THE EFFECT OF SODIUM FLUORIDE INTOXICATION ON THE ESTROUS CYCLE AND OVARIAN HORMONES IN RATS

Varsha Dhurvey,[a,*] Mangala Thakare[a]

Nagpur, India

ABSTRACT: The objective of this study was to investigate the toxic effects of different concentrations of sodium fluoride (0 [control], 5, 10, 15, and 20 mg NaF/kg body weight/day) administered in drinking water for 30 days, on estrous cycle and ovarian hormones of adult female albino rats (*Rattus norvegicus*). The rats in the two higher dose groups (15 and 20 mg NaF/kg body weight/day) showed clinical signs of toxicity unlike those exposed to NaF at a concentration of 5 mg NaF/kg body weight/day. Body weight (bw) was significantly reduced in the rats ingesting 10 ($p \leq 0.05$), 15 ($p \leq 0.001$), and 20 ($p \leq 0.001$) mg NaF/kg bw/day and ovarian weight was significantly reduced in the rats ingesting 15 ($p \leq 0.05$) and 20 ($p \leq 0.001$) mg NaF/kg bw/day. Vaginal secretions from the 30 female rats were collected every morning for a month and the changes in the estrous stages observed. The duration of the proestrous phase was significantly increased in the 10 mg ($p \leq 0.05$), 15 ($p \leq 0.001$), and 20 mg ($p \leq 0.001$) NaF/kg bw/day groups. In the 15 and 20 mg NaF/kg bw/day groups there were significant decreases in the diestrous ($p \leq 0.001$), estrous ($p \leq 0.05$), and metaestrous ($p \leq 0.05$) phases. The marked alteration in the estrous cycle was caused by decreased hormonal concentrations of luteinising hormone (LH) ($p \leq 0.05$ in the 15 and 20 NaF/kg bw/day groups), follicle-stimulating hormone (FSH) ($p \leq 0.001$ in the 10, 15, and 20 NaF/kg bw/day groups), and estrogen ($p \leq 0.05$ in the 10 NaF/kg bw/day group; $p \leq 0.001$ in the 15 and 20 NaF/kg bw/day groups). These hormones are responsible for ovulation. The results indicate that exposure of female albino rats to NaF in drinking water might have some immediate harmful effects on the reproductive system.

Keywords: Body weight; Estrous cycle; Follicle-stimulating hormone (FSH); Luteinising hormone (LH); Ovarian hormones; Ovarian weight; Rats (*Rattus norvegicus*).

INTRODUCTION

Prolonged exposure to hydrogen fluoride and the fluoride ion (F) through water, air, and soil results in its accumulation in the body, predominantly in the teeth and bones with dental mottling (dental fluorosis) and bone deformities (skeletal fluorosis) in both man[1-3] and domestic animals.[4-8] Besides these osteal abnormalities, nonskeletal changes due to chronic exposure to F have also been observed including gastrointestinal disturbances, neurological disorders, reproductive dysfunctions, apoptosis, excitotoxicity, genotoxicosis, and teratogenic effects.[9] An epidemiological study to assess whether F could affect human birth rates using a US database of drinking water systems showed an association between decreasing total fertility rate and increasing F levels.[10] These studies have suggested that F toxicity may cause adverse effects in the reproductive system of males living in fluorosis endemic areas.[11] A decreased fertility rate due to sodium fluoride (NaF) toxicity has been found in the female rat (*Rattus norvegicus*).[12] Although there are a number of studies regarding the toxic effects of NaF exposure on the male reproductive system in humans,[13] rats,[14,15]

[a]Department of Zoology, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur - 440033, India; *For correspondence: Dr Varsha Dhurvey, Associate Professor, Department of Zoology, Rashtrasant Tukadoji Maharaj Nagpur University, Nagpur - 440033, India. Email: varshadhurvey@yahoo.com.

and mice,[16] studies on the female reproductive system are very limited. In female rats, estrous cycles are characterized by morphological changes in the ovaries, uterus, and vagina.[17] The rat vagina can be considered to be an indicator of ovarian function and to reflect the activity of the sex hormones.[18] Although estrous cycles are influenced by light, seasons of the year, and life circumstances, they occur without seasonal influence in rats submitted to environmental control under laboratory conditions.[19] Thus, the present study was undertaken to investigate the effect in female albino rats of NaF exposure for 30 days on the estrous cycle, ovarian changes, and serum concentrations of the gonadotropin hormones, follicle-stimulating hormone (FSH), and luteinising hormone (LH).

