Effects of chronic fluoride toxicity on the morphology of ductus epididymis and the maturation of spermatozoa of rabbit

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Summary. This study used light and scanning electron microscopy to observe the effect of chronic fluoride toxicity on the structure of the ductus epididymis, testis and spermatozoa in rabbit. The rabbits were treated with 10 mg NaF/kg body weight/day for 20 and 23 months. Serum fluoride was estimated by the fluoride ion-specific electrode method. Fluoride levels in the sera of both 20 and 23-month treated rabbits were significantly increased (P < 0.001). Loss of stereocilia, significant decrease (P < 0.001) in the height of the pseudostratified columnar epithelium and significant increase (P < 0.001) in the diameter of both the caput and cauda ductus epididymis were observed only in the 23-month fluoride treated rabbits. The decreases in the epithelial cell height (P < 0.01) and the tubular diameter (P < 0.001) of the testis were significant only in 23-month treated animals. Spermatozoa in the lumen of the testis of both treated groups of animals and in the caput and cauda ductus epididymis of 20-month treated animals appeared normal, but spermatozoa in the caput and cauda ductus epididymis of 23-month treated animals were fragmented. In the 23-month fluoride treated rabbits, the weights of the caput and cauda epididymis were significantly reduced (P < 0.025) and there was also a reduction in the number of secretory granules in these organs. The structural changes observed in the caput and cauda ductus epididymis might adversely affect the maturation of spermatozoa.

Keywords: fluoride, epididymis, stereocilia, spermatozoa, testis, ultrastructure

Fluorides are known environmental pollutants. A positive correlation between infertility in animals and fluoride toxicity has long been observed (Shulz & Lamb 1925; Udall & Kellers 1952). Infertility has been reported among married males in a highly endemic area for

fluorosis in India (Neelam *et al.* 1987). An increase in the occurrence of oligospermia and azoospermia in male workers suffering from industrial fluorosis has also been noticed (Tarinsky 1972).

There are many conflicting reports on the effects of fluoride toxicity on the testes of different animals. Experiments on chickens given rations supplemented with fluoride (as sodium fluoride) at levels of 150, 300 and 600 p.p.m. (1 p.p.m. = 1 mg/l) showed that as little as 600 p.p.m. fluoride delayed initiation of spermatozoa

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(Mehdi et al. 1983). In mice fed on 125, 250 and 500 p.p.m. fluoride, spermatogenesis has been reported to be impaired (Ridha et al. 1978). Degenerative changes, such as atrophy and necrosis of seminiferous tubules and lack of differentiation and muturation of spermatocytes, have been shown in the testes of mice that received higher doses of 500 and 1000 p.p.m. fluoride, but at 10 p.p.m. there were no changes (Kaur & Singh 1980). Testicular cells of adult rats given oral NaF up to 84 mg/kg body weight did not show DNA strand breaks (Skare et al. 1986). However, reports on the effects of fluoride toxicity on the ductus epididymis are few. A light microscopic study by Chinoy and Sequeira (1989a) has shown adverse effects of fluoride ingestion on the histoarchitecture of the testis and epididymis of adult mice given 10 and 20 mg NaF/kg body weight for 30 days. Changes in the morphology and metabolism of cauda epididymal spermatozoa of rabbits fed on 20 and 40 mg NaF/kg body weight for 30 days have been reported to reduce the fertility rate significantly (Chinoy et al. 1991).

Fluorides are being used as anticariogenic (caries preventing) and anaesthetic agents and fluorosteroids are prescribed as medications for arthritis and allergic diseases, fluorouracil for cancer, sodium fluoride for osteoporosis and otosclerosis, fluoride containing tranquilizers, diuretics and antibiotics, e.g. the fluoroquinolones among which ciprofloxacin is one of the most commonly available and widely used (Susheela 1985; Jowsey et al. 1972; Susheela et al. 1992; Pradhan 1993). The present study was designed in view of the above reports and the paucity of data on the effects of fluoride on male reproductive organs, particularly the ductus epididymis. To evaluate the effects of fluoride toxicity on the testis, ductus epididymis and their spermatozoa, the rabbits were treated with fluoride for 20 and 23 months. The durations of 20 and 23 months were specifically chosen because Susheela and Kumar (1991) have reported no appreciable change in the testis of 18-month fluoride treated rabbits, but they observed cessation of spermatogenesis in rabbits given 10 mg NaF/kg body weight for 29 months; consequently, durations between 18 and 29 months were selected.

