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SODIUM FLUORIDE-INDUCED UTERINE REDOX IMBALANCE AND STEROIDOGENIC HAZARDS: DOSE DEPENDENT RESPONSE

Pikash Pratim Maity,[a] Lipi Rani Jana,[a] Bimal Deb,[a] Hasina Perveen,[a] Moulima Maity,[a] Shamima Khatun, [a] Sandip Chattopadhyay[a,*]

Midnapore, India

ABSTRACT: The effect of sodium fluoride (NaF) on uterine oxidative stress and ovarian steroidogenesis was investigated in an animal model using 100 ppm and 200 ppm of doses for a period of 28 days. Ovarian and uterine wet weights were measured along with the measurement of steroidogenesis, uterine superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). This dose dependency treatment resulted in a marked degree ($p<0.001$) of diminishing ovarian and uterine wet weight and ovarian steroidogenesis along with a significant fall in plasma levels of luteinizing hormone (LH), follicle stimulating hormone (FSH), and estradiol, especially at a dose of 200 ppm of NaF. Uterine malondialdehyde (MDA) and conjugated diene (CD) levels were elevated with a concomitant inhibition in the activities of oxidative stress remission enzymes e.g., SOD, CAT, and GPx at both the doses of NaF but 200 ppm of NaF treatment revealed a greater degree of alteration in these parameters. NaF (200 ppm)-treated rats showed a lengthy diestrus phase in their estrous cycle in contrast to control rats who had a regular 4 day estrous cycle pattern. It can be concluded that the dose of NaF treatment is the critical factor for the development of adverse effects on ovarian and uterine activities.

Keywords: Antioxidant enzymes; Ovary; Oxidative stress; Steroidogenesis; Uterus.

INTRODUCTION

It is well documented that long-term exposure of fluoride (F), either through fluoridated drinking water or industrial fluoride pollution, results in osteo-dental and non-skeletal fluorosis in both humans and domestic animals.[1-8] An anionic form of F in drinking water easily travels through the intestinal mucosa in combination with calcium ions (Ca^{2+}). F opposes normal cell metabolism and also interferes with hydrogen bonding present in biomolecules.[9] Previous study revealed that F-treatment in animals significantly increases the intracellular levels of reactive oxygen species (ROS) and serum lactate dehydrogenase with a simultaneous inhibition in the activities of glutathione peroxidase.[10] Excess F toxicity interferes with glycolysis and oxidative phosphorylation. It inhibits Na^+/K^+-ATPase, which may lead to hyperkalemia due to the extracellular release of potassium and decreased acetyl cholinesterase.[11] F induces hepatic cell damage due to elevation of serum aspartate transaminase and alanine transaminase.[12] F exposure in animal models proposed impaired glucose tolerance and insulin dependent hyperglycemia.[13] The outcome of chronic F exposure is associated with an upregulation of thyroid stimulating hormone (TSH) with decreased levels of T_3.[14] Studies proposed that F leads to male reproductive dysfunction characterized by structural and functional deficiencies of spermatozoa[15] and epididymis,[16] reduced spermatogenesis,[17]and sterility.[18] F leads to histopathological changes in uterus,[19] hampers ovulation, estrous cycle, and

[a]Department of Biomedical Laboratory Science and Management, and Clinical Nutrition and Dietetics Division (UGC Innovative Department), Vidyasagar University, Midnapore-721102, West Bengal, India; *For correspondence: Dr Sandip Chattopadhyay, Department of Biomedical Laboratory Science and Management, and Clinical Nutrition and Dietetics Division (UGC Innovative Department), Vidyasagar University, Midnapore-721102, West Bengal, India; E-mail: sandipdoc@yahoo.com, sandipdoc@mail.vidyasagar.ac.in.

ovarian hormones secretion.[20] Considering the above facts our objectives were to outline the adverse effect of NaF in an *in vivo* model in a dose dependent manner and to explore the antioxidant status of the uterus along with the manifestation of F on ovarian steroidogenesis.

MATERIALS AND METHODS

Animal selection, treatment, study of estrous cycle, and sample collection: Wistar strain female albino rats of 100–120 g were well maintained under a standard laboratory atmosphere in polycarbonate cages with standard pellet and water *ad libitum*. This study was performed following the guidelines of the Institutional Ethical Committee (Reference number: IEC/7-3/C-3/16 dated 26.08.2016). Rats were randomly distributed in a control group, and 100 ppm of NaF- and 200 ppm of NaF-treated groups. The treatment was continued for 28 days (7 estrous cycle lengths of the rats).The phases of estrous cycles were monitored microscopically by staining the vaginal smear with Leishman's stain. On day 29, prior to sacrifice, the final body weights were recorded, and blood and organs were collected, weighed, and preserved at –20ºC until use. Six rats in each group were considered in the study and the results of the experiment were analysed in triplicate.

