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Research Article

EFFICACY OF *BOERHAAVIA DIFFUSA* L. ON DISRUPTION OF GONADOTROPINS AND TESTOSTERONE IN FLUORIDE INTOXICATED MALE RATS

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ABSTRACT

Objective: The present study explored the influence of fluoride exposure on gonadotropins and reproductive hormones.

Methods: Male Wistar albino rats weighing 100-150 g were administered with 100, 200, and 300 ppm/kg body weight of sodium fluoride daily for 20 and 40 days. The control group was given 1 ml deionized water/kgbw/day for the same period. At the end of the experimental period, half of the animals were sacrificed and blood serum was collected and testis sample was taken concurrently. Remaining half rats were administrated with two doses (250 mg and 500 mg/kg body weight) of leaf extract of *Boerhaavia diffusa* L. for another 20 days. Serum level of follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and intratesticular level of testosterone were evaluated.

Results: It was found that fluoride intoxication resulted in significant increase in the level of FSH ($p<0.01$) and LH ($p<0.0001$) while significant decrease in the serum ($p<0.01$) and testis ($p<0.0001$) level of testosterone. A significant ($p<0.0001$) positive correlation was found between level of serum fluoride and gonadotropins: FSH (20 days, $r=0.708$ and 40 days, $r=0.795$) and LH (20 days, $r=0.779$ and 40 days, $r=0.891$). However, there was significant ($p<0.0001$) negative correlation existed between serum fluoride and testosterone levels in serum (20 days, $r=-0.780$ and 40 days, $r=-0.862$) as well as testis (20 days, $r=-0.915$ and 40 days, $r=-0.938$).

Conclusion: It was found that administration of *B. diffusa* L. alleviated the adverse effects of fluoride on reproductive endocrine functions.

Keywords: *Boerhaavia diffusa* L., Fluoride, Follicle-stimulating hormone, Luteinizing hormone, Testosterone.

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INTRODUCTION

Epidemiological studies on environmental exposure to fluoride have been associated with male infertility and low birth rates of people living in endemic areas of fluorosis [1].

The adult male reproductive function is considered to be controlled by the hypothalamus-pituitary-testicular axis through a negative feedback mechanism [2]. It played a key role in maintaining the sex hormones secretion. In this system, gonadotropin-releasing hormone (GnRH) from hypothalamus controls the synthesis and the release of pituitary gland hormones, follicle-stimulating hormone (FSH), and luteinizing hormone (LH). Then, they activate FSH and LH receptors in testis regulating the development of testes, steroid biosynthesis, and spermatogenesis [3]. It has been observed in male rats that fluoride could affect hormone levels of each layer of the hypothalamus-hypophysis-testis axis, which then may disturb the reproductive endocrine function [4].

Recently, mounting alertness in finding natural antioxidant phytoconstituents from plants has drawn more concern. Plant materials and products are a rich repository of an array of biologically active compounds such as antioxidants and free radical scavengers. To the best of our knowledge, this is the first study concerning the effect of *Boerhaavia diffusa* L. against fluoride-induced reproductive toxicity.

However, fewer studies have been conducted to explore the relationship between serum fluoride and male sex hormone levels. Hence, the present study has been designed to evaluate the ameliorative role of *B. diffusa* on fluoride-induced alteration in the concentration of gonadotropins and testosterone.

METHODS

Young male Wistar rats weighing between 100 and 150 g were housed in polypropylene cages with stainless steel grill tops and fed with standard rat pellet diet (Hindustan Lever Limited, India) and maintained in an air conditioned animal house facility with controlled temperature (22-25°C), 12 hrs light/dark cycle, and humidity. The water was given *ad libitum*. The experiments were performed under the approval of Institutional Animal Ethics Committee, Punjabi University Patiala (Approval number 107/99/CPCSEA-2012-11).

