Ultrastructural Studies on the Leydig Cells of Rabbits Exposed to Chronic Fluoride Toxicity

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The present communication addresses the effect of chronic fluoride toxicity on the structure of rabbit Leydig cells using light, scanning and transmission electron microscopy. An ion-specific electrode method was used for the determination of fluoride in sera. The levels of fluoride in the sera of fluoride exposed rabbits were significantly increased ($p<0.001$) and in treated rabbits, transmission electron microscopy revealed dilatation of the smooth endoplasmic reticulum and mitochondrial cristae of the Leydig cells. Leydig cells with lower numbers of lipid droplets and smooth endoplasmic reticulum compared with Leydig cells of unexposed rabbits were seen. Some mitochondria were pyknotic whereas some revealed large electron-dense granules in their matrix. Intranuclear filamentous inclusions were also observed. The degeneration of the cytoplasm as seen using transmission electron microscopy is in agreement with the significant decrease ($p<0.001$) in the diameter of Leydig cell and nucleus as observed using light microscopy and with the degeneration of interstitial tissue of the testis observed using scanning electron microscopy. Thus, the extensive degenerative changes (which are progressive) seen in the Leydig cells due to fluoride toxicity may lead to a decrease in testosterone production resulting initially in regression of seminiferous tubules and structural damage of the epididymis and finally cessation of spermatogenesis.
1. Introduction

The high rates of perinatal deaths and congenital anomalies in dogs affected with skeletal fluorosis due to the incorporation of 460 ppm fluoride in their commercial dog food has been reported by Schellenberg et al.\(^{(1)}\) However, their investigation on experimental animals did not produce convincing evidence that fluoride adversely affected reproduction and the cause of the reproductive problems observed remained unresolved. The data collected by Tao and Suttie\(^{(2)}\) on mice do not support the claims by Messer et al.\(^{(3)}\) regarding the essentiality of fluoride in reproduction. The adverse effects of fluoride on the testes, epididymides and vasa deferentia of rabbits and mice have been reported.\(^{(4-7)}\) Initiation of spermatogenesis in chickens fed on NaF incorporated in their basal diet has been reported to be impaired.\(^{(8)}\) A clear relationship between fluorosis and testis damage was observed in mice administered sodium fluoride in drinking water.\(^{(9)}\) Impaired spermatogenesis in mice given fluoride in feed has also been reported.\(^{(10)}\) Infertility has been described as one of the most common sequelae of fluorosis in cattle.\(^{(11)}\) Impaired reproduction is reported to be a more sensitive indicator of fluoride toxicity in rats than the suppression of growth rates.\(^{(12)}\) In rats given drinking water containing NaF, a decrease in the serum testosterone level and hepatic tissue cholesterol level with no change in testis cholesterol level has been reported.\(^{(13)}\) However, data on the effects of fluoride on the structure and function of Leydig cells are few. Although, in human subjects, an increase in the symptoms of oligospermia and azoospermia in male workers suffering from industrial fluorosis compared with healthy men of the same age has been reported\(^{(14)}\) and an association between fluorosis and hypogonadism has been identified.\(^{(15,16)}\) The residents of Kizilcaoren, an endemic area of fluorosis in Turkey have been reported to suffer from severe depression because of their early impotence (as cited by Yiamouyianis).\(^{(17)}\) A history of impotency in males has been reported in 6 out of 12 cases of fluorosis patients from an endemic region of India.\(^{(18)}\) Prevalence of infertility in 70% of the married males affected with fluorosis in endemic districts in India has also been reported.\(^{(19)}\)

Considering the above reports and paucity of data on the effect of fluoride toxicity on the Leydig cells, the present investigation was carried out in rabbits treated with NaF at a dose of 10 mg/kg body weight for 18 and 23 months. These two periods of treatment were chosen because earlier studies in rabbits using the dose of 10 mg/kg body weight NaF for 18 months have shown no appreciable change in the seminiferous tubules using light and scanning electron microscopy, but transmission electron microscopy revealed several abnormalities in the spermatids and spermatozoa.\(^{(4,20)}\) Furthermore, a decrease in the tubular diameter and epithelial cell height of seminiferous tubules after 23 months and complete cessation of spermatogenesis after 29 months treatment using the same dose was observed in rabbits.\(^{(6,5)}\) The dose of 10 mg NaF/kg body weight is equivalent to 40 ppm fluoride. In India, people in endemic regions have been reported to consume water containing fluoride at concentrations up to 40 ppm.
2. Materials and Methods