MATERIALS AND METHODS

Animals: Thirty female albino rats (*Rattus norvegicus*) were selected for the study, weighing about 180–200 g. Each rat were caged separately, and raised in a ventilated animal house of the Department of Zoology, RTM Nagpur University, Nagpur, India, under the controlled temperature of 25±2ºC on a 12 hr light /dark cycle. The animals were acclimatized for 7 days prior to the beginning of the study.

Treatments: The rats were randomly divided into 5 groups with each group consisting of 6 animals. The first group, I, served as a control and was provided with saline/defluoridated water while the remaining animals in groups II, III, IV, and V were treated with doses of NaF (Sigma chemical company, USA) at 5, 10, 15, and 20 mg NaF/kg bw/day, respectively, orally in their drinking water for 30 days (Table 1).

Table 1. Experimental protocol

Groups	Treatments	Duration of exposure (days)	No. of animals	Day of autopsy
I (control)	Saline / defluoridated water	30	6	31st
II	5 mg NaF/kg bw/day	30	6	31st
III	10 mg NaF/kg bw/day	30	6	31st
IV	15 mg NaF/kg bw/day	30	6	31st
V	20 mg NaF/kg bw/day	30	6	31st

The experimental protocol was approved by the Institutional Animal Ethics Committee (Register number 478/01/a CPCSEA) of the RTM Nagpur University, Nagpur, prior to the commencement of the study. At the end of the protocol the animals were sacrificed and the ovaries excised and weighed. Blood samples were collected by cardiac puncture for hormonal assessment.

Assessment of estrous cycle: During the 30 days of treatment, every morning between 9:00 to 10:00 am, vaginal secretions were collected with a plastic pipette filled with 10 µL of normal saline (0.9% NaCl) by inserting the tip into the vagina of the rats. One drop was collected with a clean tip from each rat and placed on a

glass slide. The slides were stained with methylene blue and observed under a light microscope with a 40× objective lens for evidence of diverse cells.

Detection of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estrogen in the serum: The blood samples from the different dose groups were centrifuged at 3,000 rpm for 10 min to obtain the serum. The serum samples were used for further analyses of the estrogen, LH, and FSH levels, by an ELISA Kit according to the manufacturer's recommended instructions.

RESULTS

Evaluation of body and ovarian weight: Compared to the control group, the final body weight of the rats was significantly reduced in the 10 ($p \leq 0.05$), 15 ($p \leq 0.001$), and 20 ($p \leq 0.001$) mg NaF/kg bw/day groups. There was no significant body weight change in the 5 mg NaF/kg bw/day group (Figure 1).