Spectrophotometric measurement of oxidative stress markers: Uterine tissue was used to measure malondialdehyde (MDA) and conjugated dienes (CD). Uterine MDA was determined by the reaction against thiobarbutaric acid.[21] The CD were measured by standard technique[22] where lipids were extracted with chloroform and methanol mixture. Uterine non-protein thiol (NPSH) was determined by standard 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) method with a slight change.[23] The superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gpx) were measured following the previous method.[24-26]

Electrozymographic measurement of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and lactate dehydrogenase (LDH) by native gel-electrophoresis: Uterine tissue was homogenized (20%; w/v) with 0.1 M ice cold phosphate buffer saline (PBS; pH 7.4) and then centrifuged at 10,000 × *g* for 20 minutes at 4ºC. Supernatant containing 60 µg proteins was run on 12% native polyacrylamide gel electrophoresis (PAGE) to separate SOD. To evaluate catalase activity, 60 µg proteins were electrophoresed on 8% PAGE using same tissue extract.[27] Uterine GPx activity was determined by the removal of peroxide that was required for the transformation of potassium ferricyanide to ferrocyanide between samples.[27]For the detection of serum lactate dehydrogenase (LDH), 8.0%, agarose gel was used.[28] Finally, densitometric analysis of band intensity was executed by using ImageJ software.

Uterine DNA fragmentation analysis and Comet assay: DNA was extracted from the supernatant using a combination of phenol:chloroform at 1:1. Ethidium bromide was added with 0.8% agarose gel and the gel was run at 65 V and documented in gel documentation system.[29] The uterine Comet assay was executed by Singh and colleagues' method with some minor modifications.[30] The slides were seen under a fluorescence microscope (Nikon, Eclipse LV100 POL), with the VisComet (Impulsvbildanalyse) software.

Ovarian steroidogenic activities and tissue histo-morphology: Ovarian hydroxysteroid dehydrogenases (HSD) enzymes were considered in this study, where Δ^5,3β-HSD, and 17 β-HSD activities were measured following the previous method.[31, 32] Serum levels of LH (luteinizing hormone) Cat no. ER1123), FSH (follicle stimulating hormone) (Cat no. ER0960), and estradiol (Cat no. ER1507) were measured by ELISA kits according to the procedures recommended by the manufacturers (Wuhan Fine Test, China). The ovarian and uterine sections were fixed in formalin, embedded in paraffin, and 5.0 µm thick tissues were stained with Harris hematoxylin and eosin and observed under microscope (Olympus, CX21i, magnification × 100).

RESULTS

Feeding habit, body growth, and organ weights: Throughout the experimental duration there were significant differences in the body weight between controls and NaF-treated groups. After 28 days of treatment, there was a significant loss of ovarian and uterine weights at both the doses of NaF in contrast to control (Table 1).

Table 1. Represents the changes of body growth and reproductive-organo-somatic indices in response to NaF ingestion. The organo-somatic indices are expressed in terms of the whole body weight.

Group	Initial body wt. (g)	Final body wt. (g)	Ovarian wt. in pair (mg)	Uterine wt. (mg)
Control	94.83±2.06	103.5±1.65	0.076±0.003	0.267±0.02
NaF 100 ppm	90.33±1.66	86.83±1.79*	0.063±0.003**	0.264±0.009
NaF 200 ppm	97.98±1.40	90.83±1.26*	0.053±0.004***	0.165±0.008**

The data are mean±SE, N=6;
ANOVA followed by Student's t test: *p<0.05, **p<0.01, ***p<0.001.

Vaginal smear study: In case of NaF-treated group, a prolonged diestrus stage was observed (Figure 1). But no alteration of this normal cyclic pattern was found in vehicle treated-control group (Figure 1). Moreover, a higher percentage of rats in the group treated with 200 ppm of NaF achieved diestrus earlier than the 100 ppm of NaF-treated rats (Figure 1).

Effect of NaF on antioxidant status and serum LDH: In 200 ppm NaF-treated group, antioxidant enzyme activities were decreased significantly compared to the vehicle treated-control group (Table 2). In contrast, the levels of MDA and CD were increased in a dose dependent fashion.

Electrophoretic study (Figure 2) revealed that a gradual decrease in the intensity of SOD, CAT, and GPx bands, along with the increased doses of NaF, was observed in contrast to control group. However, the band intensity of LDH was more prominent in NaF-treated group as compared to the control group (Figure 2).