Chemical and diagnostic kits

Sodium fluoride (NaF) (guaranteed reagent) was purchased from Merck Specialties Pvt., Ltd., India; enzyme-linked immunosorbent assay (ELISA) kits of Testosterone by Diametra, FSH and LH ELISA kits by Weldon Biotech were purchased.

Experimental design

After acclimatization to environment for 1 week, the male rats were divided randomly into 12 equal groups with six rats in each group: Control group (C1) was administered deionized water for 20 and 40 days; the rats in experimental groups received 100, 200, and 300 ppm NaF/kg bw/day, for the same days by oral gavage. Rats treated with NaF for 40 days were post-treated with 250 and 500 mg/kg bw/day of *B. diffusa* extract for next 20 days. Positive controls were administered with 250 (C2) and 500 (C3) mg/kg bw/day of *B. diffusa* leaf extract, respectively. At the end of the experimental period, rats were fasted overnight and sacrificed. Blood samples were collected from lateral tail vein.

Preparation of plant extract

The leaf extract of *B. diffusa* was prepared by the method of Narendhirakannan *et al.* [5].

Hormonal analysis

The serum level of FSH, LH, testosterone, and intratesticular testosterone level in control and fluoride-treated rats was determined using commercial test kits by following methods given in respective datasheet.

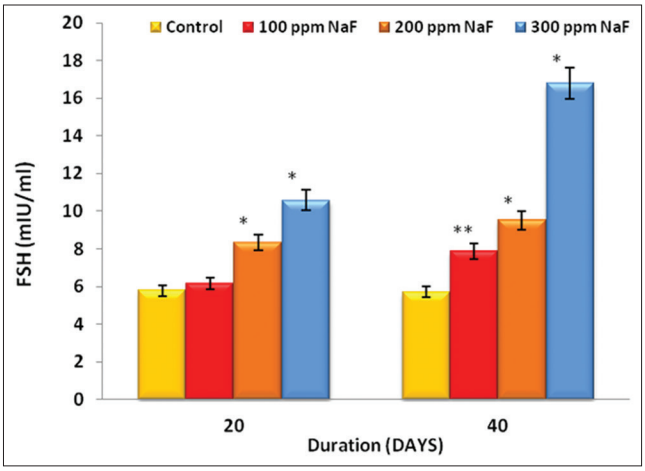

Fig. 1: Serum level of follicle-stimulating hormone in control and sodium fluoride (NaF)-treated rats. Values are expressed as mean±SD. *p<0.0001 and **p<0.01 NaF-treated groups compared with control

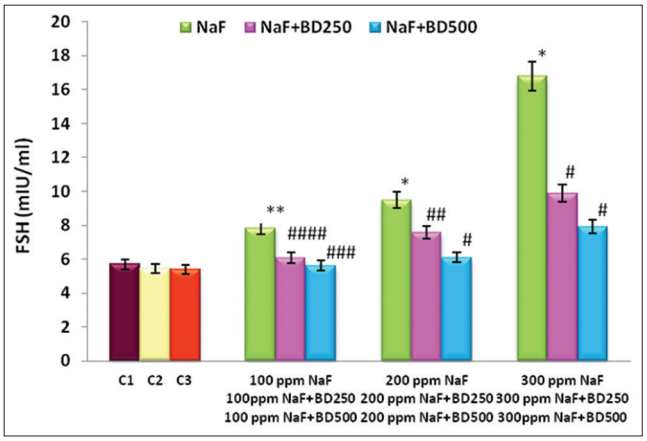

Fig. 2: Serum level of follicle-stimulating hormone in control (C1), positive controls (C2 and C3), sodium fluoride (NaF)-treated, and combination of NaF and leaf extract-treated group. Values are represented as mean±SD. *p<0.0001 and **p<0.01 compared with control (C1). #p<0.0001, ##p<0.001, ###p<0.01, and ####p<0.05 compared to respective NaF-treated group

RESULTS

FSH

The serum level of FSH of test rat showed significant (p<0.0001) increase after 20 (F=44.878) and after 40 days (F=139.127) of fluoride treatment. Maximum percentage increase of 194.09% was registered in 300 ppm NaF group (Fig. 1).