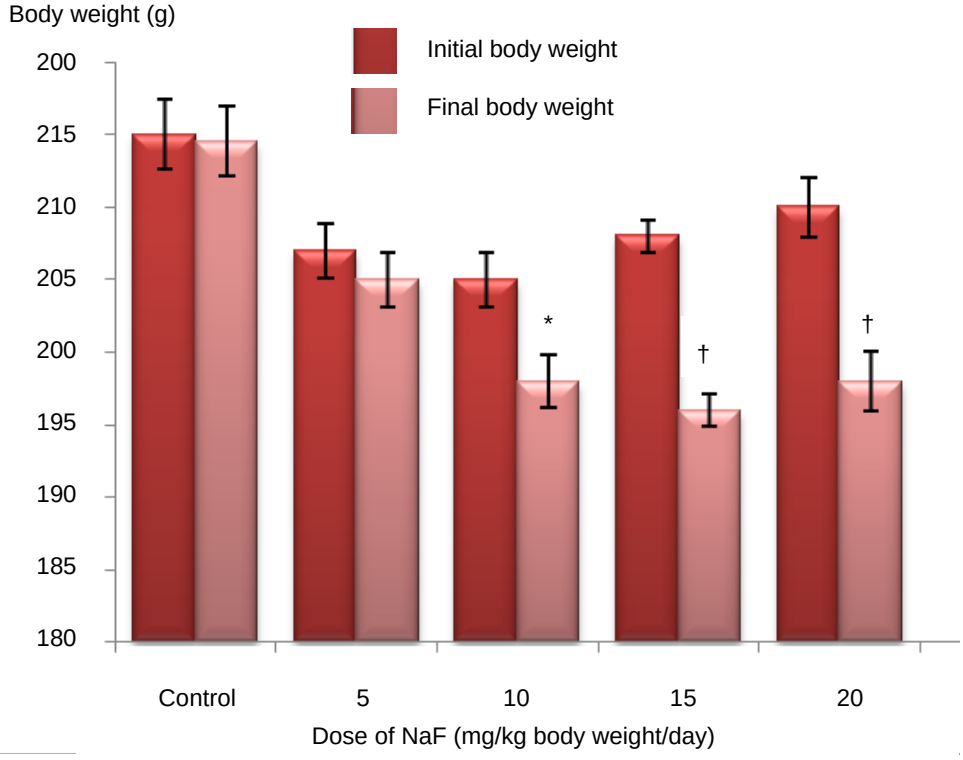

Figure 1. Body weights (g) of the control and NaF-treated rats. Values are expressed as mean±SEM (n=6 for each group). Comparing the initial and final body weights with the control group: *$p \leq 0.05$; †$p \leq 0.001$; where no symbol is shown no significant difference was present.

As shown in Table 2, the right and left ovarian weights from the control and the NaF-treated animals showed no statistically significant difference at 5 and 10 mg NaF/kg bw/day but significant decreases were present in the 15 ($p \leq 0.05$) and the 20 ($p \leq 0.001$) mg NaF/kg bw/day treatment groups as compared to the control group.

Table 2. Ovarian weight (mg/100g body weight) of the treated and control groups

Parameter	Treatment (mg NaF/kg body weight/day)				
	Control	5 mg	10 mg	15 mg	20 mg
Right ovary weight (mg/100g bw)	25.66±0.56	24.16±0.25	23.54±0.54^{ns}	22.66±0.79*	20.83±0.75†
Left ovary weight (mg/100g bw)	25.34±0.21	23.76±0.45	22.54±0.57^{ns}	21.08±0.85*	19.97±0.78†

Values are expressed as mean±SEM (n=6 for each group). Compared with control: *p≤0.05; †p≤0.001; where no symbol or ^{ns} is shown no significant difference was present.

Evaluation of estrous stage in female rats: The cyclic changes observed in the vaginal smear provided a reasonable index of ovarian activity and the hormonal synthesis of estrogen and progesterone. Compared to the control group, the NaF-treated rats showed a trend towards reduced estrous cyclicity which increased as the NaF dose increased (Figure 2). Compared to the control group, the time in the proestrous phase was significantly increased in the 10 (p≤0.05), 15 (p≤0.001), and 20 (p≤0.001) mg NaF/kg bw/day groups. Compared to the control group, the rats in the 15 and 20 mg NaF/kg bw/day groups had significant reductions in the diestrous (p≤0.001), estrous (p≤0.05), and metaestrous (p≤0.05) phases.

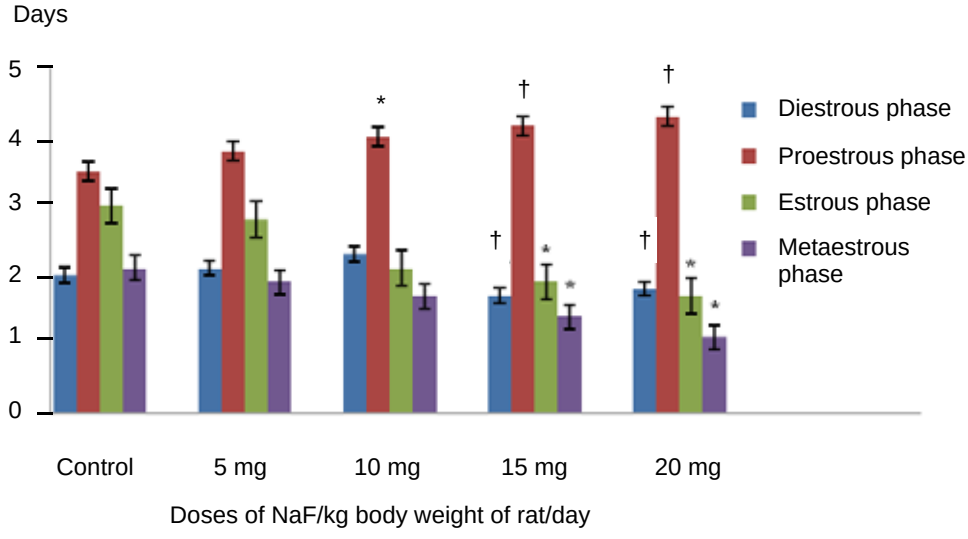

Figure 2. Effect of NaF on the estrous cycle of albino rats. Data represent the mean±SEM (n = 6 for each group). Compared to the control: *p≤0.05; †p≤0.001.