Materials and methods

Animals and treatment

Twenty-four male rabbits weighing 1000-1200 g were chosen for the study and were housed individually in

wire cages in the Central Animal Facility of the All India Institute of Medical Sciences. Animals were fed a standard animal diet (Lipton India Ltd) and were supplied with municipal water containing <0.5 p.p.m. fluoride. All animals were acclimatized for at least one month before the day of dosing. The rabbits were randomly assigned to two groups each of 12 animals and kept under identical laboratory conditions. Doses of NaF (Analar grade, BDH, Chemical Division, Glaxo Laboratory (India) Ltd, Bombay, purity 99%) at 10 mg/ kg body weight/day were given to one group of animals orally as a solution of 20 mg/ml in de-ionized distilled water. The other group was pair fed, but given no fluoride. The animals were anaesthetized with ether, 6 were sacrificed after 20 months of treatment and 6 after 23 months. Together with each treated group, 6 control rabbits were sacrificed. Thus, Control I was designated for the 20-month treated group and Control II for the 23month treated group respectively. The caput and cauda epididymis and both testes were carefully removed after opening the abdominal cavity and weighed on a Mettler (AE 163) electronic balance. The organs were washed briefly in normal physiological saline and cut into pieces. Some pieces of tissue were fixed in Bouin's fixative for 24 hours for light microscopy and others, after being washed in 0.1 M phosphate buffer, were fixed in 2.5% glutaraldehyde in the same buffer for 6 hours at 4°C temperature for scanning electron microscopy.

Light microscopy

The specimens were washed well after fixation and dehydrated through a graded series of alcohol, cleared in xylene, embedded in paraffin wax (E. Merck (India) Ltd, Bombay), and sectioned at 5μ m. The sections were stained by the standard haematoxylin and eosin staining technique. The histocytometric measurements were made at random using an ocular and micrometer scale. The shortest and longest diameters of the seminiferous and epididymal tubules were measured and the mean of the two diameters was taken. The basement membrane was included in the measurements. The epithelial height was recorded at the four points of the tubule at which the diameter was measured and the mean of the four was taken.

Scanning electron microscopy

The specimens were washed in $0.1 \,\text{m}$ phosphate buffer after fixation and then post-fixed for 2 hours in 1% osmium tetroxide in the same buffer at 4°C. After a few washes in 0.1 M phosphate buffer, the specimens were dehydrated in graded acetone solutions. Critical point drying was done using liquid CO₂ (Polaron Jumbo apparatus) and gold sputter-coating was carried out under reduced pressure in an inert argon atmosphere (Balzer SCD 020 sputter device). After sputter-coating the tissues were examined under a scanning electron microscope (Philips 501 B) operated at 15 kV.

Estimation of serum fluoride content

Blood samples were collected from the ocular plexus using heparinized capillaries for fluoride estimation. Fluoride in sera from control and treated rabbits was determined by the method of Hall *et al.* (1972) using a fluoride ion-specific electrode in an ION 85 Analyser, Radiometer (Copenhagen).

Statistics

The data recorded were analysed by Student's *t*-test and a probability P < 0.05 was taken as a minimum level of significance.

Results

Fluoride concentration in serum

The levels of fluoride in sera of control and treated animals are shown in Table 1. The results indicate that

the fluoride content in the sera of both 20 and 23-month treated rabbits was significantly higher (P < 0.001) than in controls.

Weights of the organs

The weights of the testes in both treated groups of animals and of the caput and cauda epididymis in 20month treated animals were not significantly different from the controls. But the weights of the caput and cauda epididymis in the 23-month treated rabbits were decreased significantly (P < 0.025) as compared to the control animals.

