IMPACT OF 12-WEEK INGESTION OF SODIUM FLUORIDE ON AGGRESSION, SEXUAL BEHAVIOR, AND FERTILITY IN ADULT MALE RATS

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SUMMARY: Ingestion of sodium fluoride at 100 and 300 ppm in drinking water for 12 weeks by adult male Sprague-Dawley rats was investigated for effects on territorial aggression, sexual behavior, and fertility. Body weight and absolute and relative testes weights were not affected, but the average weights of epididymis, ventral prostate, seminal vesicles, and preputial glands decreased significantly. A significant decline of spermatogenesis in testes due to a decrease in the number of spermatocytes (primary and secondary) and spermatids in the treatment group is attributed to a significant decrease in testosterone. Sperm motility and density were also significantly decreased in the cauda epididymis and in testes in both NaF-treated groups. In addition, the treatment markedly diminished aggressive and sexual behavioral parameters such as lateralization, boxing bouts, and ventral presenting postures. It also prolonged the time to the first mount, increased the intromission latency, decreased the number of intromissions, prolonged the post-ejaculatory interval, and increased the number of fetal resorptions in female rats impregnated by these males, thereby reducing their fertility.

Keywords: Aggressive behavior; Male fertility; Male rats; Reproduction; Sexual behavior; Sodium fluoride.

INTRODUCTION

In females, exposure to fluoride (F) has been associated with the earlier onset of puberty in both animals[1] and humans.[2] Fluoride is taken up by the pineal gland[1,3] with a consequent reduction in melatonin, which controls the onset of the menarche.[1] Males with fluorosis show a reduction in testosterone and elevation of follicle stimulating hormone and luteinizing hormone.[4] Lower birth rates have also been found in areas with elevated F levels in the drinking water.[5] Various laboratory studies have investigated the effects of NaF on fetal development,[6,7] spermatogenesis,[8-11] and fertility.[12-15] Reproductive toxicity of F in cows and silver foxes has also been reported.[16,17]

On the other hand, the US National Toxicology Program has concluded that the existing studies of reproductive toxicity effects of NaF in laboratory animals and epidemiological studies of F in human are inadequate to determine any potential reproductive hazards.[18]

The 2006 National Research Council (NRC) report indicated that high concentrations of fluoride have a marked adverse effect on reproduction and development. Moreover, the report also suggested that fluoride might decrease reproductive hormones and fertility and increase Down's syndrome in humans.[19]

The present study was conducted to evaluate the impact of elevated levels of NaF in drinking water on aggression and sex behavior in adult male rats. Presumably both aggressive and sexual behaviors are controlled by androgenic

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hormones.[20,21] Furthermore, the effects of NaF were also monitored on the fertility and structure of the reproductive system.

MATERIALS AND METHODS

Sixty adult male Sprague-Dawley rats weighing approximately 250 g were bred in and obtained from the Animal House Unit at Jordan University of Science and Technology, School of Medicine, between June and December 2002. Rats were maintained at a controlled temperature of 21±1°C and under a 12-hr-light:12-hr-dark schedule. Food and water were supplied *ad libitum*.

NaF (Fluka Chemical Corp., Milwaukee, WI) was dissolved in tap drinking water at concentrations of 100 and 300 ppm. Male rats were randomly assigned to control or experimental groups. Experimental male rats were provided access to drinking water containing NaF for 12 weeks. Control male rats were given tap water for the same period of time; NaF in the tap water was 1.2 ppm. Except for 4 unaccountable deaths in the 300-ppm group during the second week, all rats were healthy and continued to receive their respective drinking water throughout the experimental period.

A rectangular observation cage with a plexiglass front (45×27×40 cm: length × breadth × height) was used for aggression assessment. A stud male rat was placed in the testing arena for 10 days. A second male (control or NaF exposed) was then placed in the test arena with the stud male for 5 min, and the following parameters were recorded: lateralizations, boxing bouts, fights with stud male, and ventral presenting postures.[22,23] Animals were observed between 09:00 and 15:00 hr in the rectangular cage, lighted by a 15-W bulb above the arena. All behavioral measures were monitored by a single observer unfamiliar with the F exposed male rat group. Male rats were present with a female of the same strain, brought into estrus by sequential subcutaneous treatment with 5.0 mg of estradiol benzoate (Sigma Chemical Co. St Louis, MO, USA) 54 hr before testing and 0.5 mg of progesterone (Gift from Roussel Uclaf, Paris, France) 6 hr before testing. The hormones were dissolved in corn oil (ALFCO: Arab International Food and Oil Processing Co.) in a total volume of 0.1 mL.