Three types of cells could be recognized in the methylene blue stained smears of the vaginal secretions: (i) epithelial cells which were round and nucleated, (ii) squamous cornified cells which were irregular in shape and without a nucleus, and (iii) leukocyes which were small round cells (Figures 3 a, b, c, and d).

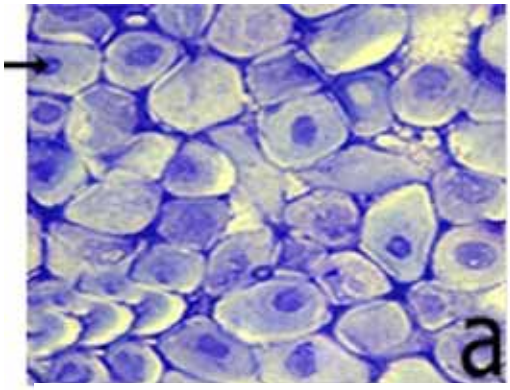

Figure 3a: proestrus

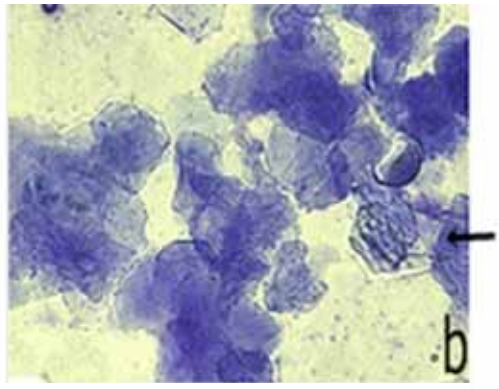

Figure 3b: estrus

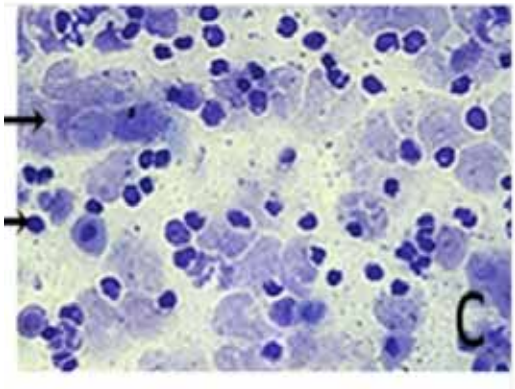

Figure 3c: metestrus

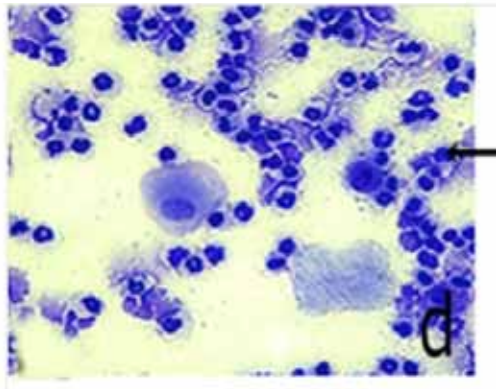

Figure 3d: diestrus

Figures 3a, 3b, 3c, and 3d. Photomicrograph of vaginal smears from female rats at different phases of the estrous cycle in the control group (methylene blue stain, 40×). 3a: proestrus, the arrow points to a nucleated epithelial cell; 3b: estrus, the arrow points to a squamous cornified cell; 3c: metestrus, the upper arrow points to a squamous cornified cell, the lower arrow points to some leucocytes; 3d: diestrus, the arrow points to some leucocytes.

The ratios of the different cell types were used as an index marker for assessing the phases of the estrous cycle. In the NaF-treated rats, in which the alteration in the phases did not follow the sequence of proestrus, estrus, metaestrus, and diestrus (or intermediates), the cycles were considered to be irregular cycles (Figures 3 e, f, g, and h).

