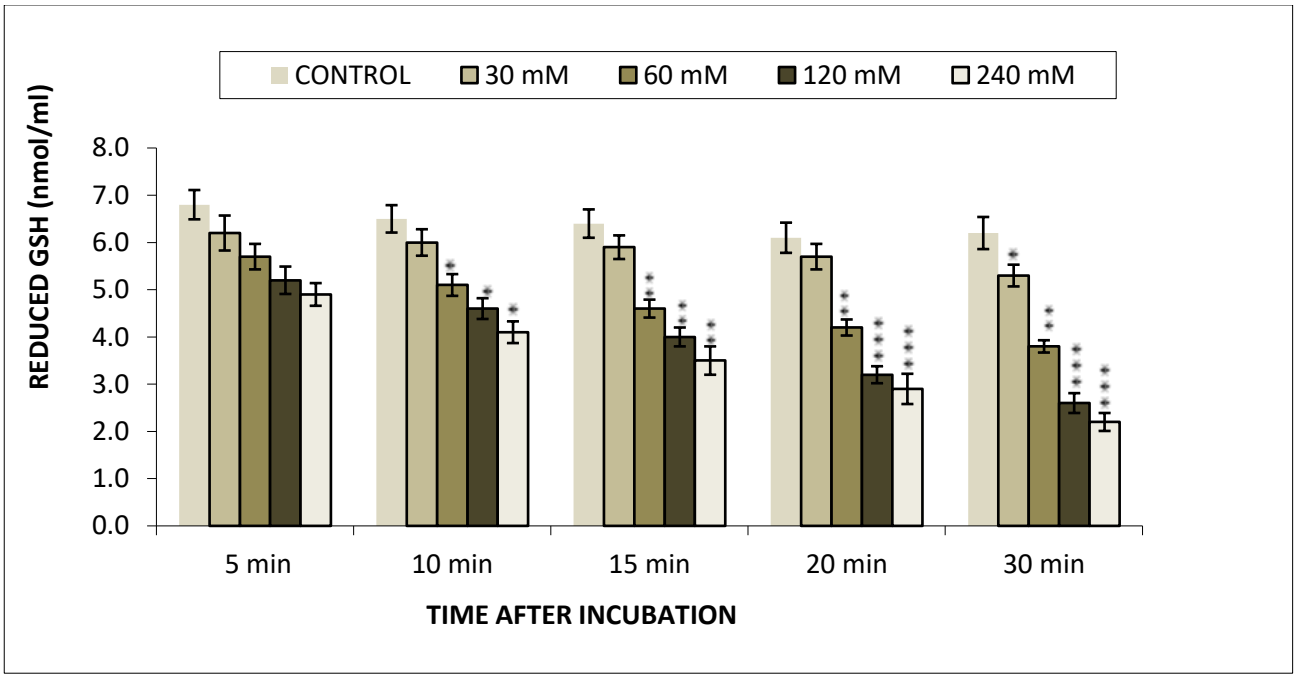

Figure 4. The Doses NaF and Red-GSH levels in The seminal plasma at different time points

Note: Values are expressed as mean ± SE
*, **, *** Statistically significant according to the control group (*p<0.05; **p<0.01; ***p<0.001)