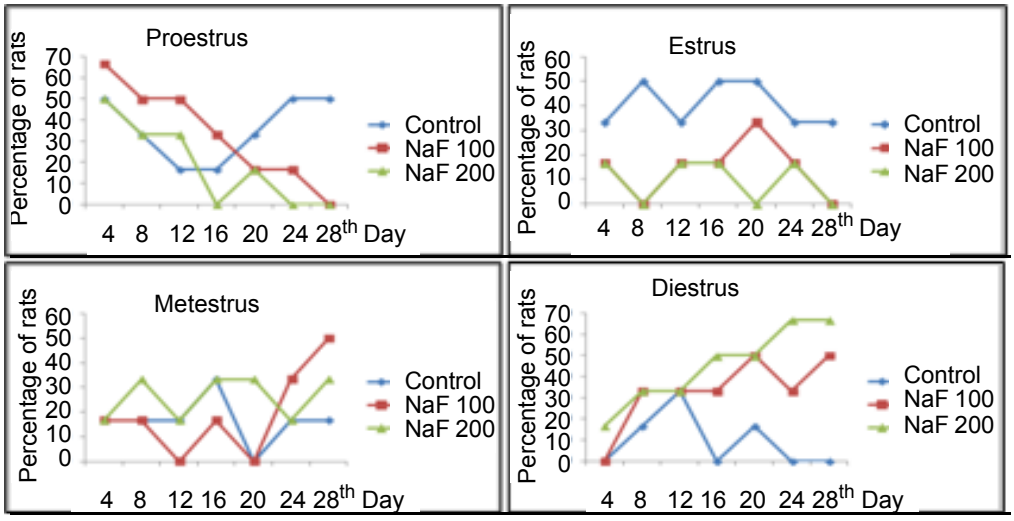


Figure 1. Estrous cycle pattern. Regularity of the estrous cycle pattern was abolished, and persistent diestrus was noticed in NaF treated rats.

Table 2. Represents the NaF mediated changes of MDA, CD, NPSH, SOD, catalase and GPx activities.

Oxidative stress markers	Control	NaF-100ppm	NaF-200ppm
MDA (nmol/g)	30.65 ± 4.5	41.32 ± 7.2*	55.67 ± 6.3**
CD (nmol/g)	21.60 ± 3.3	27.15 ± 1.5	42.24 ± 2.4*
NPSH (µg/g of protein)	42.51 ± 6.3	36.29 ± 1.5	24.90 ± 1.8*
SOD (unit/g of protein)	45.5 ± 3.7	39.2 ± 4.2	27.2 ± 3.6*
Catalase (unit/g of protein)	27.4 ± 2.4	23.5 ± 3.6	11.7 ± 1.9*
GPx (unit/g of protein)	2.2 ± 0.6	1.6 ± 0.9	0.9 ± 0.2*

The data are mean ± SE, N=6;
ANOVA followed by Student's t test: *p<0.05, **p<0.01.

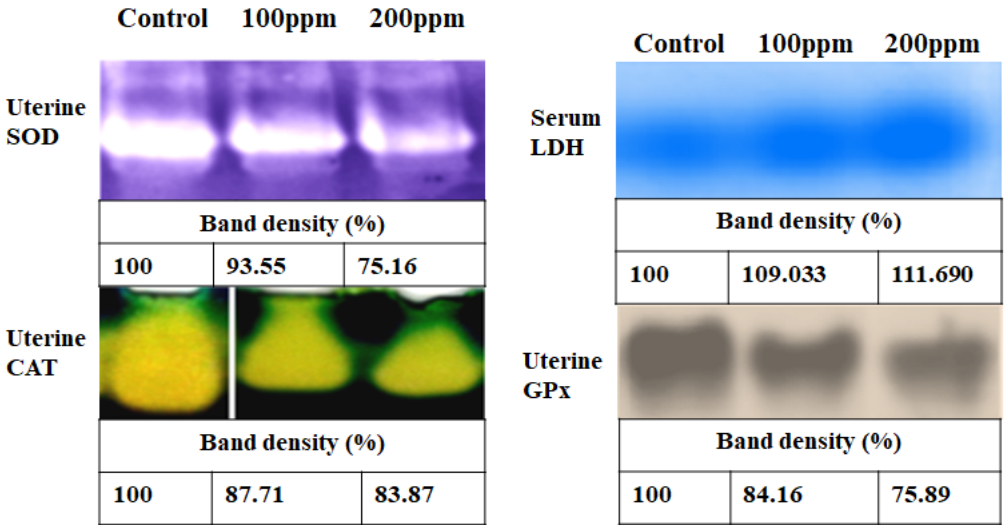
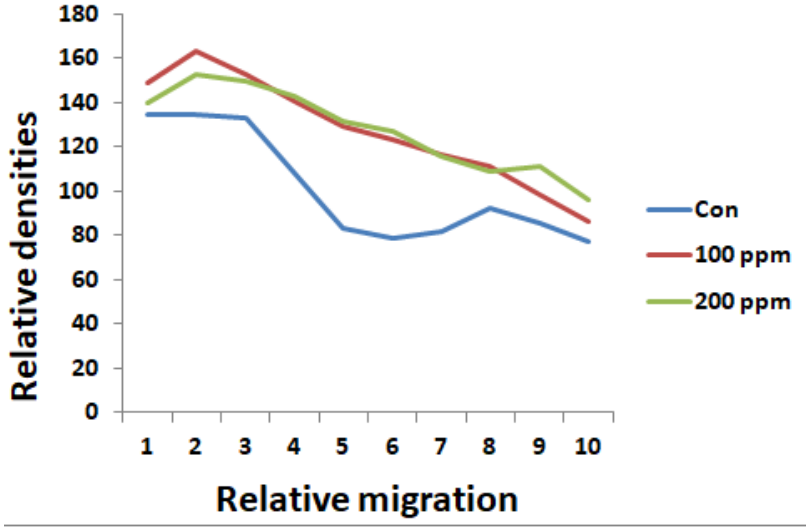