Bonferroni multiple comparison test after ANOVA demonstrated a significant increase in the FSH level between and within groups after 20 (95%, CI=−3.8920 to −0.9015, p<0.001) and 40 (95%, Cl=−10.5994 to −5.5869, p<0.0001) days of fluoride exposure.

Dunnett's t (2-sided) multiple comparison test revealed that serum FSH level was decreased significantly in all fluoridated rats post-treated with 250 (95%, Cl=−3.2976 to −5.7708, p<0.05) and 500 mg/kg bw/day (95%, Cl=−3.7456 - −7.7443, p<0.001) leaf extract of *B. diffusa* (Fig. 2).

Pearson's bivariate correlation analysis showed significant (p<0.0001) positive relationship between serum level of fluoride and FSH in experimental rats after 20 (r=0.708; Fig. 3a) and 40 days (r=0.795; Fig. 3b) of fluoride intoxication.

LH

The serum level of LH of test rat showed a significant (p<0.0001) increase after 20 (F=139.718) and 40 days (F=306.103) of fluoride treatment. More prominent (561.32%) elevation was registered in the highest dose group (Fig. 4).

Bonferroni multiple comparison test after ANOVA depicted a significant (p<0.0001) increase in the LH level between and within groups after 20 (95%, CI=−4.5567 - −2.4161) and 40 days (95%, Cl=−3.8338 to −1.1690) of fluoride exposure.

Dunnett's t (2-sided) multiple comparison test revealed that serum LH level was significantly (p<0.0001) decreased in all fluoridated rats post-treated with 250 (95%, Cl=−2.6262 to −2.2337) and 500 mg/kg bw/day (95%, Cl=−3.3587 to −4.7290) leaf extract of *B. diffusa* (Fig. 5).

Pearson's bivariate correlation analysis showed a significant (p<0.0001) positive relationship between serum level of fluoride and LH in experimental rats after 20 (r=0.779; Fig. 6a) and 40 days (r=0.891; Fig. 6b) of fluoride intoxication.

Testosterone

Serum

The serum level of testosterone in fluorotic rats showed a significant (p<0.0001) decrease after 20 days (F=15.332) and 40 days (F=39.368) as compared to control. More prominent decrease (81.49%) was registered in group treated with 300 ppm fluoride/kg b.w./day after 40 days of fluoride intoxication (Fig. 7).

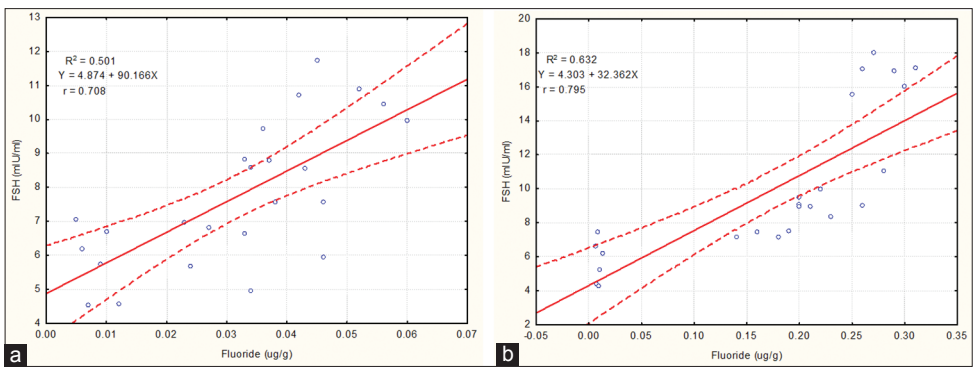

Fig. 3: Correlation and linear regression between serum level of fluoride and follicle-stimulating hormone after (a) 20 days, and (b) after 40 days of fluoride intoxication

Bonferroni multiple comparison test after ANOVA displayed a significant ($p<0.001$) decrease in the serum testosterone between and within groups treated with fluoride for 20 (95%, CI=0.6402 to 3.3608, $p<0.01$) and 40 days (95%, Cl=0.4316 to 3.3501, $p<0.05$) of fluoride treatment.