Testis

Seminiferous tubules of control animals clearly showed different stages of spermatogenesis. Bundles of spermatozoa were seen in the lumen (Figure 1). In 23-month treated rabbits there was significant decrease in epithelial cell height (P < 0.01) and tubular diameter (P < 0.001) in comparison to the controls (Table 1), but the different stages of spermatogenesis appeared normal (Figures 2 and 3). Normal-looking spermatozoa were seen in large numbers in the lumen of the seminiferous tubules. However, a few highly eosinophilic spermatogenic cells were encountered. No appreciable structural or morphometric changes were observed in the seminiferous epithelium or spermatozoa of 20-month treated animals (Table 1).

Table 1. Serum fluoride, organ weights, tubular diameters and epithelial heights in control and treated rabbits

Parameters	Control I	20-Month	Control II	23-Month fluoride treatment
		fluoride treatment		
Serum fluoride (p.p.m.)	0.103 ± 0.023	1.124 ± 0.490*	0.116 ± 0.032	1.388 ± 0.321*
Testis				
Weight (g)	2.171 ± 0.216	2.105 ± 0.307	2.386 ± 0.327	2.228 ± 0.338
Tubular diameter (μm)	221.1 ± 32.33	210.75 ± 31.04	214.56 ± 37.08	186.98 ± 28.74*
Epithelial cell height (μ m)	63.8 ± 11.37	62.3 ± 10.76	60.8 ± 11.59	$54.79 \pm 9.71 \dagger$
Caput epididymis				
Weight (g)	0.476 ± 0.067	0.429 ± 0.037	0.433 ± 0.03	$0.360 \pm 0.05 \pm$
Tubular diameter (μm)	202.05 ± 31.02	211.98 ± 48.59	210.39 ± 44.3	372.18 ± 85.43*
Epithelial cell height (μ m)	$\textbf{33.6} \pm \textbf{4.9}$	33.03 ± 4.36	$\textbf{32.64} \pm \textbf{4.36}$	$25.22 \pm 4.07^{*}$
Cauda epididymis				
Weight (g)	$\textbf{0.308} \pm \textbf{0.02}$	0.296 ± 0.024	0.297 ± 0.02	$0.249 \pm 0.035 \pm$
Tubular diameter (μ m)	407.12 ± 65.52	424.5 ± 68.76	430.27 ± 79.6	489.42 ± 99.3*
Epithelial cell height (μ m)	40.46 ± 10.6	$\textbf{37.94} \pm \textbf{7.94}$	$\textbf{39.06} \pm \textbf{9.10}$	$33.02 \pm 8.02^{\star}$

Data represents mean ± standard deviation of 6 animals.

**P* < 0.001.

†P < 0.01.

‡*P* < 0.025.

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Figure 1. Scanning electron micrograph of seminiferous tubules of control rabbit showing spermatogenic cells and bundles of spermatozoa in the lumen. ×900.



Caput ductus epididymis

In the control animals, the luminal surface of the ductus epididymis showed dense layers of stereocilia amidst which were seen protruding apical surfaces of pseudostratified columnar epithelial cells (Figures 4 and 5). Secretory granules were seen in abundance in the epithelial cells and in the lumen which had masses of healthy spermatozoa with well defined heads and tails. Parts of the tails of some spermatozoa were seen coiled forming ring-like structures. In animals treated with NaF for 23 months, stereocilia on the pseudostratified columnar epithelial cells lining the lumen of the ductus epididymis were less dense or absent in many regions giving it a bald appearance (Figures 6 and 7). The epithelial cells and the lumen of the ductus epididymis had scanty secretory granules. The lumen had spermatozoa some of which were fragmented (Figure 6). The height of the pseudostratified columnar epithelium was significantly decreased (P < 0.001) and the lumen significantly dilated (P < 0.001) in comparison with the control rabbits (Table 1). In some places the epididymal

Figure 2. Photomicrograph of the section of seminiferous tubules of 23-month fluoride treated rabbits showing different stages of spermatogenesis. H&E. $\times 550$.