The males were placed in the mating arena for 5 min before the receptive females were introduced. Mating performance of male rats and the number of ejaculations were classified as follows: Number of mounts is the number of mounts without penile intromission. Intromission latency is the time in min from the presentation of the female to the first intromission. Intromissions are the number of mounts with penile intromissions. Ejaculation latency is the time from the first intromission until ejaculation. Post ejaculatory interval (latency period) is the time from the end of ejaculation until the next intromission.

Observations were terminated if no intromission had occurred within 15 min after presentation of the female and if the male had not ejaculated within 30 min after the first intromission and if no intromission had occurred within 15 min after ejaculation following the first intromission.[24-26]

To estimate the fertility in both exposed and control male rats; each male was placed in an individual cage with two virgin untreated females of the same strain. They were left together for ten days during which two estrus cycles should have

elapsed.[27] One week after the removal of the males, the females were killed by cervical dislocation under light ether anesthesia, and the number of pregnant females, implantation sites, viable fetuses, and fetal resorptions were recorded.[22,23]

All males were sacrificed after 12 weeks of exposure and their body weight and weights of paired testes, seminal vesicles (stripped of fluid), and preputial glands were recorded. The reproductive organs of male rats including the testes, epididymides, ventral prostrate, seminal vesicle, and vas deferens were fixed in Bouin's fixative for histological studies.

The sperm motility and sperm counts of cauda epididymis were determined by the method described earlier.[22,23] Quantitative motility (%) was determined by counting both motile and immotile spermatozoa per unit area. Cauda epididymis and testicular sperm counts were performed by routine procedure and expressed as millions/mL of suspension.[23] The Bouin's fixed reproductive organs were processed for paraffin embedding, sectioning (5 μm), and staining (Harris haematoxylin and eosin).

Using Camera Lucida, 100 circular appearing seminiferous tubules were traced at ×80, and the diameter of each tubule was measured separately. The measurement was expressed in terms of mean of all the traced tubules. Similarly, Leydig cell nuclei were traced at ×800. The epithelial cell height of caput and cauda epididymis and seminal vesicle were also traced at ×360.

The spermatogonia, spermatocytes, and spermatids were counted in 5-μm thick cross sections of 10 seminiferous tubules in 10 animals of each group. All raw counts were transformed into 'true' counts by an adaptation of Abercrombie formula[25] from germ cell diameter measurement. Interstitial cell types (such as fibroblasts, immature and mature Leydig cells, and degenerating cells) were counted, applying a differential count over a 200-cell population and statistically verified by the binomial distribution.[26]

Plasma FSH (follicle stimulating hormone) and testosterone concentrations were measured by radioimmunoassay using commercial kits from Cis BIO International, Gif sur Yvette, France.

Data were expressed as mean ± SD. Differences between control and NaF exposed groups were analyzed using either the Chi-square test or Student "t" test.[28]

RESULTS

Table 1 shows the effects of 12-week ingestion of NaF on the parameters of territorial aggression in adult male rats. The exposed groups had significantly fewer lateralization and boxing bouts. Although F seemed to reduce fighting by male rats with the stud male rat, this effect was not significant. However, male rats ingesting NaF displayed a significantly lower number of ventral presenting postures. Moreover, in the control group, shortly after introducing the female rat into the observation arena, extensive exploratory activities conducted by the male rat including sniffing, nose-nose contact, genital exploring, and grooming were observed. These activities were followed by mounting and copulation.

Exploratory activities, although not quantitatively recorded, seemed to be reduced in the exposure group.

Table 1. Effect of 12-week ingestion of NaF on aggressive behavior in adult male rats
(LSM= Lateralization by stud male; BBSM = Boxing bouts with stud male; FSM = Fights with stud male; VP = Ventral presenting; n = number of animals; Results are expressed as mean ±SD)

Group	LSM	BBSM	FSM	VP
Tap water n=20	14.11 ±1.99	5.88 ±1.52	1.94 ±0.63	1.77 ±0.54
NaF 100 ppm n=20	9.95 ±1.49*	4.31 ±1.28*	1.77 ±0.499	1.65 ±0.47
NaF 300 ppm n=16	4.15 ±1.36‡	2.18 ±0.83‡	1.63 ±0.499	1.31 ±0.47*

*p<0.05, †p<0.01, ‡p<0.001 significantly different from control group (Student's "t" test).