Dunnett's t (2-sided) multiple comparison test revealed that serum testosterone level was significantly ($p<0.001$) increased in rats post-treated with 250 (95%, CI=0.8305 to 3.5822) and 500 mg/kg bw/day (95%, CI=0.6635 - 5.0547) of plant leaf extract (Fig. 8).

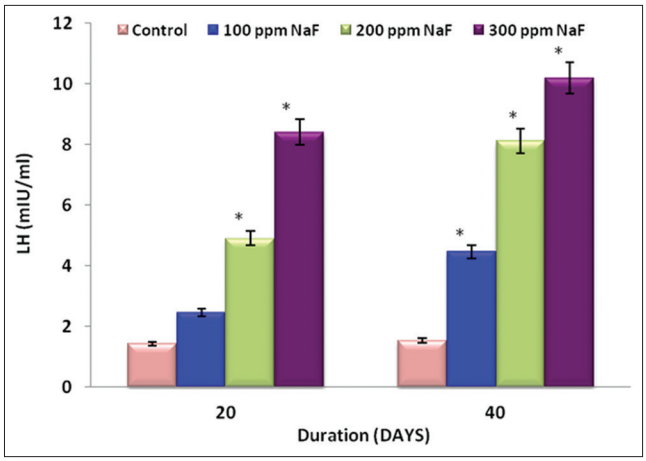

Fig. 4: Serum level of luteinizing hormone in control and sodium fluoride (NaF)-treated rats. Values are expressed as mean±SD. *p<0.0001 NaF-treated groups compared with control

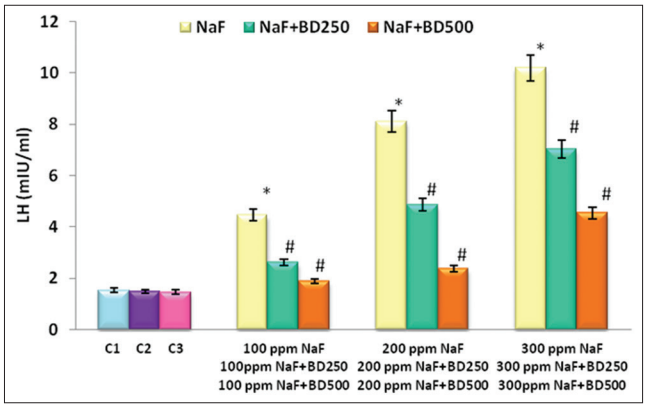

Fig. 5: Serum level of luteinizing hormone in control (C1), positive control (C2 and C3), sodium fluoride (NaF)-treated, and combination of NaF and leaf extract-treated group. Values are represented as mean±SD. *p<0.0001 compared with control (C1). #p<0.0001 compared to respective NaF-treated group

Pearson's bivariate correlation analysis showed significant ($p<0.0001$) negative relationship between serum level of fluoride and testosterone of test rats after 20 ($r=-0.780$; Fig. 9a) and 40 days ($r=-0.862$; Fig. 9b) of fluoride treatment.

Testis

The level of intratesticular testosterone in experimental rats showed a significant ($p<0.0001$) decrease after 20 ($F=128.758$) and 40 days ($F=195.441$) of fluoride treatment. The percentage decline was much higher (74.87%) in group treated with 300 ppm NaF (Fig. 10).

Bonferroni multiple comparison test after ANOVA revealed a significant ($p<0.0001$) decrease in the intratesticular testosterone level after 20 (95%, CI=2.8300 - 4.6849) and 40 days (95%, Cl=5.7126-5.6302) of fluoride exposure between and within all groups studied.

