EVALUATION OF SUBACUTE SODIUM FLUORIDE TOXICITY ON SPERMATOZOA AND TESTICULAR TISSUE OF MALE WISTAR RATS

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INTRODUCTION
Global population is increasing now a day as a result of the variations in natural and anthropogenic activities. This is leading to the contamination of various terrestrial and aquatic ecosystems with metals, nonmetals, organic and inorganic compounds. Therefore, a large part of the population in the industrialized world is exposed to variety of pollutants on daily basis, fluorides being one of them (Chouhan and Flora, 2010). Besides immunotoxic, neurotoxic and cytotoxic effects induced by fluoride, the adverse influence of fluoride on the reproductive system has become a major concern in many countries. The importance of reproductive health to offspring developments has prompted epidemiological investigations of the apparent connection between excessive fluoride exposure to male infertility and low birth rates (Sun et al., 2009). Sperm quality is one of the important indices of male reproductive function. Changes in sperm quality induced by fluoride have been demonstrated in vivo and in vitro in many species, including the rat, mouse, rabbit, gerbil, guinea pig, bank vole, chicken and even people (Wan et al., 2006). However, experimental results differ. Some reports indicate that sodium fluoride does not affect sperm quality in rats (Sprando et al., 1997; Collins et al., 2001) whereas other experimental studies suggest that fluoride can cause low sperm quality and diminished fertility (Ghosh et al., 2002; Wan et al., 2006). There are many conflicts regarding importance of fluoride for health and its toxic effects on vital physiological functions of man and animals. Therefore, in view of the aforesaid controversies, the present investigation was undertaken to study the effect of sodium fluoride on seminal characteristics and histoarchitecture of testicles of male Wistar rats.

MATERIALS AND METHODS
Experimental design, animals and test chemical
The experimental investigation was planned to adjudge the toxicopathological effects of sodium fluoride on gametes and the gonads of the male Wistar rats after obtaining approval from Institutional Animal Ethics Committee. The rats were maintained under regular supervision in controlled environment with 12 hours light dark cycle and provided with standard feed and water ad libitum throughout the experimental period. The rats were acclimatized for 10 days before commencement of the experimental study. Twelve healthy male Wistar rats weighing 220 - 235g were randomly divided into four different groups having 3 male rats each. Rats of group I served as control and was given only distilled water, orally. Rats of group II, III and IV were administered NaF orally, dissolved in distilled water, at the dose rate of 5.0, 25.0 and 50.0 mg/kg body weight for 28 days in 1mL of distilled water once daily at morning hours between 7-8 am. The test chemical sodium fluoride of 99% purity was obtained from Merck Limited, Mumbai, India- 400018.
Evaluation of Spermatozoa for Male Reproductive Toxicity

At the end of the experimental period, the rats were humanely sacrificed under diethyl ether anaesthesia and the testicles were withdrawn so as to visualise cauda epididymis.

**Epididymal sperm count**

Epididymal sperm collection and sperm counting were carried out as per the procedure suggested by Khan and Sinha (1996). Firstly, both distal cauda epididymis were collected from rat in petridish and after several stabs on cauda with 18 G needle, it was placed in a pre-labelled and pre-warmed (37°C) test tube containing 4mL normal saline. Then the sperms were allowed to disperse for 5 minutes and sperm counts were made by transferring small amount of the diluted suspension in the Neubauer’s chamber using a Pasteur pipette by touching the edge of the cover slip allowing each chamber to be filled by capillary action. Sperms were observed at high power in five large squares.

**Sperm motility**

Epididymal sperm motility was carried out as per the procedure outlined by Saalu et al. (2010). Briefly, 10 - 20µL of cauda epididymal sperm suspension in normal saline was taken on a prewarmed slide. After applying a cover slip, the sperm motility and progressive sperm motility were recorded in percentage (%) at low power followed by high power objective lens.