Figure 2. Catalase, SOD, and glutathione peroxidase activity in uterine tissue on native gel. The uterine tissue extract protein in each lane was electrophoresed on 8.0%, 12%, and 8.0% native gel followed by substrate specific development of catalase, SOD, and GPx bands respectively. Electrophoresed serum protein on agarose gel was followed by substrate specific yield of LDH bands.

DNA fragmentation and comet assay: Graphical representation of relative migration and relative densities of uterine DNA showed that NaF was able to damage the uterine DNA (Figures 3A and 3B). This evidence was further supported by the Comet assay results where the broken DNA was prominently shown by the appearance of the comet (Figures 3A and 3B).

Serum gonadotrophins and ovarian steroidogenesis: NaF treatment for 28 days resulted in a significant down regulation in plasma level of FSH, LH, and estrogen in comparison to controls (Table 3). We found a significant alteration of these gonadotrophins and ovarian steroids at the dose of 200 ppm of NaF treatment. NaF at both two doses, 100 and 200 ppm, also significantly suppressed the steroidogenic enzymes Δ^5,3β-HSD and 17β-HSD.

Figure 3A. The band intensity of DNA at its different positions on the agarose gel was evaluated by ImageJ software, expressed in relative/ normalized values, and plotted on a graph.



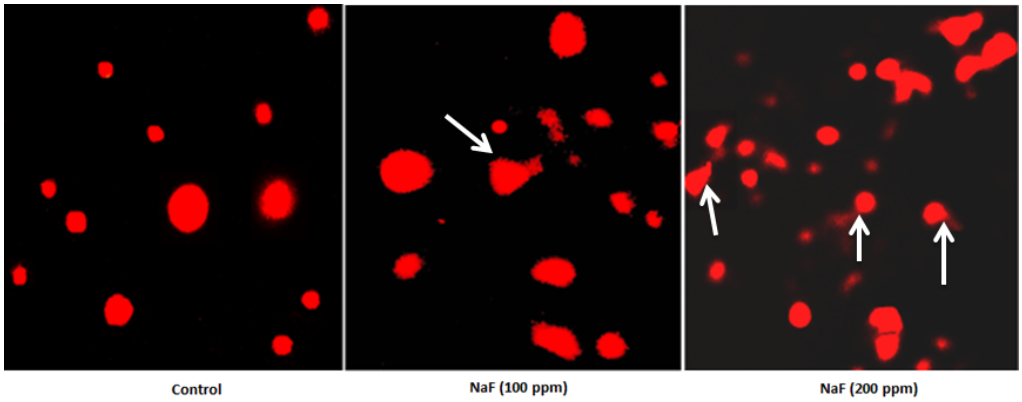


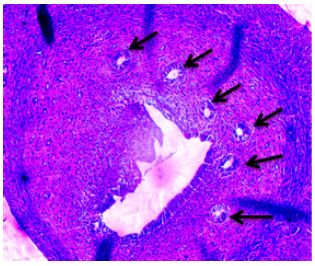
Figure 3B. NaF-induced severe DNA breakage which was noticed in single cell apoptotic damage. Arrows show comet formation in uterine horn cells following NaF ingestion.