Dunnett's t (2-sided) multiple comparison test illustrated a significant increase in intratesticular testosterone level in all fluoridated rats post-treated with 250 (95%, Cl=1.3256 - 6.1637, $p<0.01$) and 500 mg/kgbw/day (95%, Cl=3.7171 - 8.7114, $p<0.0001$) of plant leaf extract (Fig. 11).

Pearson's bivariate correlation analysis showed significant ($p<0.0001$) negative relationship between serum fluoride and intratesticular testosterone level in test rats after 20 ($r=-0.915$; Fig. 12a) and 40 days ($r=-0.938$; Fig. 12b) of fluoride treatment.

DISCUSSION

The neuroendocrine system of the hypothalamus-pituitary-gonadal axis regulates reproduction in vertebrates and can be influenced by toxins and therefore affects the reproductive system. The activity of the hypothalamic-pituitary- gonadal axis is controlled by GnRH [6]. GnRH is a hypothalamic neuronal secretory decapeptide that plays a pivotal role in mammalian reproduction [7].

During the present study, data revealed elevated levels of FSH and LH, associated with significantly reduced serum testosterone level in rats exposed to fluoride, indicating inhibition of androgen synthesis in fluoridated rats. There was a significant increase in the serum level of FSH ($p<0.01$) and LH ($p<0.0001$) as compared to control group, but the disruption in the level of these hormones was more in rats after 40 days of fluoride intoxication. The serum level of these hormones in the groups that were given *B. diffusa* leaf extract in association with fluoride was demonstrated to be declined significantly for the level of FSH ($p<0.05$) as well as LH ($p<0.0001$). The findings are in agreement with the study of Chen *et al.* [8] who explored the effects of high fluoride exposure on the reproductive function of 31 male adults in a high fluoride area with 26 subjects in normal area serving as a control group and revealed that

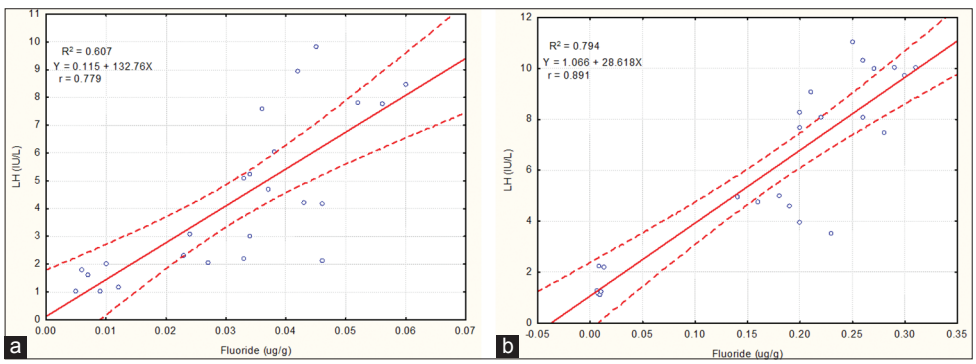

Fig. 6: Correlation and linear regression between serum fluoride level and luteinizing hormone after (a) 20 days, and (b) 40 days of fluoride intoxication

the levels of serum LH and FSH were markedly increased (p<0.001) among the subjects in the high fluoride area and that the serum level of testosterone was significantly decreased (p<0.01) as compared to the control group. Reddy *et al.* [9] observed that exposure of rats to fluoride during early stages of development to a concentration of 4.5 and 9.0 ppm administered in drinking water resulted in elevated levels of FSH and LH and significantly reduced serum testosterone levels in rats exposed to fluoride.