Male rabbits (New Zealand white) weighing 1000 to 1400 g were randomly distributed in two groups of 12 animals each and were housed in individual wire cages under constant climatic conditions (12 h light: 12 h darkness and ambient temperature 26 ± 2°C) in the Central Experimental Animal Facility of All India Institute of Medical Sciences. The animals were obtained from the animal supply division of the Central Animal Facility of All India Institute of Medical Sciences, New Delhi. A standard animal diet (Lipton India Ltd.) and drinking water (fluoride content less than 0.5 ppm) were provided ad libitum. All animals were acclimatized for at least one month before the day of dosing. Solutions (2%) of NaF (Analar grade, BDH, Glaxo Laboratory, Bombay, purity 99%) in deionized distilled water and at a dose of 10 mg/kg body weight were administered orally to one group of rabbits for 18 and 23 months and the other group served as a control. The animals were anesthetized with ether and sacrificed after 18 and 23 months treatment along with age-matched controls I and II, respectively. The abdominal cavity was opened and testes were dissected out and washed briefly in normal physiological saline. The organ was cut into pieces and some pieces were fixed in Bouin’s fixative for 24 h for light microscopy and others, after being washed in cold 0.1M phosphate buffer (pH 7.2), were fixed in 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd., Birkshire, England) in the same buffer for six hours at 4°C temperature for electron microscopy. Phosphate buffer was prepared with sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate (BDH, E. Merck (India) Ltd., Worli, Bombay).

2.1 Light microscopy

After fixation, the specimens were washed well, dehydrated using a graded series of alcohol, cleared in xylene and embedded in paraffin wax (E. Merck (India) Ltd., Bombay). Sections were cut at 5 μm thickness and stained with the standard hematoxylin and eosin staining technique. The cytometric measurements of the Leydig cells and nucleus were made at random using an ocular eye piece and micrometer scale. The diameters were measured at two different axes and the mean of the two was taken. A total of 150 Leydig cells and nuclei were counted from rabbits of control I, II and 18- and 23-months treatment groups. Semithin sections of 0.5 μm were cut from araldite-embedded tissue (described under TEM) using an ultracut E (Reichert, Austria) microtome and stained with 1% toluidine blue in 1% borax.

2.2 Transmission electron microscopy (TEM)

The small pieces of tissue were washed in cold 0.1M phosphate buffer after fixation and post-fixed for two hours in 1% osmium tetroxide in the same buffer at 4°C. After several washes in 0.1M phosphate buffer, the specimens were dehydrated using graded acetone solutions and embedded in CY 212 araldite. Ultrathin sectioning of the tissue blocks was carried out using an Ultracut E (Reichert, Austria) microtome. The sections were stained in alcoholic uranyl acetate and lead citrate and viewed under a transmission electron microscope (Philips CM 10) operated at 60 kv.
2.3 Scanning electron microscopy

Post-fixation and dehydration of the specimens were carried out as for TEM. The critical point drying was performed using liquid CO₂ (Bio-Rad apparatus, UK) and sputter coating with gold was carried out under vacuum in an inert atmosphere using argon gas (Balzer SCD 020 sputter device). The specimens were then viewed under a scanning electron microscope (Philips 501B) operated at 15 kv.

2.4 Serum fluoride determination

The ionic fluoride contents of sera from control and treated rabbits were measured in parts per million (ppm) by the method of Hall et al. using a fluoride ion-specific electrode in an ION85 ion analyzer (Radiometer, Copenhagen).

2.5 Statistical calculations

The data recorded were compared using Student’s t-test and a probability, p, of less than 0.05 was considered as a minimum level of significance.

3. Results

3.1 Fluoride concentration in serum

The levels of fluoride in sera of control and treated rabbits are shown in the Table 1. The results indicate that the fluoride contents in the sera of both 18- and 23-months-treated animals were significantly higher ($p<0.001$) than in the sera of the controls.