**Percentage viability assay/live and dead sperm count**

The microscopic identification of live and dead spermatozoa was carried out following the method suggested by Roberts (1971). A drop of the epididymal sperm suspension was placed near the frosted edge of a clean prewarmed glass slide and mixed gently with small drop of eosin (3%) - nigrosin (10%) stain in 3% sodium citrate dihydrate as buffer. After a few seconds smears were made to study the stained (dead, eosinophilic) and unstained (live) spermatozoa in entire slide over a dark background produced by nigrosin. Two hundred spermatozoa of each rat were counted at high power and live and dead spermatozoa counts were recorded in percentage (%).

**Organosomatic index of testis**

Following humane sacrifice the testes were carefully removed, blotted free of blood and weighed (in grams) over electronic digital balance. The organo-somatic index (OSI) was calculated by using the following formula as per Chattopadhyay et al. (2011).

\[
\text{Organosomatic Index (OSI)} = \frac{\text{Organ weight (g)}}{\text{Live body weight (g)}} \times 100
\]

**Histopathology of testis**

The testicular tissue samples were collected in modified Davidson’s fluid as outlined by Latendresse et al. (2002) in pre-labeled tissue sampling containers for histopathological examination. The tissues were thoroughly washed in running water; dehydrated in ascending grades of alcohol; cleared in benzene and embedded in paraffin at 58°C. The paraffin embedded tissue sections of 4 - 5µm were obtained as described by Luna (1972) and stained with haematoxylin and eosin (H and E) as per the method described by Bancroft and Stevens (1990) with slight modifications. The stained sections were examined under light microscope and the lesions were recorded, if any.

**Statistical analysis**

Statistical analysis was done using complete randomized design (CRD) -single factor analysis of variance by Snedecor.
and Cochran (1968). The mean values between treatment and control groups were tested for critical difference (CD), if any. The results are expressed as Mean ± S.E.

RESULTS

Epididymal sperm evaluation

Intoxication of NaF to Wistar rats of group II, III and IV caused substantial (p ≤ 0.01) decrease in epididymal sperm motility, progressive sperm motility, sperm concentration and live spermatozoa (%) along with simultaneous increase in dead spermatozoa (%) as compared to the rats of control group (group I). The dose dependent increase in percent morphological abnormalities (head, tail and multiple) of spermatozoa were highly significant (p ≤ 0.01) in group III and group IV as compared to rats of group I and group II. Further, the spermatozoa of group II rats showed non significant increase in percent tail and multiple sperm abnormalities but significantly (p ≤ 0.05) increased head abnormality as compared to the rats of control group (group I). Overall, the maximum mean values for various sperm abnormalities (%), dead spermatozoa (%) and lowest mean values for sperm motility, epididymal sperm concentration and live spermatozoa (%) were recorded in group IV rats exposed to the repeated dose subacute oral toxicity of NaF (250mg/kg body weight). The consequences of daily oral administration of NaF on seminal characteristics of male Wistar rats is summarised in Table 1. The spermatozoa vitality and various morphological abnormalities recorded in the present study have been shown in Fig. 1, 2 and 3 respectively.

Organosomatic index of testsis

When compared with the control rats of group I, the OSI of testsis was significantly decreased in (p < 0.05) in group II. The decrease (p < 0.01) was more pronounced in group III and IV. The results have been summarised and presented in Fig. 4.

Pathomorphology of testsis

No appreciable gross lesions were seen in testes of all the experimental groups. However, there were significant pathomorphological changes in histoarchitecture of testicles of intoxicated rats. The microscopic lesions in testsis of group IV rat showed moderate necrotic changes in different layers of seminiferous tubules along with their defoliation. Abnormal seminiferous tubules having increased size of lumen with clumped spermatids were also noticed. Congestion in testicular vessels was observed (Fig. 5 and 6). Similar but, moderate to milder changes were also observed in the histologic sections of group III rats (Fig. 7 and 8). The histological sections of testsis in group II (Fig. 9) and group I rats was apparently normal.