Table 3. The effect of NaF on the steroidogenic enzymes and hormones

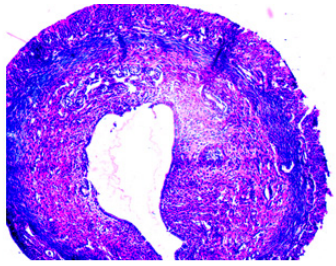
	Control	NaF-100 ppm	NaF-200 ppm
Δ^5, 3β-HSD (unit/mg of tissue)	29.43 ± 5.5	18.88 ± 4.2**	9.63 ± 1.6***
17β–HSD (unit/mg of tissue)	20.22 ± 1.9	16.76 ± 2.9*	11.47 ± 2.4*
LH (mIU/mL)	7.79 ± 0.91	5.31 ± 0.79	2.09 ± 0.63**
FSH (mIU/mL)	18.64 ± 2.41	14.46 ± 3.21	6.96 ± 0.24**
Estradiol (pg/mL)	465 ± 19	393 ± 27	225 ± 24**

The data are mean ± SE, N=6;
ANOVA followed by Student's t test: *p<0.05, **p<0.01, ***p<0.001.

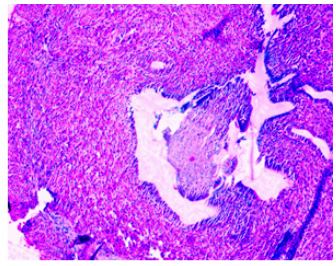
Ovarian and uterine histomorphology: A distinguishing decline in the count of primary classes of preantral and antral follicles was detected in the ovaries of fluorosed rats in a dose dependent manner. A significantly diminished number of Graafian follicles, with a higher grade of follicular atresia or follicular regression, was also apparent after F ingestion compared with the control rats (Figure 4). Uterine secretory glands were lost in fluoridated rats (Figure 4). Uterine myometrium and endometrium degenerations were characterized by a remarkable distortion of these layers (×100 magnification) following NaF exposure with the 100- and 200-ppm doses compared to the untreated group (Table 4).



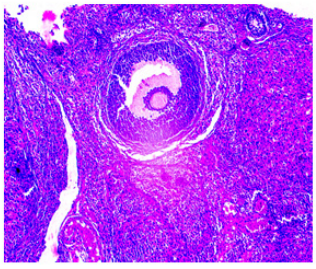
Control uterus. The arrows indicate secretory glands.



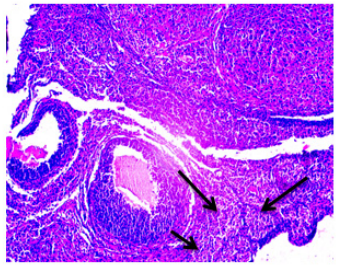
Uterus 100 ppm F exposure



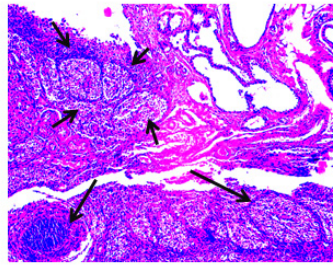
Uterus 200 ppm F exposure



Control ovary



Ovary 100 ppm F exposure. The arrows indicate follicular atresia.



Ovary 200 ppm F exposure. The arrows indicate follicular atresia.

Figure 4. Uterine and ovarian tissue were implanted in paraffin, serially sectioned laterally at 5 µM, stained with eosin and hematoxylin (Harris), and observed under a microscope (×100). The upper row shows the loss of uterine secretory cells along with distortion of the endometrial layer in the F-exposed groups. The lower row shows an increase in the number of atretic follicles in the ovary of the NaF-exposed group.

Table 4. The changes in the number of ovarian follicles and the thickness of the uterine bed following the administration of NaF in different doses

	Control	NaF-100 ppm	NaF-200 ppm
SPAF (n)	10.32 ± 2.45	8.16 ± 1.96	3.97 ± 1.98**
LPAF (n)	9.12 ± 1.62	5.67 ± 0.22**	4.83 ± 0.54*
SAF (n)	7.63 ±0.91	4.33 ± 0.72**	3.06 ± 0.63**
MAF (n)	3.66 ± 0.47	1.05 ± 0.36*	0.97 ± 0.51*
LAF (n)	1.97 ± 0.33	1.00 ± 0.24	0.87 ± 0.23*
GF (n)	2.76 ± 0.42	0.91 ± 0.12**	0.79 ± 0.32**
ATF (n)	1.12 ± 0.49	18.36 ± 3.24***	25.51 ± 2.63***
Endometrium (µm)	320.22 ± 11.78	199.28 ± 16.29***	165.72 ± 7.28***
Myometrium (µm)	115.25 ± 3.27	94.32 ± 1.32**	79.19 ± 1.65***

SPAF = small preantral follicles (< 94 µm); LPAF = large preantral follicles (94–260 µm);
SAF = small antral follicles (261–350 µm); MAF = medium antralfollicles (351–430 µm);
LAF = large antral follicles (431–490 µm)GF = Graafian follicles (> 491 µm);
AF = atretic follicles. The data are mean ± SE, N=6;
ANOVA followed by Student's t test: *p<0.05, **p<0.01, ***p<0.001.