Results in Table 2 indicate that the NaF-exposed groups had a significantly prolonged time to the first mount. However, the treatment had no significant effect on the number of mounts compared to the control group, but it affected the initiation of copulatory behavior as indicated by the significantly prolonged intromission latency. On the other hand, the treatment significantly decreased the number of intromissions preceding ejaculation. Moreover, ingestion of NaF had no significant effect on ejaculatory latency compared to the control group, and the treatment also significantly prolonged the post-ejaculatory interval.

Table 2. Effect of l2-week ingestion of NaF on sexual behavior in adult male rats
(TFM = Time to the first mount; NM = Number of mounts; IL = Intromission latency; NI = Number of intromissions; EL = Ejaculatory latency; PEI = Postejaculatory interval; PME = % of male ejaculation; n = number of animals; Results are expressed as mean ±SD)

Group	TFM (min)	NM	IL (min)	NI	EL (min)	PEI (min)	PME
Tap water n=20	1.05 ±0.59	15.94 ±2.75	2.22 ±0.64	8.72 ±1.96	15.21 ±1.57	4.72 ±0.95	80 %
NaF 100 ppm n=20	1.22 ±1.56*	16.12 ±1.98	2.83 ±1.65*	6.77 ±2.36*	15.07 ±0.78	9.33 ±1.33*	48.84 %
NaF 300 ppm n=16	1.84 ±1.34*	16.72 ±1.53	3.78 ±1.12‡	4.22 ±2.54‡	14.88 ±0.78	25.11 ±1.26‡	43.75 %

*p<0.05, †p<0.01, ‡p<0.001 significantly different from control group (Student's "t" test).

Table 3 shows that F exposure significantly reduced fertility as indicated by the smaller number of pregnant females impregnated by the NaF-exposed males.

However, the number of implantations and the number of viable fetuses were not statistically different from those in the control group. The total number of resorptions was also significantly increased in females impregnated by NaF-exposed males. Body weight and absolute and relative testes weights of these males were not affected when compared to those of control group. However, their absolute and relative weights of seminal vesicles and preputial gland were significantly reduced.

Table 3. Effect of 12-week ingestion of NaF on fertility of adult male rats
(n = number of male animals; Results are expressed as mean ±SD)

Group	No. of males	No. of Females	No. of pregnant females	No. of Implantations	No. of viable fetuses	No. of resorptions/ Total no. of implantations
Tap water n=20	20	40	35/40 (87.5%)	8.80 ±2.03	7.84 ±1.83	4/308 (1.3%)
NaF 100 ppm n=20	20	40	27/40* (67.5%)	8.13 ±2.15	7.78 ±1.91	9/220‡ (4.1%)
NaF 300 ppm n=16	16	32	19/32* (59.37%)	7.93 ±1.79	7.65 ±1.91	21/151‡ (13.9%)

*p<0.05, †p<0.01, ‡p<0.001 significantly different from control value (Chi-square test).

Table 4. Effect of 12-week ingestion of sodium fluoride on body and organ weight in adult male rats
(n = number of male animals; Results are expressed as mean ±SD)

Group	Body weight (g)	Testes (g)	Seminal vesicle (g)	Preputial Gland (g)	Water consumption mL/day	Dose of NAF from water (mg/kg/day)	No. of Dead males
Tap water n=20	374.27 ±10.63	3.47 ±0.10	1.22 ±0.08	0.20 ±0.01	39.8 ±1.71	0.17 ±0.06	0
NaF 100 ppm n=20	377.36 ±07.01	3.46 ±0.04	1.15 ±0.03*	0.18 ±0.003‡	34.6 ±1.10	14.6 ±1.08	0
NaF 300 ppm n=16	377.36 ±07.01	3.46 ±0.04	1.03 ±0.06‡	0.18 ±0.005‡	18.6 ±1.31	19.23 ±1.17	4

*p<0.05, †p<0.01, ‡p<0.001 significantly different from control group (Student's "t" test).

Table 5 shows that the motility of sperm in the cauda epididymis was significantly decreased in NaF-treated males as compared to the controls ($p<0.01$). In addition, their sperm density, seminiferous tubule diameter, and Leydig cell nuclear diameter were significantly decreased, as was the epithelial cell height in epididymides (cauda and caput and seminal vesicle).

Administration of NaF caused a significant decrease in the germinal cell population (Table 6): spermatocytes (primary and secondary) and spermatids were decreased to a significantly lower level. Similarly, the numbers of immature and mature Leydig cells were also significantly decreased. The number of degenerating cells, however, was significantly increased.