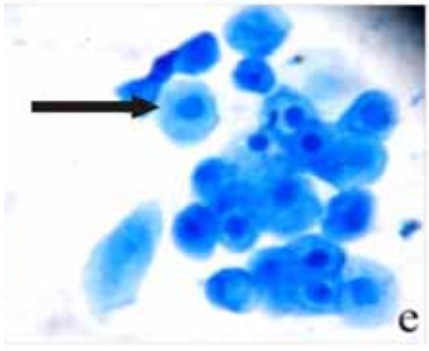

Figure 3e: proestrus

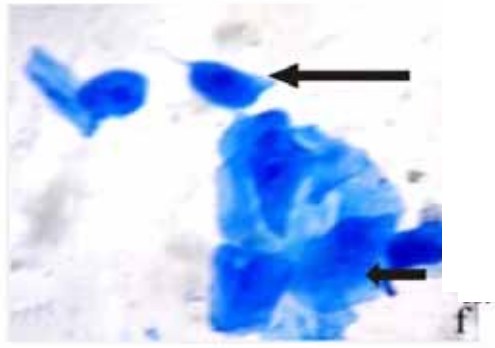

Figure 3f: estrus

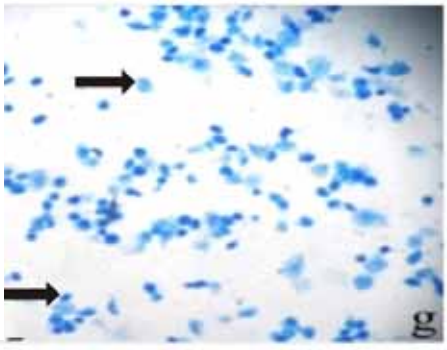

Figure 3g: metaestrus

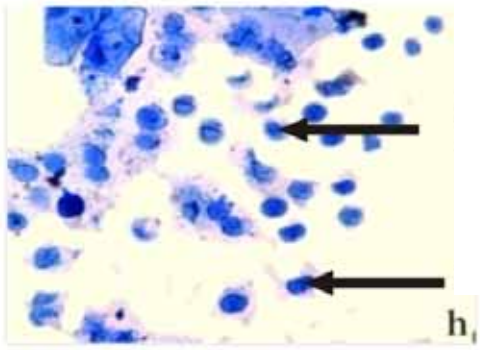

Figure 3h: diestrus

Figures 3e, 3f, 3g, and 3h. Photomicrograph of vaginal smears from female rats at different phases of the estrous cycle in the NaF-treated groups (methylene blue stain, 40×). 3e: proestrus, the arrow points to a nucleated epithelial cell; 3f: estrus, the upper arrow points to a nucleated epithelial cell, the lower arrow points to a squamous cornified cell; 3g: metestrus, the upper arrow points to a nucleated epithelial cell, the lower arrow points to some leucocytes; 3h: diestrus, the upper arrow points to a nucleated epithelial cell, the lower arrow points to some leucocytes.

Estrogen level in the serum of female rats: Compared to the control group, the estrogen levels were significantly lower in the 10 ($p \leq 0.05$), 15 ($p \leq 0.001$), and 20 ($p \leq 0.001$) mg NaF/kg bw/day groups (Figure 4). No significant difference was present in the 5 mg NaF/kg bw/day group compared to the control group.

Concentration of FSH and LH in the serum of female rats: Compared to the control group, the FSH levels were significantly lower ($p \leq 0.001$) in the 10, 15, and 20 mg NaF/kg bw/day groups (Figure 5).

Compared to the control group, the LH levels were significantly lower ($p \leq 0.05$) in the 15 and 20 mg NaF/kg bw/day groups (Figure 6). No significant difference was present in the 5 and 10 mg NaF/kg bw/day groups compared to the control group.