3.2 Light microscopy and cytometric measurements

The Leydig cell and nuclear diameter were significantly ($p<0.001$) reduced in animals treated with NaF for 23 months compared to controls. In rabbits treated for 18 months, the diameter of the Leydig cell and nucleus was not significantly different from that of controls (Table 1).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control I</th>
<th>18 months treatment</th>
<th>Control II</th>
<th>23 months treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum fluoride (ppm)</td>
<td>0.13 ± 0.04</td>
<td>1.25 ± 0.15</td>
<td>0.118 ± 0.03</td>
<td>0.98 ± 0.20</td>
</tr>
<tr>
<td>Leydig cell diameter (µm)</td>
<td>11.53 ± 2.55</td>
<td>11.65 ± 3.35</td>
<td>12.013 ± 2.47</td>
<td>10.48 ± 2.47</td>
</tr>
<tr>
<td>Nuclear diameter (µm)</td>
<td>6.79 ± 0.98</td>
<td>6.86 ± 0.94</td>
<td>6.95 ± 1.12</td>
<td>6.14 ± 0.94</td>
</tr>
</tbody>
</table>

Table 1
Serum fluoride content and diameter of the Leydig cell and nucleus of the treated rabbits compared with age-matched controls.

a: Data represents mean ± standard deviation for 6 animals.
b: $p<0.001$
Sections 5 μm thick stained with H and E were used for the cytometric measurements because the complete outline of the Leydig cells was clearly seen due to eosinophilic cytoplasm. Leydig cells with round to oval shaped nuclei were seen in large clusters in the interstitial space between the seminiferous tubules of the testes of control rabbits (Fig. 1). In animals treated with NaF for 23 months, Leydig cells were found isolated or in small clusters and lower in number compared to the control rabbits (Fig. 2). The Leydig cells of animals treated for 18 months were similar in number to those of controls.

3.3 Scanning electron microscopy

The cells of interstitial tissues of the testes were conspicuous and in large groups in the control rabbits (Fig. 3). In rabbits treated with NaF for 23 months, the cells of interstitial tissues looked degenerated (Fig. 4) in contrast to those of the controls. The Leydig cells of rabbits treated for 18 months were similar to those of the controls.

Fig. 1 Photomicrograph of the section of testis of control rabbit showing large clusters of Leydig cells (arrowheads) in the vicinity of blood capillaries and between the seminiferous tubules. Semithin section, toluidine blue staining. Scale bar = 30 μm.
3.4 Transmission electron microscopy

The Leydig cells of control rabbits had a characteristic abundance of smooth endoplasmic reticulum and lipid droplets (Figs. 5 and 6). The abundant cytoplasm contained large numbers of mitochondria and a few electron-dense granules. The mitochondria contained small electron-dense granules in the matrix and tubular and vesicular cristae. In rabbits treated with NaF for 23 months, the Leydig cells had a lower number of lipid droplets and smooth endoplasmic reticulum most of which were dilated and there was general degeneration of the cytoplasm (Figs. 7a, b, c and 8) compared with that of the control rabbits. Leydig cells showing dilated smooth endoplasmic reticulum were also found in company with others having smooth endoplasmic reticulum similar to that in the control rabbits. The Leydig cell nuclei contained a small amount of cytoplasm containing electron-dense granules. Some mitochondria had large electron-dense granules in the matrix and some were pyknotic. Cytolysosomes containing disintegrating mitochondrial remnants and other structures were frequently seen in rabbits treated for 23 months (Figs. 7a, b) in comparison to the control animals. Dilatation of the mitochondrial cristae was also
Fig. 3 Scanning electron micrograph of the testis of control rabbit showing a large group of conspicuous cells (arrows) of interstitial tissue. Scale bar = 5 µm.

Fig. 4 Scanning electron micrograph of the testis of rabbit treated with fluoride for 23 months showing degenerated and shrunken cells (arrows) of interstitial tissue. Scale bar = 5 µm.
Fig. 5  Transmission electron micrograph of the testis of control rabbit showing abundant lipid droplets (arrowheads) in the cytoplasm of the Leydig cell in close vicinity of a blood capillary. Arrow indicates a portion of the RBC, l = lumen of the capillary, e = endothelium of the capillary, n = Leydig cell nucleus with normal chromatin pattern. Scale bar = 1 μm.

Fig. 6  Transmission electron micrograph of the Leydig cell of control rabbit showing lipid droplets (arrow), abundant smooth endoplasmic reticulum (arrowhead), large numbers of mitochondria and a few electron-dense granules in the cytoplasm. The nucleus (n) shows a normal chromatin pattern and a nucleolus in the upper left corner. Scale bar = 0.5 μm.
Fig. 7a. Transmission electron micrograph of the Leydig cell of rabbit treated with fluoride for 23 months showing a small amount of cytoplasm surrounding the nucleus (n) due to degeneration. Note that the nucleus has more heterochromatin than euchromatin and a bundle of packed filaments (arrowhead). Arrow indicates a cytolysosome containing remnants of disintegrating mitochondria. Scale bar = 1 µm.