DISCUSSION

Uterine and ovarian tissue damage was shown in the rats due to the treatment with NaF at the doses of 100 ppm and 200 ppm. F is primed to produce hydrogen peroxide, superoxide anion, hydroxyl radical, and peroxynitrite.[33] Exposure to F significantly decreases the activity of GPx in different cells[34] and this was supported by the results of the present investigation. The reduced GSH/GSSG ratio is one of the causative factors of oxidative stress and apoptosis.[35] Another study suggested that 10 ppm NaF considerably increases the circulating level of ROS followed by a decline in glutathione (GSH) level and GSH/GSSG ratio.[36] F has a tendency to decrease the activity of SOD and CAT.[37] Inhibition of SOD by NaF is likely to be the consequence of bonding of F^- ion with the active site of Cu on SOD.[38] The diminution in catalase activity found in the present study in response to F may be explained by the interaction of F with the trivalent metals (Fe^{3+}) located in the active site of catalase.[39] F as a pro-oxidant preoccupies a hydrogen atom from a methylene group in polyunsaturated fatty acids (PUFA) and produces lipid radicals, conjugated dienes (CD), and oxidized fragments of fatty acids.[40] Hydrogen peroxide radicals due to NaF may oxidize non-protein thiol (NPSH) and yield sulfonic acid. F may generate thiol or thiyl radicals ($RS^{\bullet}$) due to the abstraction of hydrogen from thiols. Thiol radicals are responsible for the formation of deoxyribonucleic acid, the building blocks for DNA.[41] Increased lactate dehydrogenase (LDH) activity with F exposure is supported by several studies in albino rats treated with NaF.[42] Extracellular activity of serum LDH is implicated under the condition of oxidative stress that indicates cell necrosis or cell death. This may be due to the destruction of cell membrane as a consequence of lipid peroxidation that allows the leakage of this enzyme from damaged tissue.[43] Electronegative F has a strong affinity with the uracil and amide bonds of DNA by –NH---F- interactions and this virtually leads to genotoxicity.[44] Moreover, the normal structure of DNA is affected following the free radical formation and lipid peroxidation that can initiate direct damage of DNA strands.[45] The process of DNA replication or repair may be arrested by F in response to its inhibitory effects on DNA polymerase.[46]

Reduction in the uterine and ovarian somatic indices may be due to low plasma levels of LH, FSH, and estrogen. Normally, gonadotrophins regulate ovarian weight,[47] whereas estradiol regulates uterine weight.[48] The interruption in the estrous cycle with NaF treatment may probably be due to the inhibitory action of NaF on the ovarian hormonal function. A report stated that decrease in the activity of Δ^5,3β-HSD in adrenal gland of fluoridated rabbits implies that during F intoxication adreno-cortico steroidogenesis would also be impaired.[49] Inhibition in the activities of ovarian Δ^5,3β-HSD and 17β-HSD in rats in the present investigation may be due to the result of low levels of plasma LH and FSH, since these gonadotrophins are responsible for regulating the activities of these steroidogenic enzymes.[50] The significant drop in the number of primary, secondary, and Graafian follicles following F ingestion seen in the current study could be due to the unavailability of proteins necessary for growth, cell division, and differentiation of germ cells in the process of oogenesis. NaF causes development of atretic follicles in ovary.[51] Another group of investigators also suggested that exposure of NaF decreases the successful rate of pregnancy because NaF has an adverse fetotoxic effects.[52] A previous investigation considered that reproductive hormones were diminished at the doses of

100, 150, and 200 ppm of NaF along with the degeneration of endometrium[19]and these results are also supported by the present study.

CONCLUSIONS

In conclusion, the data reported here show that 100 ppm and 200 ppm NaF treatments were associated with uterine oxidative stress and inhibition of the activities of enzymatic antioxidants. This oxidative stress finally led to the reduction in the activities of ovarian steroidogenic dehydrogenases and the levels of FSH, LH, and estrogen. In addition, our data suggest an possible effect of NaF at the pituitary level. However, more information is necessary to better understand the effect of F on the functional physiology of the female reproductive system.

REFERENCES

1 Choubisa SL, Sompura K, Bhatt SK, Choubisa DK, Pandya H, Joshi SC, et al. Prevalence of fluorosis in some tribal villages of Dungarpur district of Rajasthan. Indian J Environ Health 1996; 38(2):119-26.

2 Choubisa SL. Endemic fluorosis in southern Rajasthan (India). Fluoride 2001;34(1):61-70.

3 Choubisa SL, Choubisa L, Choubisa D. Osteo-dental fluorosis in relation to nutritional status, living habits and occupation in rural areas of Rajasthan, India. Fluoride 2009;42(3):210-15.