Table 5. Effect of 12-week ingestion of NaF on hypsometrical parameters and sperm dynamics of adult male rats
(SM = sperm motility; SD = sperm density; STD = seminiferous tubule diameter; LCND = Leydig cell nuclear diameter; TSN = testosterone; FSH= follicle stimulating hormone; Ten rats were included per group; Results are expressed as mean ±SD)

Group	SM %	SD million/mL		STD μm	LCND μm	TSN nmol / L	FSH IU /L
	Cauda	Testes	Cauda				
Tap water	74.1 ±1.94	4.75 ±0.47	56.0 ±1.94	290.6 ±3.2	6.45 ±0.96	14.4 ±2.53	21.87 ±0.47
NaF 100 ppm	68.3 ±1.08†	4.65 ±0.25*	37.185 ±1.44†	282.27 ±2.83†	5.79 ±1.06†	11.94 ±1.88*	18.35 ±0.38†
NaF 300 ppm	63.26 ±1.08†	4.55 ±0.14*	37.18 ±1.08†	266.27 ±2.35†	4.23 ±0.762†	7.06 ±1.88‡	15.83 ±0.38‡

*p<0.05, †p<0.01, ‡ p<0.001, significantly different from control group (Student's "t" test).

For comparison, the Figure shows typical testes of the three groups: control (1), 100 ppm NaF (2), and 300 ppm NaF (3). Marked atrophy is evident in the testes in the F-exposed rats (2 and 3). Plasma levels of FSH and testosterone were also significantly decreased in the NaF-treated groups when compared with the control group (Table 6).

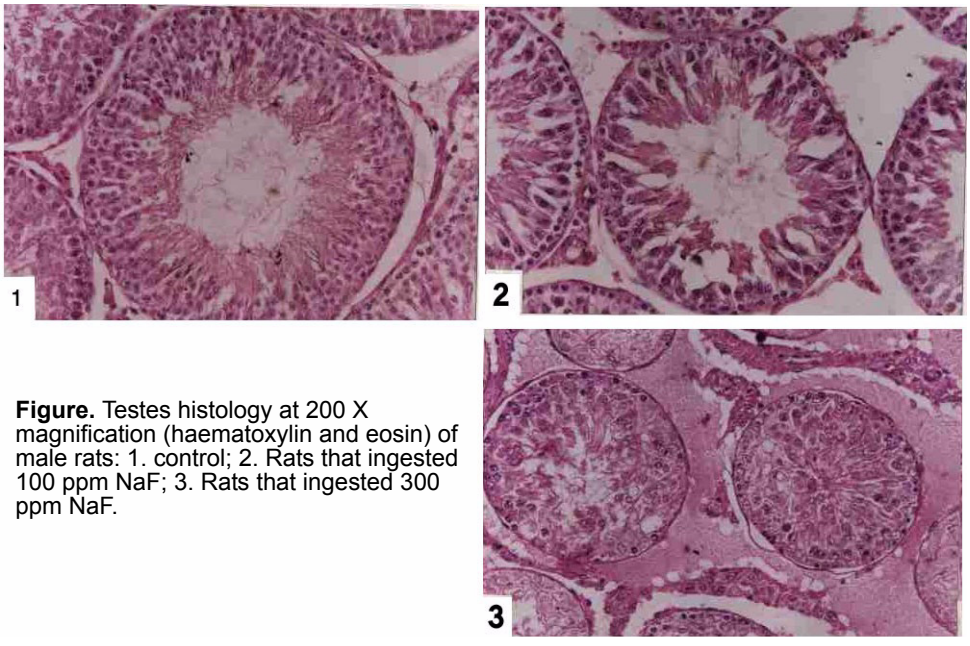

Figure. Testes histology at 200 X magnification (haematoxylin and eosin) of male rats: 1. control; 2. Rats that ingested 100 ppm NaF; 3. Rats that ingested 300 ppm NaF.