DISCUSSION

Epididymal sperm evaluation

The results of our study are in accordance with Huang et al. (2007) who reported that exposure to high concentrations of NaF (200ppm and 300ppm) in drinking water lead to decrease sperm count, sperm motility, sperm survival and an increased sperm abnormality in male mice. Wan et al. (2006) also reported a decline in sperm viability, sperm density and a significant increase of sperm abnormalities by NaF at 150mg/L in the drinking water when given to male Wistar rats for 50, 80, 100 and 120 days. Akin to our findings, Kour and Singh (1980) also reported that, the testicular spermatogenic process was affected in mice administered with fluoride at a dose of 500 and 1000ppm in drinking water. On the other hand, Li et al. (1987) claimed that fluoride did not have adverse effects on spermatogenesis or sperm morphology. Similarly, Collins et al. (2001) also indicated that fluoride does not adversely affect spermatogenesis or endocrine function at 25, 100, 175 and 250ppm in the drinking water of male rats. However, Cui et al. (2003) found that NaF at the dose rate of 150ppm in drinking water for 10 weeks caused a significant decrease in sperm count and sperm motility of male rats.

Table 1: Effect of daily oral administration of sodium fluoride on seminal characteristics of male Wistar rats (n = 3)

<table>
<thead>
<tr>
<th>Various Sperm Parameters</th>
<th>Experimental Groups</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm Motility (%)</td>
<td></td>
<td>81.0 ± 0.58</td>
<td>76.0 ± 1.15</td>
<td>61.0 ± 0.58</td>
<td>53.0 ± 0.58</td>
</tr>
<tr>
<td>Sperm Progressive motility (%)</td>
<td></td>
<td>67.67 ± 0.33</td>
<td>54.33 ± 0.33</td>
<td>41.67 ± 0.88</td>
<td>36.67 ± 0.33</td>
</tr>
<tr>
<td>Spermatozoa Concentration (millions/ml)</td>
<td></td>
<td>84.33 ± 0.88</td>
<td>75.67 ± 0.88</td>
<td>60.33 ± 0.88</td>
<td>36.0 ± 0.58</td>
</tr>
<tr>
<td>Live Spermatoza (%)</td>
<td></td>
<td>80.0 ± 0.58</td>
<td>77.33 ± 0.33</td>
<td>71.33 ± 0.33</td>
<td>57.33 ± 0.33</td>
</tr>
<tr>
<td>Dead Spermatoza (%)</td>
<td></td>
<td>20.0 ± 0.58</td>
<td>22.67 ± 0.33</td>
<td>28.67 ± 0.33</td>
<td>42.67 ± 0.33</td>
</tr>
<tr>
<td>Sperm Head abnormalities (%)</td>
<td></td>
<td>6.0 ± 0.00</td>
<td>6.67 ± 0.33</td>
<td>10.67 ± 0.33</td>
<td>13.0 ± 0.00</td>
</tr>
<tr>
<td>Sperm Tail abnormalities (%)</td>
<td></td>
<td>6.67 ± 0.88</td>
<td>10.0 ± 0.58</td>
<td>12.00 ± 1.00</td>
<td>12.33 ± 0.33</td>
</tr>
<tr>
<td>Multiple Sperm abnormalities (%)</td>
<td></td>
<td>7.00 ± 0.58</td>
<td>7.33 ± 0.67</td>
<td>11.67 ± 1.45</td>
<td>23.0 ± 0.58</td>
</tr>
</tbody>
</table>

Values indicate Mean ± S.E. Superscripts may read row wise for comparison of means. Mean values with similar superscripts do not differ significantly from each other. *Indicates significant at p ≤ 0.05 and **indicate significant at p ≤ 0.01 from control.
The effect of fluoride toxicity on spermatogenesis might be because fluoride reduces the testosterone levels and by reducing the testicular zinc levels, it impairs angiotensin-converting enzyme activity and hence causes inhibition of spermatogenesis (Ghosh et al., 2002). However, Wan et al. (2006) reported that epidermal growth factor and its receptor, which plays an important role in male reproductive functions of rat, were significantly decreased in Leydig cells, spermatogonia and spermatocytes on exposure to 68ppm fluoride for 10 days.