4 Choubisa SL, Choubisa D. Status of industrial fluoride pollution and its diverse adverse health effects in man and domestic animals in India. Environ Sci Pollut Res 2016;23(8):7244-54.

5 Choubisa SL. Fluorosis in dromedary camels of Rajasthan, India. Fluoride 2010;43(3):194-9.

6 Choubisa SL. Fluoride toxicosis in immature herbivorous domestic animals living in low fluoride water endemic areas of Rajasthan, India: an observational survey. Fluoride 2013;46(1):19-24.

7 Choubisa SL. Industrial fluorosis in domestic goats (*Capra hircus*), Rajasthan, India. Fluoride 2015;48(2):105-15.

8 Choubisa SL. A brief and critical review on hydrofluorosis in diverse species of domestic animals in India. Environ Geochem Health2018;40:99-114.

9 Emsley J, Jones DJ, Miller JM, Overill RE, Waddilove RA. An unexpectedly strong hydrogen bond: *ab initio* calculations and spectroscopic studies of amide-fluoride systems. J Am Chem Soc1981;103(1):24-8.

10 Zhang M, Wang A, He W, He P, Xu B, Xia T, et al. Effects of fluoride on the expression of NCAM, oxidative stress, and apoptosis in primary cultured hippocampal neurons. Toxicology 2007 17;236(3):208-16.

11 Rani B, Trivedi A, Pathak RK, Maheshwar RK. Fluoride ion: an inexplicable hydro toxicant. J Ind Pollut Contr 2014;30:23-32.

12 Bouaziz H, Ketata S, Jammoussi K, Boudawara T, Ayedi F, Ellouze F, et al. Effects of sodium fluoride on hepatic toxicity in adult mice and their suckling pups. Pestic Biochem Physiol 2006;86(3):124-30.

13 Menoyo I, Rigalli A, Puche RC. Effect of fluoride on the secretion of insulin in the rat. Arzneimittelforschung 2005;55(08):455-60.

14 Bachinskii PP, Gutsalenko OA, Naryzhniuk ND, Sidora VD, Shliakhta AI. Action of the body fluorine of healthy persons and thyroidopathy patients on the function of hypophyseal-thyroid the system. Probl Endokrinol (Mosk)1985;31(6):25-9.

15 Chinoy NJ, Sharma A. Amelioration of fluoride toxicity by vitamins E and D in reproductive functions of male mice. Fluoride 1998;31(4):203-16.

16 Kumar A, Susheela AK. Effects of chronic fluoride toxicity on the morphology of ductus epididymis and the maturation of spermatozoa of rabbit. Int J Exp Pathol 1995;76(1):1-11.

17 Ghosh D, Das S, Maiti R, Jana D, Das UB. Testicular toxicity in sodium fluoride treated rats: association with oxidative stress. Reprod Toxicol 2002;16(4):385-90.

18 Elbetieha A, Darmani H, Al-Hiyasat AS. Fertility effects of sodium fluoride in male mice. Fluoride 2000;33(3):128-34.

19 Zhou Y, Zhang H, He J, Chen X, Ding Y, Wang Y, et al. Effects of sodium fluoride on reproductive function in female rats. Food ChemToxicol 2013;56:297-303.

20 Dhurvey V, Thakare M. The effect of sodium fluoride intoxication on the estrous cycle and ovarian hormones in rats. Fluoride 2016;49(3):223-32.

21 Devasagayam TP, Boloor KK, Ramasarma T. Methods for estimating lipid peroxidation: an analysis of merits and demerits. Indian J Biochem Biophys 2003;40:300-8.

22 Recknagel RO, Glende Jr. E. Spectrophotometric detection of lipid conjugated dienes. Methods Enzymol 1984;105:331-7.

23 Mieyal JJ, Gallogly MM, Qanungo S, Sabens EA, Shelton MD. Molecular mechanisms and clinical implications of reversible protein S-glutathionylation. Antioxid Redox Signal2008;10(11):1941-88.

24 Pattichis K, Louca LL, Glover V. Quantitation of soluble superoxide dismutase in rat striata, based on the inhibition of nitrite formation from hydroxylammonium chloride. Anal Biochem. 1994;221(2):428-31.

25 Hadwan MH. New method for assessment of serum catalase activity. Indian J Sci Technol 2016;9(4):1-5.

26 Paglia DE, ValentineWN.Studies on quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med1967;70:158-69.

27 Weydert CJ, Cullen JJ. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat Protoc 2010;5(1):51-66.

28 Brandt RB, Laux JE, Spainhour SE, Kline ES. Lactate dehydrogenase in rat mitochondria. Arch Biochem Biophys 1987;259(2):412-22.