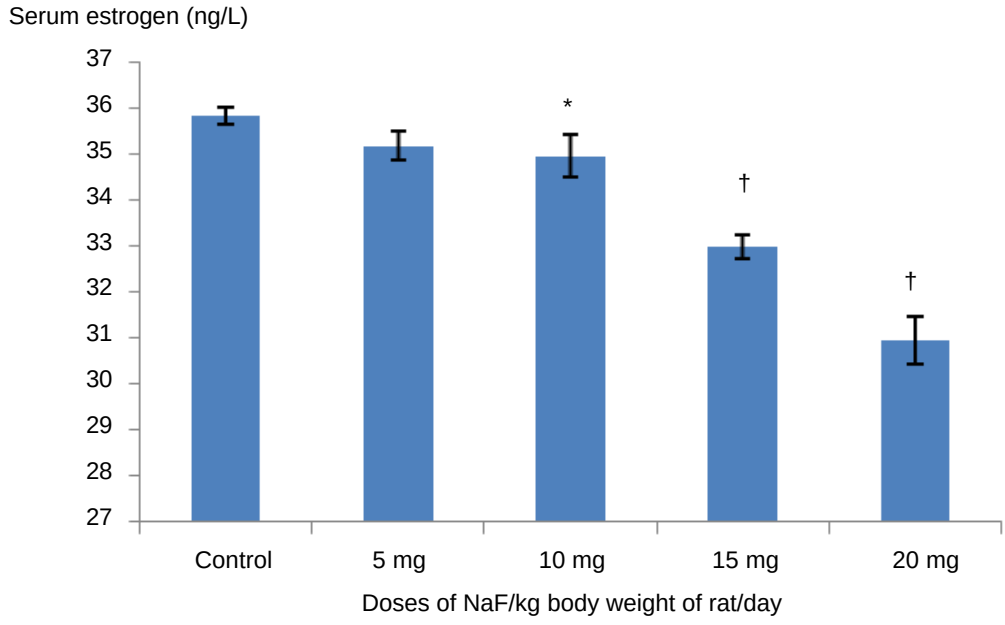

Figure 4. Effect of different doses of NaF on the serum estrogen of female albino rats. Data represent the mean±SEM (n = 6 for each group). Compared to the control: *$p \leq 0.05$; †$p \leq 0.001$.

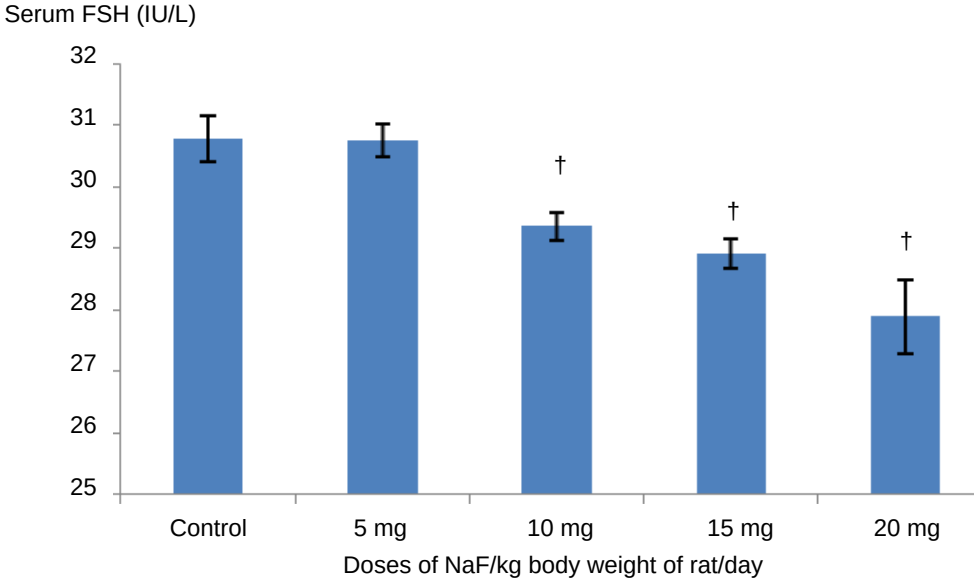

Figure 5. Effect of different doses of NaF on the serum FSH of female albino rats. Data represent the mean±SEM (n = 6 for each group). Compared to the control: †$p \leq 0.001$.