Fig. 7b. Higher magnification of a portion of the same Leydig cell shown in Fig. 7a revealing a lower number and dilatation of smooth endoplasmic reticulum (arrowhead). Note the large electron-dense granule in a mitochondrion (arrow) and absence of lipid droplets. Scale bar = 0.5 µm.
Fig. 7c. Higher magnification of another portion of the same Leydig cell shown in Fig. 7a revealing dilatation of the mitochondrial cristae (arrowhead) and smooth endoplasmic reticulum (arrow). Note the presence of more large sized electron-dense granules in the cytoplasm and absence of lipid droplets. Scale bar = 0.5 μm.

Fig. 8 Transmission electron micrograph of Leydig cell of rabbit treated with fluoride for 23 months showing pyknosis of mitochondria (arrowheads) and degeneration of cytoplasm. A portion of the nucleus (n) reveals the presence of more heterochromatin than euchromatin. Scale bar = 0.25 μm.
observed in rabbits treated for 23 months. The Leydig cell nucleus contained more heterochromatin than euchromatin as compared with those of the control rabbits in which the nucleoplasm contained more euchromatin than heterochromatin (Figs. 5, 6, 7 and 8). Intranuclear inclusions composed of bundles of closely packed filaments were also observed in Leydig cells of rabbits treated for 23 months. These structurally altered Leydig cells were seen amidst normal looking Leydig cells. In rabbits treated with NaF for 18 months, the Leydig cell had abundant smooth endoplasmic reticulum and lipid droplets as in control rabbits, but some ring-shaped mitochondria were frequently observed (Fig. 9). In some ring-shaped mitochondria, electron-dense granules were seen inside the hollow space. A few mitochondria had a more electron-dense matrix than those of controls. However, some mitochondria of normal size with a lucent area inside the organelle, giving it a patchy appearance, were found in both the control and treated rabbits.

4. Discussion

The present study demonstrated that the administration of NaF to rabbits at a dose of 10 mg/kg body weight caused significant elevation in serum fluoride levels indicating its rapid absorption into/retention in the body and extensive damage to the Leydig cells. In rabbits
trreated for 23 months, the significant decrease ($p<0.001$) in the size of the Leydig cells and their presence in small clusters or in isolation indicated the degeneration of the Leydig cells as seen under light and scanning electron microscopy. This finding was further supported by the results of the transmission electron microscopic study showing the marked degeneration of the cytoplasm, dilatation of the smooth endoplasmic reticulum, dilatation of the mitochondrial cristae and mitochondrial pyknosis. The observation of ring-shaped mitochondria and mitochondria with a more electron-dense matrix in rabbits treated for 18 months is suggestive of the initiation of the Leydig cell degeneration. The presence of ring-shaped mitochondria has been reported in the liver after administration of various drugs, such as alcohol.$^{(22-24)}$ The findings of the present study support our earlier observation$^{(5)}$ that fluoride-induced damages are progressive.

There is clear evidence that Leydig cells produce androgenic steroid hormones, principally testosterone, necessary for the germ cells to proliferate and differentiate. It has been reported that high concentrations of testosterone are required for normal spermatogenesis.$^{(25)}$ Ethane dimethane sulfonate (EDS), a specific Leydig cell cytotoxin, causes maximum disruption of spermatogenesis, reduction in the number of germ cells and extensive vacuolation of the seminiferous epithelium in rats,$^{(26)}$ which, after supplementation with testosterone esters in another experiment by Sharpe et al.$^{(27)}$ showed that the degenerative changes in the seminiferous epithelium could be reversed. Thus, the extensive degeneration of Leydig cells seen in the present study may lead to inhibition of steroidogenesis and be responsible for the decrease in the epithelial cell height and diameter of the seminiferous tubules, structural abnormalities in the epididymides in rabbits treated with NaF for 23 months and the complete cessation of spermatogenesis in rabbits treated for 29 months.$^{(4,5)}$ NaF administered orally to the rats at a daily dose of 10 mg/kg body weight for 50 days caused a significant decrease in serum testosterone levels and in Leydig cell and nuclear diameter.$^{(28)}$ In an experiment on male mice ingesting NaF at doses of 10 mg and 20 mg/kg body weight for 30 days, a 40% decrease in the serum testosterone level which was not however significant, was reported with no change in Leydig cell and nuclear diameter.$^{(6,7)}$ The fluoride ion, as the primary toxic contaminant of the commercially produced perfluorochemical emulsion, oxyph erot E.T., has been shown to cause decreased amounts of testosterone secretion from rat testes.$^{(29)}$ Tokar and Savchenko$^{(16)}$ reported decreased serum testosterone content and elevated serum FSH content in male patients suffering from fluorosis: blood LH content increased only in those patients who had been exposed to fluorine for a long period of over 15 years. Investigation carried out in male patients with fluorosis showed a decreased urinary testosterone content and increased excretion of epitestosterone, estrogen, and estriol, with a decreased ratio of urinary testosterone to androstenedione.$^{(30,31)}$ Serum testosterone levels in skeletal fluorosis patients and in males living in fluorosis endemic regions and consuming the same water as patients but not exhibiting symptoms of skeletal fluorosis, were observed to be significantly decreased in comparison to those of normal subjects from non-endemic areas.$^{(32)}$