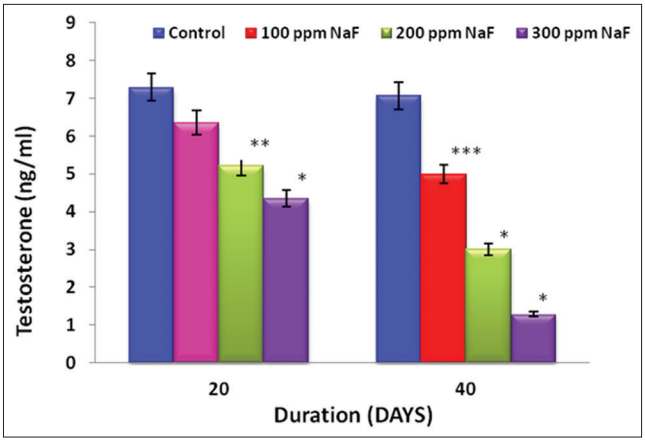

Fig. 7: Serum level of testosterone in control and (sodium fluoride) NaF-treated rats. Values are expressed as mean±SD. *p<0.0001, **p<0.001and ***p<0.01 NaF-treated groups compared with control

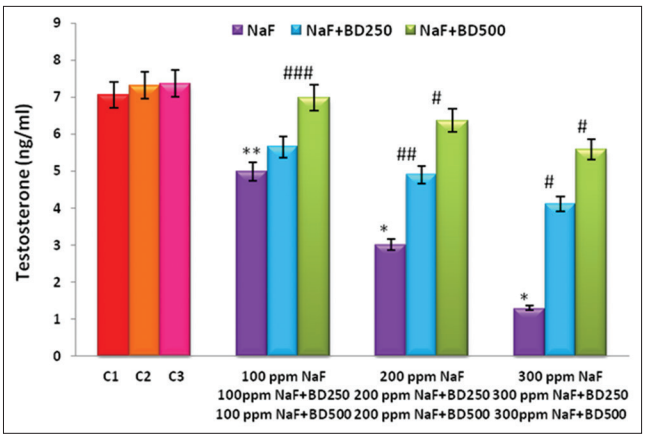

Fig. 8: Level of testosterone in serum of control (C1), positive control (C2 and C3), sodium fluoride (NaF)-treated, and combination of NaF and leaf extract-treated group. Values are represented as mean±SD. *p<0.0001 and **p<0.01 compared with control (C1). #p<0.0001, ##p<0.001, and ###p<0.01 compared to respective NaF-treated group

The FSH and LH are dimeric proteins which are secreted by anterior pituitary gonadotropes. It acts on the gonad in sequential and synergistic manner for the initiation of sexual maturation [12]. The main indicator of dysfunctioning of spermatogenesis is alteration of FSH, LH, and testosterone. For spermatogenesis initiation and spermatozoa maturation, FSH is necessary. It is reported that elevated levels of serum FSH indicate impairment of spermatogenesis and reflect the germ cell loss or damage to Sertoli cell, thereby affecting the feedback regulation of FSH secretion [13].

The testosterone is an important androgen produced in the testis and is involved in the initiation of spermatogenesis [14]. Many studies have reported the male reproductive defects induced by fluoride, such as the deleterious effects of NaF on testosterone level, spermatogenesis, sperm motility and morphology, and fertilizing ability [1,15,16].

In the present study, it was found that there was a significant decrease in serum (p<0.01) and testis (p<0.0001) testosterone level after the treatment of rats with NaF as compared to control. Decrease was more prominent after 40 days of fluoride intoxication as compared to 20 days. With the supplementation of leaf extract, there was a significant (p<0.01) increase in the serum and testicular level of testosterone.

Testosterone synthesis and production is the result of a series of complex biochemical interactions involving the hypothalamus, pituitary, and the testis. In the present study, the serum testosterone levels were decreased significantly (p<0.0001) as the concentration of fluoride was increased. Decreased serum testosterone levels have also been reported in human population exposed to fluoride at doses of 3-27 mg/day compared with a group of individuals exposed to fluoride at low doses of 2-13 mg/day [17]. The decrease in serum testosterone observed in the present study could also be due to fluoride-induced damage of Leydig cells [18].