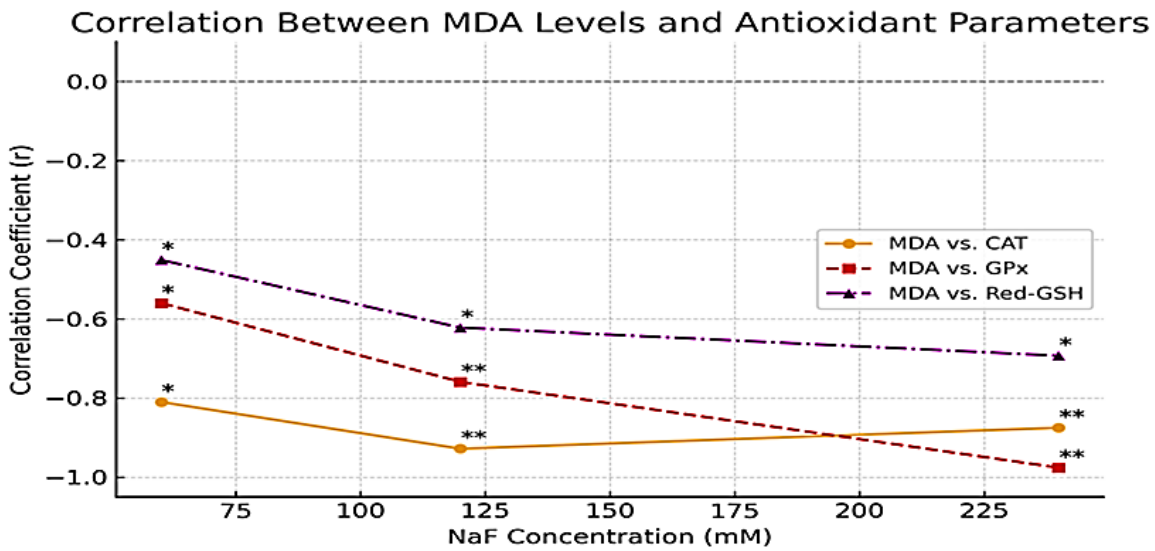

Figure 5. Correlation curve between MDA levels in seminal plasma and antioxidant parameters after NaF incubation

Notes:
*p < 0.05; **p < 0.01; **p < 0.001 (levels of statistical significance)
Correlation coefficients (r) indicate a negative relationship between the MDA levels and CAT, Se-GPx, and Red-GSH

DISCUSSION

Disturbances in the reproductive cycle stemming from fluoride and other external environmental pollutants raise significant ethical concerns, given the intricate biological mechanisms involved in reproduction. Existing evidence indicates a correlation between fluoride levels in food and drinking water and an increased incidence of adverse reproductive effects. The literature documents effects such as impaired sperm morphology, reduced motility, and increased LPO, an indicator of oxidative stress. Consequently, the impact on male fertility in regions with high fluoride levels in groundwater poses a significant global public health concern.

Oxidative stress is considered a primary mechanism underlying the adverse effects of fluoride on the reproductive system[26]. During fluoride exposure, superoxide radicals and harmful signals associated with cellular metabolism are generated[27]. Consistent with this, our study identified strong negative correlations between fluoride exposure and antioxidant enzyme activities[15,16]. These findings strongly support the notion that fluoride is a potent reproductive toxicant. This study establishes a robust foundation for a more detailed interpretation and generalization of fluoride-induced reproductive disorders. Toxicity assays are of paramount importance for enhancing our understanding of biological processes. Future research on fluoride should prioritize elucidating cellular-level mechanisms and developing protective strategies. Longitudinal studies involving human and animal populations exposed to fluoride levels below endemic thresholds have become a national imperative.

Fluoride-mediated ROS disrupt cellular homeostasis, triggering a cascade of adverse effects including ATP depletion, reduced sperm motility, decreased phosphorylation of axonemal proteins, and increased membrane permeability[28,29]. Prolonged fluoride exposure exacerbates endocrine disruption via oxidative stress, thereby impairing reproductive functions[30]. The high content of polyunsaturated fatty acids (PUFAs) in sperm cells renders them particularly susceptible to oxidative stress due to ROS-induced membrane damage[31,32]. The dose- and time-dependent increase in oxidative stress parameters observed in bovine seminal plasma following NaF exposure in this study aligns with these findings. The significant elevation of MDA levels after fluoride exposure indicates increased LPO, which negatively impacts membrane integrity and function. Similarly, the observed decrease in the activities of antioxidant enzymes such as GPx and CAT provides further evidence of fluoride-induced oxidative stress. The reduction in GSH levels suggests a diminished capacity of the antioxidant defense system to cope with ROS, thereby rendering sperm cells more vulnerable to oxidative damage. These results corroborate previous studies indicating that fluoride reduces GSH levels, disrupts the GSH/GSSG ratio, and induces damage in sperm cells via oxidative stress and apoptosis.