Table 6. Effect of 12-week ingestion of NaF on testicular cell population dynamics of adult male rats (SPG= spermatogonia; SPT= spermatocyte; SPD= spermatids; FBT= fibroblast; ILC = immature Leydig cell; MLC = mature Leydig cell; DGC= degenerating cell; Ten rats were included per group; Results are expressed as mean ±SD)

Group	Germinal cell types				Interstitial cell types			
	SPG	SPT		SPD	FBT	ILC	MLC	DGC
		primary	secondary					
Tap water	23.99 ±0.93	18.85 ±0.80	64.126 ±3.51	147.71 ±4.87	63.83 ±1.64	65.195 ±3.47	70.64 ±1.03	18.34 ±1.67
NaF 100 ppm	19.25 ±7.15*	16.35 ±2.36†	36.54 ±3.73†	65.66 ±7.14*	53.36 ±2.33*	41.66 ±1.65†	46.66 ±0.78†	8.9 ±0.76‡
NaF 300 ppm	17.05 ±4.44*	12.96 ±2.41†	17.97 ±3.73‡	9.32 ±6.82‡	38.66 ±1.33†	41.66 ±1.65†	46.66 ±0.78†	8.9 ±0.76‡

*p<0.05, †p<0.01, ‡p<0.001, significantly different from control group (Student's "t" test).

DISCUSSION

In this study, effects of long-term exposure of adult male rat to NaF administered in drinking water on aggression, sex behavior, fertility, and reproductive system were investigated. This animal model has been previously used to assess adverse effects of various metal salts ingestion on behavior and fertility in small laboratory animals without compromising the health of those animals. [22,23,29]

The dosage levels of 100 and 300 ppm of NaF in drinking water were selected because of the reported toxicity potentials of higher doses of this compound, including decreased body weight, water consumption, and clinical signs of toxicity, such as dehydration, lethargy, and hunched posture.[7,18] Nonetheless, 4 animals that received 300 ppm NaF died during the second week of exposure (Table 4), which could be due to the toxicity of the compound. Most animal species require much higher fluoride levels before toxicity effects can be observed. Lower doses of NaF apparently do not impair spermatogenesis and endocrine function of male rats.[9-11]

One of the main findings of the present work is that ingestion of NaF greatly diminished or abolished aggressive behavior postures. The other main finding is that the ingestion of NaF by adult male rats resulted in marked suppression of sexual performance as evidenced by prolongation of the intromission latency, decrease in the number of intromissions, and a marked increase in post-ejaculatory interval. The data suggest that aggressive and sexual behaviors are very susceptible to the toxicity produced by NaF and could be explained by the direct or indirect effect of this compound on the testes and the influence on androgen biosynthesis. Therefore, this might affect these two types of behavior. An agent acting directly on the brain, hypothalamus or anterior pituitary gland will affect the testes and will possibly affect sexual behavior.[27]

The results show that ingestion of NaF reduced the fertility of adult male rats. Fertility impairment has been observed in NaF exposed Swiss male mice and

Holtzman male rats.[14,15] Furthermore, a recent study showed that consumption of NaF in drinking water caused impairment of sperm quality as well as histological changes in the seminiferous epithelium of testicular tissues.[30] Chinoy and coworkers reported that the toxic effect of NaF on fertility in mice could be curbed by an increased protein diet.[31] On the other hand, several workers reported that oral administration of 75 ppm and 250 ppm NaF had no effect on spermatogenesis in mice and rats, respectively.[8-11,32]

The adverse effect of NaF on male rat fertility observed in this work may suggest a disturbance of reproductive endocrine functions along the hypothalamic-pituitary-gonadal axis. Fluoride toxicity is also due to the inhibition of enzymes particularly those in which divalent metal cations act as cofactors.[33] At the molecular level, fluoride affects G-proteins and thus adenylate cyclase system of signal transduction[34] causing inhibition of enzymes involved in cell growth and protein synthesis.[35] This might affect male fertility by lowering the release of hormones and sperm motility.[36,37] The increase in the total number of resorptions seen in female rats impregnated by males exposed to NaF might be attributed to an increase in the incidence of perimplantation mortality of fertilized ova.

The 2006 National Research Council (NRC) report highlighted the adverse effect of high concentrations of fluoride on reproductive and development. The report also noted that fluoride might diminish reproductive hormones and fertility and increase the incidence of Down's syndrome births in younger mothers.[19]

The weight, size, and secretory function of testes, epididymis, seminal vesicles, ventral prostate, and vasa deferentia are closely regulated by androgens.[38,39] The testosterone levels were decreased due to a decline in the number of Leydig cells. The decline in hormone levels influenced the weight of those organs and affected the process of spermatogenesis. The size and activity of preputial gland in rodents are clearly influenced by steroid hormones.[40] Preputial gland produces behavior modulating pheromones that may alter fighting and other behaviors.[41]

These results confirm that long-term NaF ingestion produces adverse effects on aggression, sexual behavior, fertility, and the reproductive system in adult male rats. However the exact mode of action requires further studies. Moreover, these findings emphasize the necessity of investigating other parameters of fertility and etiology to monitor the toxic potentials of various xenobiotics.

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