29 Sigmon J, Larcom LL. The effect of ethidium bromide on mobility of DNA fragments in agarose gel electrophoresis. Electrophoresis 1996;17(10):1524-7.

30 Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res1988;175(1):184-91.

31 Talalay P. [69] Hydroxysteroid dehydrogenases: Hydroxysteroid+ DPN+(TPN^+) Ketosteroid DPNH (TPNH)+ H^+ [chapter 69]. Methods Enzymol 1962;5:512-26.

32 Jarabak J, Adams JA, Williams-Ashman HG, Talalay P. Purification of a 17β-hydroxysteroid dehydrogenase of human placenta and studies on its transhydrogenase function. J Biol Chem 1962;237(2):345-57.

33 Barbier O, Arreola-Mendoza L, Del Razo LM. Molecular mechanisms of fluoride toxicity. Chem Biol Interact 2010;188(2):319-33.

34 Inkielewicz-Stepniak I, Czarnowski W. Oxidative stress parameters in rats exposed to fluoride and caffeine. Food ChemToxicol 2010;48(6):1607-11.

35 Franco R, Cidlowski JA. Apoptosis and glutathione: beyond an antioxidant. Cell Death Differ2009;16(10):1303-14.

36 Agalakova NI, Gusev GP. Molecular mechanisms of cytotoxicity and apoptosis induced by inorganic fluoride. ISRN Cell Biol 2012;2012:1-16.

37 Ozbey U, Deger Y, Yur F, Çambay Z, Ozbeye G. Investigation of blood antioxidant enzyme levels and glutathione peroxidase, catalase, and superoxide dismutase gene polymorphism in sheep with fluorosis. Fluoride 2017;50(3):374-81.

38 Lawson PB, Yu MH. Fluoride inhibition of superoxide dismutase (SOD) from the earthworm Eiseniafetida. Fluoride 2003;36(3):143-51.

39 Yamaguti PM, Simões A, Ganzerla E, Souza DN, Nogueira FN, Nicolau J. Effects of single exposure of sodium fluoride on lipid peroxidation and antioxidant enzymes in salivary glands of rats. Oxid Med Cell Longev 2013;2013:1-7.

40 Halliwell BA, Gutteridge JM, Cross CE. Free radicals, antioxidants, and human disease: where are we now? J Lab Clin Med1992;119(6):598-620.

41 Stubbe J, Nocera DG, Yee CS, Chang MC. Radical initiation in the class I ribonucleotide reductase: long-range proton-coupled electron transfer? Chem Rev 2003;103(6):2167-202.

42 Zhan XA, Wang M, Xu ZR, Li JX, Li JX. Toxic effects of fluoride on kidney function and histological structure in young pigs. Fluoride 2006;39(1):22-6.

43 Drent M, Cobben NA, Henderson RF, Wouters EF, van Dieijen-Visser M. Usefulness of lactate dehydrogenase and its isoenzymes as indicators of lung damage or inflammation. Eur Respir J 1996;9(8):1736-42.

44 Li Y, Heerema NA, Dunipace AJ, Stookey GK. Genotoxic effects of fluoride evaluated by sister-chromatid exchange. Mutat Res 1987;192(3):191-201.

45 Wang AG, Xia T, Chu QL, Zhang M, Liu F, Chen XM, et al. Effects of fluoride on lipid peroxidation, DNA damage and apoptosis in human embryo hepatocytes. Biomed Environ Sci 2004;17(2):217-22.

46 Aardema MJ, Tsutsui T. Sodium fluoride-induced chromosome aberrations in different cell cycle stages. Mutat Res 1995;331(1):171-2.

47 Reiter EO, Kulin HE, Hamwood SM. Preparation of urine containing small amounts of FSH and LH for radioimmunoassay: comparison of the kaolin-acetone and acetone extraction techniques. J Clin Endocrinol Metab1973;36(4):661-5.

48 Edman CD. The effects of steroids on the endometrium. Seminars in Reproductive Endocrinol 1983;1(03):179-87.

49 Mindnich R, Möller G, Adamski J. The role of 17 beta-hydroxysteroid dehydrogenases. Mol Cell Endocrinol 2004;218(1-2):7-20.

50 Rao K, Susheela AK. Effect of sodium fluoride on adrenal gland of rabbit. I. Studies on ascorbic acid and delta 5-3 beta hydroxysteroid dehydrogenase activity. Fluoride 1979;12(2):65-71.

51 Dhurvey V, Patil V, Thakare M. Effect of sodium fluoride on the structure and function of the thyroid and ovary in albino rats (*Rattus norvegicus*). Fluoride 2017;50(2):235-46.

52 Al-Hiyasat AS, Elbetieha AM, Darmani H, Irbid J. Reproductive toxic effects of ingestion of sodium fluoride in female rats. Fluoride 2000;33(2):79-84.