The present observations are supportive of previous findings in which significant (p<0.05) decline in the serum level of testosterone in men of fluoride polluted areas as compared to control [19]. Dong *et al.* [20] also reported decreased concentration of serum testosterone in guinea pigs. The decline in level of testosterone was 23.67% compared with control group. When male mice were treated with 0, 25, 50, 100 mg/L of NaF in drinking water for 11 weeks, respectively, compared with the control group, Cao *et al.* [21] observed a significant increase in fluoride content and injury to the structure of testes. The testosterone contents

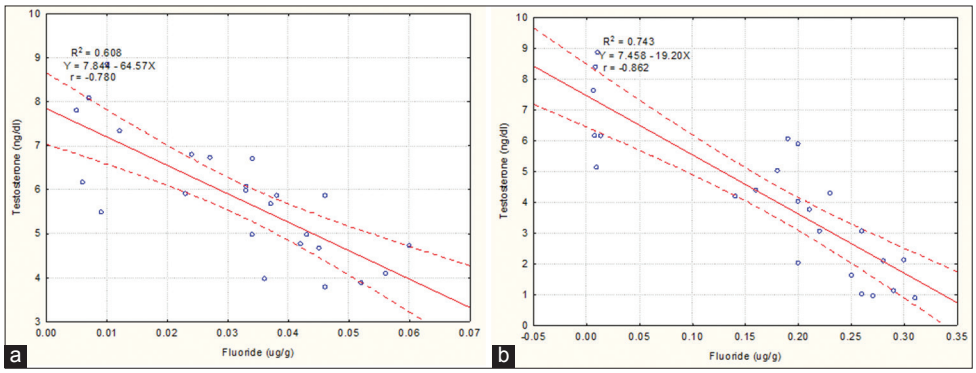

Fig. 9: Correlation and linear regression between level of serum fluoride and testosterone after (a) 20 days, and (b) 40 days of fluoride intoxication

in serum and testis were lower than that of control group. The serum testosterone decreased by 61.22% in 100 mg/L NaF groups, and the testis testosterone was remarkedly reduced by 44.72% and 43.95% in 50 and 100 mg/L NaF groups. A similar decrease in serum and testis testosterone level on fluoride exposure has also been observed by Narayana and Chinoy [22] and Gupta *et al.* [23].

Fluoride interferes with spermatogenesis by modifying important cell signal transducers called G-protein-coupled receptors [24] which are used by the pituitary neurohormone-luteinizing hormone [25]. LH is an important regulator of testosterone production in Leydig cells. Therefore, F-induced modification of G-proteins could inhibit the release of testosterone, and since testosterone is essential for the initiation of spermatogenesis, this inhibition would eventually lead to low levels of testosterone, thereby impairing spermatogenesis [15,26]. It is reported that fluoride is known to accumulate in the pineal gland [27] and to inhibit the release of melatonin by the pineal gland [28]. Since melatonin has an anti-gonadotropic effect, it is conceivable that fluoride inhibition of melatonin indirectly but significantly increases the level of gonadotropins [29]. Under normal circumstances, elevated levels of gonadotropic hormones would result in increased testosterone levels. If there is an inability to increase testosterone due to fluoride interference, elevated gonadotropic hormones may be sustained, without eliciting a compensatory elevation of testosterone [1].

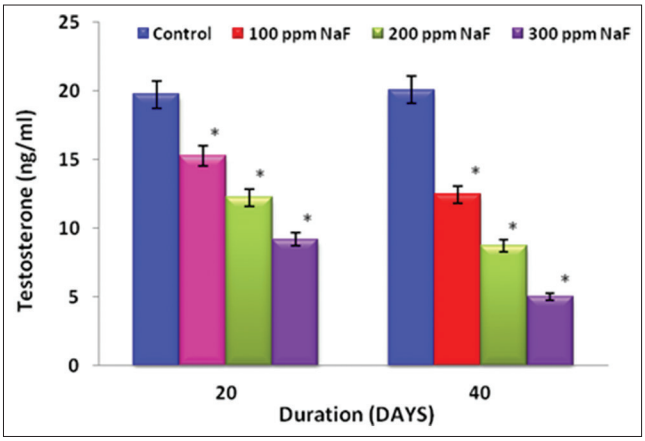

Fig. 10: Level of intratesticular testosterone of control and sodium fluoride (NaF)-treated rats. Values are expressed as mean±SD. *p<0.0001 NaF-treated groups compared with control