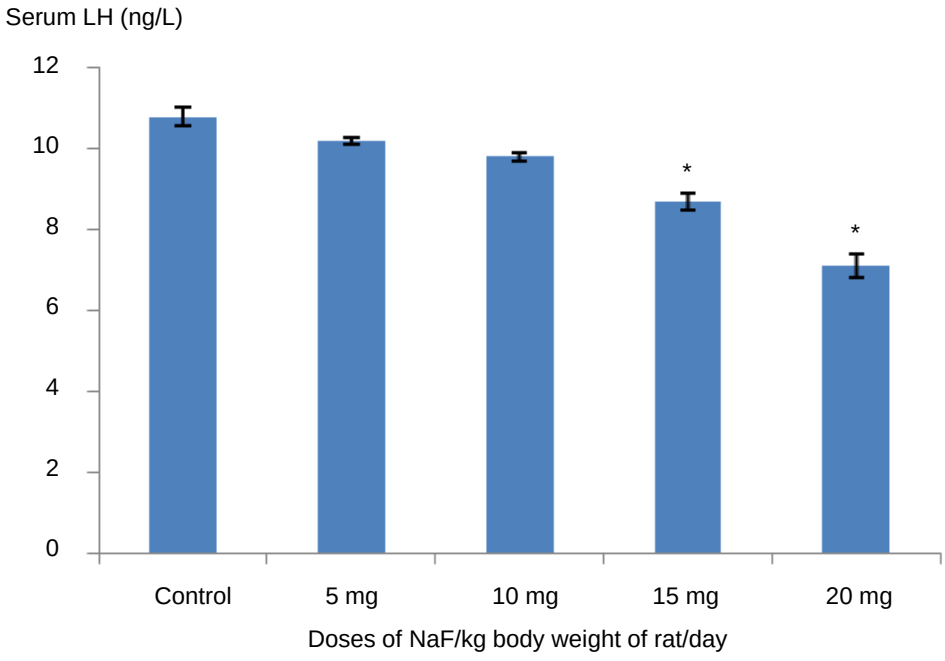

Figure 6. Effect of different doses of NaF on the serum LH of female albino rats. Data represent the mean±SEM (n = 6 for each group). Compared to the control: *p≤0.05.

DISCUSSION

In the present investigation, a significant reduction was found in the body and ovarian weights of the NaF-treated rats as compared to the control group. Similar results have been reported in rats and mice and may be due to an adverse effect of F on metabolism or physiology.[20-23]

We found that the phases of the estrous cycle in the experimental groups showed a pattern of dose dependent changes, with a prolongation of the proestrous phases of the cycle in the 10, 15, and 20 mg NaF/kg bw/day groups, when compared with the control group, which is suggestive of an antifertility effect (Figure 2).[24-26] During the estrous cycle, prolactin, LH, and FSH remain low until increasing in the afternoon of the proestrous phase.[27] The estradiol levels begin to increase at the metestrous phase, reach peak levels during the proestrous phase, and return to the baseline at the estrous phase.[27] Progesterone secretion increases during metestrus and diestrus and then decreases before rising again to reach a second peak towards the end of proestrus.[27,28] The delay in the estrous cycle with NaF treatment is probably due to the NaF inhibiting the ovarian hormonal function, possibly through compromising cellular integrity and function.

The present study also revealed that the ovarian secretion of estrogen was significantly decreased in the groups treated with 10, 15, and 20 mg NaF/kg bw/day. It is accepted that the secretion of the reproductive hormones from the ovary is regulated by the release of LH and FSH from the anterior pituitary gland.[29] We

found that both FSH and LH secretion were significantly reduced in the groups treated with 15 and 20 mg NaF/kg bw/day and that FSH secretion was also significantly reduced in the group treated with 10 mg NaF/kg bw/day. This marked decrease of LH and FSH secretion could explain the blockade of ovulation and the estrous cycle by the NaF treatment. Many investigators have demonstrated that the LH release surges at the proestrous stage are responsible for ovulation.[30-32] The inhibition of this release by NaF could disrupt ovulation by decreasing the number of mature follicles or inducing an estrous cycle disruption at a rest stage. These results may point to one of the causes of the low fertility observed in the NaF-treated groups. More similar studies will help to further evaluate the aetiology of infertility in female animals with prolonged F exposure.

CONCLUSION

NaF may disrupt ovulation and the estrous cycle in rats by reducing LH and FSH secretion. Further similar research will help to further the understanding of the aetiology of infertility in female animals with prolonged F exposure.

ACKNOWLEDGEMENT

The authors wish to thank Dr SL Choubisa, Regional Editor for India for *Fluoride,* for his valuable suggestions.