Figure 3. Bundles of normal-looking spermatozoa in the lumen and spermatogenic cells of seminiferous tubule of 23-month fluoride treated rabbits. × 1800.

tubule was found collapsed to varying degrees. No appreciable change was observed in the ductus epididymis of the 20-month treated group either structurally or morphometrically (Table 1).

Cauda ductus epididymis

The pseudostratified columnar epithelial lining of the cauda ductus epididymis was thrown into many folds (Figures 8 and 9) in comparison to the caput ductus epididymis where such folds were rarely observed. In the control animals, the epithelial cells lining the lumen had dense layers of stereocilia amidst which were seen the protrusion of the apical surface of the cells (Figures 8 and 9). Secretory granules were seen in abundance in the lumina containing masses of healthy spermatozoa with well defined heads and tails. The coiling of a part of the tail of spermatozoa, seen in the caput ductus epididymis, was rarely seen in the cauda region. The 23-month treated rabbits displayed

less dense or an absence of stereocilia at many points of the pseudostratified epithelial cells lining the lumen of the ductus epididymis, giving it a bald appearance (Figure 10). The cell boundaries were not clear in some regions imparting a smooth appearance to the luminal surface. The lumen had fewer spermatozoa, some of which were fragmented (Figure 10) as seen in the caput ductus epididymis of 23-month treated rabbits (Figure 6). There was a reduction in the number of secretory granules. The height of the pseudostratified columnar epithelium was reduced significantly (P < 0.001) and the tubular diameter was significantly increased (P < 0.001) (Table 1). At some places the epididymal tubule was found collapsed to varying degrees. There was no appreciable change in the morphology of the cauda ductus epididymis of the 20month treated rabbits. The height of the pseudostratified columnar epithelium and tubular diameter were not significantly changed compared with control rabbits (Table 1).



Figure 4. Luminal surface of the caput ductus epididymis of control rabbits showing abundant stereocilia amidst which are some bulging epithelial cells (arrow-heads). Spermatozoa are seen in the lumen. Note the secretory granules (small arrows) and coiled flagellum of the spermatozoa (large arrow). ×3600.



Figure 5. Photomicrograph of a section of caput ductus epididymis of control rabbit showing pseudostratified columnar epithelium with stereocilia (arrow-heads) and spermatozoa in the lumen. Note the secretory granules in the epithelial cell cytoplasm (arrows). H&E. ×500.



Figure 6. Luminal surface of the caput ductus epididymis of 23-month fluoride treated rabbit showing total loss of stereocilia and denuded epithelial cells. Note the fragmented spermatozoa (arrows). ×3600.



Figure 7. Photomicrograph of a section of caput ductus epididymis of 23-month fluoride treated rabbit showing loss of stereocilia (small arrows) and flattening of the tubule. Detachment of the pseudostratified columnar epithelium (arrow-head) from the basement membrane may be an artefact. H&E. ×390.



Figure 8. Scanning electron micrograph of the luminal surface of the cauda ductus epididymis of control rabbit showing tortuous folds of the epithelium having a dense layer of stereocilia and numerous spermatozoa. Arrows indicate scretory granules. ×925.



Figure 9. Photomicrograph of a section of cauda ductus epididymis of control rabbit showing pseudostratified columnar epithelium with stereocilia (arrow-heads) thrown into many folds and spermatozoa in the lumen. H&E. ×360.



Figure 10. Luminal surface of the cauda ductus epididymis of 23-month fluoride treated rabbit showing absence of stereocilia at one side (stars) and a spermatozoon with fragmented tail (arrow). Note that the cell outline is not clear giving a smooth appearance to the luminal surface (stars). ×7200.