The Leydig cells synthesize and release the male sex hormone, testosterone, which is necessary for spermatogenesis in the seminiferous tubules and maintenance of functions of the accessory sex glands of the male reproductive tract. In the Leydig cells, cholesterol is either synthesized de novo from acetate or derived from the circulating cholesterol pool.
The smooth endoplasmic reticulum contains the necessary enzymes to synthesize cholesterol from acetate and to transform pregnenolone into androgens. The mitochondria have the necessary enzymatic equipment to cleave the cholesterol side chain and produce pregnenolone. Hence, the lower number of lipid droplets and smooth endoplasmic reticulum and dilatation of the smooth endoplasmic reticulum observed in the Leydig cells of treated rabbits during the present investigation may result in the inhibition of testosterone production, which may be further aggravated by the mitochondrial pyknosis, dilatation of the cristae, and large electron-dense granules also observed in the matrix. Swollen mitochondria, obliterated cristae and the large electron-dense granules in the mitochondrial matrix have also been reported in the mitochondrial sheath of the epididymal spermatozoa of fluoride-treated rabbits. In rabbits exposed to NaF, a reduction in ascorbic acid content and in delta-5-3-beta hydroxysteroid dehydrogenase activity, implying that in fluoride toxicity steroid production is impaired in the adrenal gland, has been reported. In rabbits administered NaF subcutaneously in doses of 5, 10, 20 and 50 mg/kg body weight for 100 days, abnormal quantities of lipids, phospholipids, triglycerides, cholesterol and free fatty acids accumulated in the testes, indicating an imbalance between the synthesis and breakdown of lipids in this organ. Phenylmethylsulfonyl fluoride injected subcutaneously to male rats once a day for 10 days at a dose of 10 mg/day has been reported to cause an increase in the concentrations of free and esterified cholesterol in the testes, a decrease in activities of cholesteryl ester synthesis and hydrolysis but no change in the activity of the cholesterol side-chain cleavage enzyme; however, serum testosterone and LH levels were significantly decreased.

The present observation of heterochromatization of the nuclear chromatin in the Leydig cell due to fluoride toxicity may cause partial or complete suppression of some genetic activity and repression of active transcription and replication of DNA leading to inhibition of protein and DNA synthesis. This is in good agreement with the earlier reports on rabbits in which DNA and protein synthesis have been shown to be markedly inhibited in vitro when the concentration of fluoride was increased from 4 to 5 mM in the culture medium. Fluoride is also reported to inhibit protein synthesis in vivo in various soft tissues including testes in rabbits. A decrease in rRNA content and dry weight of Leydig cells of mice with fluorosis, reflecting the disturbances in protein synthesis and explaining the hormonal imbalance in this disease, have been reported. It has also been shown that fluoride has genotoxic effects which cause DNA and chromosomal damage. However, the significance of the presence of intranuclear filamentous inclusions in Leydig cells of treated rabbits, observed during the present study, could not be ascertained.

Thus, it is evident from the results of the present study that the extensive structural damage revealed, particularly in the smooth endoplasmic reticulum and mitochondria of the Leydig cells, due to chronic fluoride toxicity may cause alterations in the normal enzymatic profile of steroidogenesis leading to a reduction in testosterone production. These changes in the Leydig cells may be initially responsible for defective spermiogenesis in rabbits treated with NaF for 18 months, regression of seminiferous tubules and structural damage in the epididymides in rabbits treated with NaF for 23 months and finally for complete cessation of spermatogenesis as observed in rabbits treated with NaF for 29 months. This is the first detailed ultrastructural investigation of Leydig cells under...
fluoride toxicity and the findings may have a direct bearing on human beings living in areas endemic for fluorosis, among whom the prevalence of infertility and impotence have been reported.

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References