REFERENCES

1 Choubisa SL, Choubisa DK, Joshi SC, Choubisa L. Fluorosis in some tribal villages of Dungarpur district of Rajasthan, India. Fluoride 1997;30(4):223-8.
2 Choubisa SL. Endemic fluorosis in southern Rajasthan, India. Fluoride 2001;34(1):61-70.
3 Choubisa SL. Fluoride in drinking water and its toxicosis in tribals, Rajasthan, India. Proc Natl Acad Sci India Sect B Biol Sci 2012;82(2):325-30.
4 Choubisa SL. Osteo-dental fluorosis in domestic horses and donkeys in Rajasthan, India. Fluoride 2010;43(1):5-12.
5 Choubisa SL. Fluorosis in dromedary camels in Rajasthan, India. Fluoride 2010;43(3):194-9.
6 Choubisa SL, Modasiya V, Bahura CK, Sheikh Z. Toxicity of fluoride in cattle of the Indian Thar Desert, Rajasthan, India. Fluoride 2012;45(4):371-6.
7 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.
8 Choubisa SL. Bovine calves as ideal bio-indicators for fluoridated drinking water and endemic osteo-dental fluorosis. Environ Moni Asses 2014;186 (7):4493-8.
9 Choubisa SL. Status of fluorosis in animals. Proc Natl Acad Sci India Sect B Biol Sci 2012;82(3):331-9.
10 Freni SC. Exposure to high fluoride concentrations in drinking water is associated with decreased birth rates. J Toxicol Environ Health 1994;42(1):109-12.
11 Susheela AK, Jethanandani P. Circulating testosterone levels in skeletal fluorosis patients. Fundam Appl Toxicol 1996;34:183-9.
12 Al-Hiyasat AS, Elbetieha AM, Darmani H. Reproductive toxic effects of ingestion of sodium fluoride in female rats. Fluoride 2000;33(2):79-84.
13 Chinoy NJ, Narayana MV. *In vitro* fluoride toxicity in human spermatozoa. Reprod Toxicol 1994;8(2):155-9.
14 Wang JL, Zhang YM, Zhang HJ, Zhang K, Zhang ZW, Li J. Toxic effects of fluoride on reproductive ability in male rats: sperm motility, oxidative stress, cell cycle, and testicular apoptosis. Fluoride 2009;42(3):174-8.
15 Spittle B. Fluoride and fertility [editorial]. Fluoride 2008;41(2):98-100.

16 Sun Z, Niu R, Su K, Wang B, Wang J, Zhang J, et al. Effect of sodium fluoride on hyper activation and Ca^{2+} signalling pathway in sperm from mice: an *in vivo* study. Arch Toxicol 2010;84(5):353-61.
17 Goldman JM, Murr AS, Cooper RL. The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. Birth Defects Res B Dev Reprod Toxicol 2007;80(2):84-97.
18 Houssay BA, Cardeza AF, Houssay AB, Pinto RM. Estrogen phenomena and adrenal tumors in ovariectomized rats. Rev Soc Argent Biol 1951;27(7-8):315-23.
19 Suckow MA, Weisbroth SH, Franklin CL, editors. The laboratory rat. 2nd ed. New York: Academic Press; 2006.
20 Vani ML, Reddy KP. Effects of fluoride accumulation on some enzymes of brain and gastrocnemius muscles of mice. Fluoride 2000;33(1):17-26.
21 Chawla SL, Yadav R, Shah D, Rao MV. Protective action of melatonin against fluoride-induced hepatotoxicity in adult female mice. Fluoride 2008;41(1):44-51.
22 Basha PM, Rai P, Begum S. Evaluation of fluoride induced oxidative stress in rat brain: in multi generation study. Biol Trace Elem Res 2011;142(3):623-37.
23 Sharma JD, Solanki M, Solanki D. Sodium fluoride toxicity in reproductive organs of female albino rats. Asian J Exp Sci 2007;21(2):359-64.
24 Marconda FK, Bianchi FJ, Tannon AP. Determination of estrous cycle phases of rats: some helpful considerations. Braz J Biol 2002;62(4A):609-14.
25 Mustapha AR, Bawa EK, Ogwu D, Abdullahi US, Kaikabo AA, Diarra SS. Effects of ethanolic extract of *Rhynchosia sublobata* (Schumach) Meikle on estrous cycle in Wistar rats. Int J Med Arom Plants 2011;1(2):122-7.
26 OluyemiK A, Oyewo OO, Okanlawon AA. Effect of methanolic extract of *Abrus precatorius* linn seeds on the estrous cycle, ovulation and body weight of adult cyclic Sprague-Dawely rats. Int J Endocrinol 2008;4(2):86-90.
27 Spornitz UM, Socin CD, Dravid AA. Estrous stage determination in rats by means of scanning electron microscopic images of uterine surface epithelium. Anat Rec 1999;254:116-26.
28 Smith MS, Freman ME, Neil JD.The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 1975;96(1):219-26.
29 Kumar TR, Wang Y, Lu N, Matzuk MM. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 1997;15(2):201-4.
30 Campbell BK, Scaramuzzi RJ, Webb R. Control of antral follicle development and selection in sheep and cattle. J Reprod Fertil 1995;49:335-50.
31 Hunter MG, Robinson RS, Mann GE, Webb R. Endocrine and paracrine control of follicular development and ovulation rate in farm species. Anim Reprod Sci 2004;82-83:461-77.
32 Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. Physiol Rev 2000;80(1):1-29.