The present study demonstrates that excess fluoride exposure can induce endocrine hormone disruption over the hypothalamic-pituitary-testis axis by influencing the regulation of reproductive hormones, hence causing deleterious effects on spermatogenesis and alters sperm and semen quality. Furthermore, the results suggest that the leaf extract of *B. diffusa* is effective in alleviating the toxic effects of NaF on male reproductive hormones.

ACKNOWLEDGMENT

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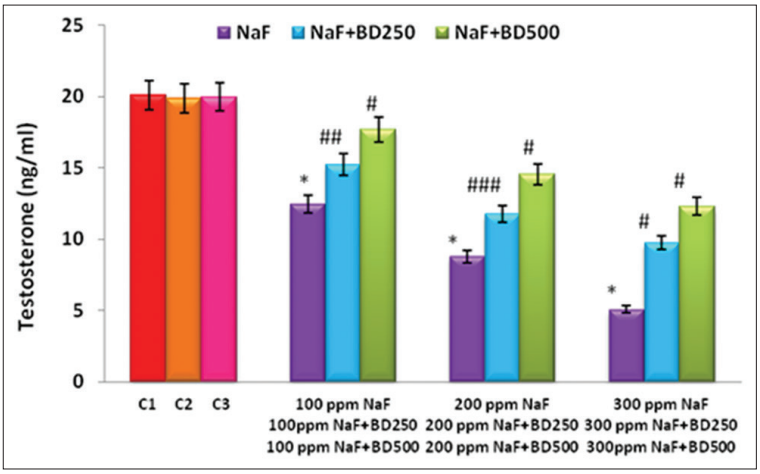

Fig. 11: Level of intratesticular testosterone in control (C1), positive control (C2 and C3), sodium fluoride (NaF)-treated, and combination of NaF and leaf extract-treated group. Values are represented as mean±SD. *p<0.0001 compared with control (C1). #p<0.0001, ##p<0.001, and ###p<0.01 compared to respective NaF-treated group

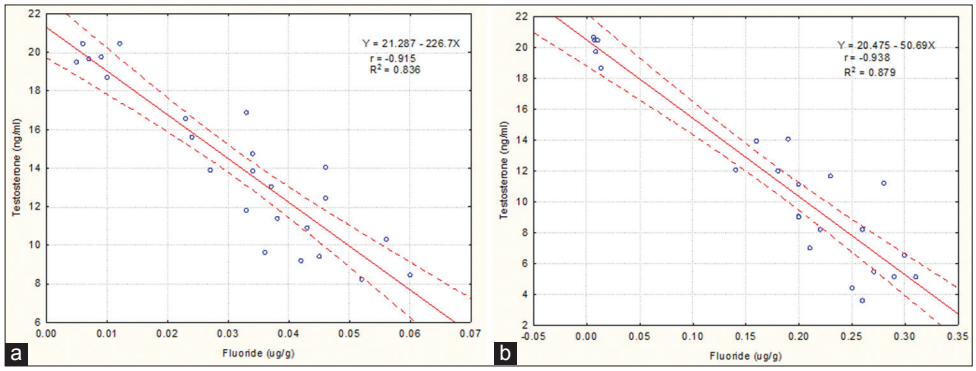

Fig. 12: Correlation and linear regression between level of testicular fluoride and testosterone after (a) 20 days, and (b) 40 days of fluoride intoxication

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