However, some studies have reported conflicting results regarding the role of fluoride in oxidative stress. For instance, Sharma et al.[33] indicated that low doses of NaF might exert protective effects on human

spermatozoa by reducing MDA levels and enhancing antioxidant enzyme activities. Likewise, Das Sarkar et al.[34] observed an increase in GPx and CAT activity in testicular tissues following NaF exposure when supplemented with antioxidant sources, suggesting a potential link to dose-dependent endocrine mechanisms. Chinoy and Narayana[35], on the other hand, reported no significant alterations in MDA levels and antioxidant enzyme activities with moderate in vitro NaF exposure, suggesting the possibility of variable responses depending on physiological conditions. Some recent studies propose that fluoride may play a protective role against oxidative stress mechanisms through specific interactions, thereby underscoring the need for more standardized research on this topic. These discrepancies may be attributed to variables such as the animal species used, routes of administration, and experimental designs. The literature also highlights that the toxic effects of fluoride can vary across species[36-40].

Chronic fluoride exposure has been shown to reduce semen quality, disrupt hormonal balance, and increase oxidative stress in humans[37]. Studies conducted in India and China have clearly demonstrated that fluoride exposure leads to a reduction in sperm motility and an increase in DNA damage[38,41,42]. Studies involving testicular fluoride exposure in mice and rats have demonstrated impaired spermatogenesis, compromised blood-testis barrier integrity, and disrupted mitochondrial function[26,36-39]. In female rats, high fluoride exposure has been shown to reduce embryo weight, decrease implantation rates, and lower the number of live fetuses. These findings indicate that fluoride-induced reproductive disorders can manifest in both sexes, underscoring the significance of addressing fluoride exposure as a major global public health concern36. Similarly, Liu et al.[2] observed a decrease in testicular weight, a reduction in sperm count, and an increase in oxidative stress markers in male rats exposed to high doses of fluoride.

Emerging research indicates that fluoride induces cell death in male germ cells by triggering caspase activation via mitochondrial dysfunction[43,44]. These findings suggest that fluoride-induced testicular damage might be at least partially mitigated by antioxidant supplementation. However, the long-term effects of such damage in human studies can be far more severe[45-48]. This situation underscores the necessity of conducting comparative studies across different species in basic research, thereby enabling a clearer understanding of the effects of fluoride on the reproductive system and its implications for human health[48]. Indeed, these data further strengthen the arguments for reducing fluoride exposure in high-risk communities, particularly those residing in regions where fluorosis is endemic[30,49,50]. Therefore, in regions with drinking water high in fluoride content, implementing measures for water defluoridation, alongside the use of antioxidant-rich dietary supplements, is essential. In clinical practice, the reproductive health of individuals exposed to fluoride should be prioritized, and antioxidant treatment options should be considered. Clinical studies have demonstrated that supplementation with substances such as vitamin E, selenium, and coenzyme Q10 reduces oxidative damage to sperm and improves overall reproductive health[51-53]. Indeed, a study conducted on male workers occupationally exposed to fluoride showed that the group receiving antioxidant supplementation exhibited higher sperm motility and lower oxidative stress markers. This further suggests the potential effectiveness of dietary and pharmacological interventions against fluoride-induced reproductive toxicity. At the molecular level, fluoride reduces the activities of antioxidant enzymes like Se-GPx and CAT by binding to their active sites.

CONCLUSIONS

In conclusion, NaF exposure disrupts redox homeostasis, leading to oxidative stress in bovine sperm cells. This effect manifests as a dose-dependent increase in MDA levels and a decrease in the activity of antioxidant enzymes (CAT and GPx). Indeed, high concentrations of NaF negatively impact sperm motility and viability, consequently reducing fertility potential. These findings underscore the necessity of developing strategies to minimize NaF exposure in livestock breeding. Future research should prioritize evaluating the epigenetic effects of chronic NaF exposure and investigating protective dietary antioxidant-based interventions against fluoride-induced reproductive toxicity.

FUNDING

Not applicable.

CONFLICTS OF INTEREST

None.

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