According to Pati and Bhunya (1987), NaF also induces cytogenetic damage in bone marrow and sperm cells viz. chromosomal aberrations, micronuclei and alterations in sperm morphology respectively. Dominok and Miller (1990) also reported that NaF induces recessive lethal mutations in the germ cells of male fruitfly (Drosophila melanogaster).

Epididymis is the site for maturation of spermatozoa. Spermatozoa acquire motility and fertilizability in epididymis. Therefore, normal internal milieu of the epididymis is necessary for maintenance of structural and functional integrity of sperms. NaF treatment disturbs the homeostasis in the epididymis by hampering sialomucoproteins (essential for maturation of spermatozoa and maintenance of structural integrity of sperm) leading to alteration in sperm motility, viability and morphology along with a resultant decrease in fertilizability of spermatozoa. By and large, epididymis is very prone to NaF induced alteration causing lowered semen quality (Chinoy et al. 1995; Chinoy et al. 1997).

Dose dependent reduction in values of cauda epididymal spermatozoa concentration by NaF might be due to the adverse effect of NaF on epididymis and resulting malformations. The decreased vitality of spermatozoa with an increase occurrence of dead (necrosed) spermatozoa and abnormal spermatozoa in our study might be due to increased lipid peroxidation, mutagenicity and cell membrane toxicity to gonadal cells by fluoride. The increased abnormalities of spermatozoa observed in our study suggest that NaF might lead to genotoxic damage in germ cells. A more severe level of NaF produced higher levels of abnormal sperm, showing a dose dependent response in the present study.

Pathomorphology of testis

The findings on the altered histoarchitecture of testis corroborates well with the earlier reports. Chinoy and Sequeira (1989) reported that 30 days of treatment with NaF (10 mg/kg body weight) to mice resulted in defoliation of the spermatogenic cells in the luminal region of seminiferous tubules of the testis leading to disorganisation of their epithelium. This caused a complete absence of spermatogenesis in the testis. Wan et al. (2006) also found that NaF (150mg/L of drinking water) caused disorganization, denudation and reduction in germinal epithelial cells of the seminiferous tubules and an accompanying absence of sperm in the lumina in histological sections of rats.

Sekhar et al. (2011) found that cypermethrin and NaF - treated mice testes exhibited clumped spermatozoa, vacuolation, severe necrosis and degenerative changes with increased lumen of seminiferous tubules. Beside these degenerative changes in spermatids, atrophied seminiferous tubules, necrotic changes in theca albuginea and scattered spermatids were also reported. Sakr and Azab (2001) reported abnormal seminiferous tubules with many vacuoles, marked reduction in spermatogenic cells and degenerated Leydig cells in albino rats inhaling pyrethroids. In addition, Manna et al. (2004) found edema between seminiferous tubules, vacuolization and hyalinization in the tubules of the testes of rats exposed to a-cypermethrin. In another study Ghosh et al. (2002) reported...
a decrease in luminal sperm and apparent dilation of tubules and opined that oxidative stress might be associated with testicular damage from NaF in rats. The histopathologic findings in the present study, justifies the findings noted during cauda epididymal spermatozoa evaluation. Therefore, it might be concluded that NaF in higher doses acts as a potential reproductive toxicant leading to significant alteration in internal milieu of testicular tissue, altered semen characteristics and various morphological abnormalities in spermatozoa. The cytotoxicity of spermatogonial cells and spermatozoa by fluoride; is an important finding in the present study in view of the increasing endemicity of fluorosis in Indian as well as the global scenario.

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REFERENCES


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