Discussion

Highly significant increases in the serum fluoride levels of both treated groups as compared to the controls indicate that fluoride is readily absorbed into the body. The present studies revealed that oral administration of NaF to rabbits at a dose of 10 mg/kg body weight daily for 23 months resulted in significant decrease in epithelial cell height, significant dilatation of the tubule and loss of stereocilia and secretory granules in both the caput and cauda ductus epididymis. These changes, it is proposed, adversely affected the structure and maturation of spermatozoa passing through the ducts. The weights of the caput and cauda epididymis were also significantly reduced. These observations are in compliance with earlier reports that the normal structure and function of the mammalian epididymis provides the necessary favourable environment for maturation, viability and motility of spermatozoa (Bedford 1975; Orgebin-Crist et al. 1975; Prasad & Rajalakshmi 1976; Courot 1981; Eddy 1988). The coiling of a part of the tail of the spermatozoa seen in the caput

ductus epididymis during the present studies may be one of the physiological processes of sperm maturation. The essential role of epididymal fibronectin in human sperm maturation has been reported (Miranda & Tezon 1992). It has also been reported that a protein secreted in primates by the epithelial lining cells of the ductus epididymis has the capacity to activate forward motility (Hoskins et al. 1979). Alterations in the histology of both the caput and cauda epididymis of male mice treated with 10 and 20 mg NaF/kg body weight for 30 days has been reported (Chinoy & Sequeira 1989a). During the present investigation, however, no appreciable change in the structure and internal milieu of the caput and cauda ductus epididymis and in the spermatozoa passing through was observed in 20-month fluoride treated rabbits. The previous transmission electron microscopic studies by Kumar and Susheela (1994) revealed abnormalities in the flagellum, the acrosome and the nucleus of the spermatids and spermatozoa of the testis and epididymis respectively of rabbits fed on 10 mg NaF/ kg body weight for 18 months. The fragmentation of spermatozoa observed in the ductus epididymis of

23-month fluoride treated rabbits during the present studies and in the vas deferens of 18 and 29-month fluoride treated rabbits in earlier studies by Susheela and Kumar (1991) may result from the above mentioned ultrastructural damage rendering the spermatozoa weak. The fragmentation might be potentiated when the weakened spermatozoa pass through the hostile environment of the post-testicular ducts in which structural and functional alterations arising from fluoride toxicity has been shown in the present as well as previous investigation (Susheela & Kumar, 1991). Light microscopic study by Li et al. (1987) did not show any significant difference in the frequency of abnormal sperm obtained from the cauda epididymis of mice treated with different doses of NaF daily for 5 days, thus supporting the view that fluoride has no adverse mutagenic effects. Chinoy et al. (1991) have reported light microscopically observed deflagellation, agglutination, acrosomal damage and decrease in the motility and number of spermatozoa obtained from the cauda epididymis of rabbits given 20 and 40 mg/kg body weight of sodium fluoride for 30 days. They have also shown a decrease in activities of some enzymes on sperm, viz. adenosine triphosphatase, succinate dehydrogenase and acid phosphatase, as well as reduction in Na⁺ and K⁺ levels in the spermatozoa. In human subjects suffering from endemic and industrial fluorosis, a decrease in the sperm count and increase in the incidence of oligospermia and azoospermia have been reported (Neelam et al. 1987; Tarinsky 1972).

Light and scanning electron microscopic studies by Susheela and Kumar (1991) showed no appreciable change in the seminiferous epithelium and its spermatozoa of rabbits treated with 10 mg NaF/kg body weight for 18 months but they reported cessation of spermatogenesis and clumping of seminiferous epithelium in 29-month fluoride treated rabbits. However, they found de-ciliation of the luminal surface of ductuli efferentis and loss of stereocilia in the vas deferens as well as mucosal damage in both 18 and 29-month fluoride treated groups. Damage to the gastrointestinal mucosa in humans, drinking naturally fluoridated water and undergoing therapy for otosclerosis with 30 mg NaF daily for 3-12 months, has been reported (Susheela et al. 1992; Das et al. 1994). In the present investigation the seminiferous tubule showed a significant decrease in epithelial cell height and tubular diameter only in 23month fluoride treated rabbits and not in 20-month treated animals. The differences in the parameters investigated in the caput and cauda epididymis of 20month fluoride treated rabbits from their controls were not significant; however, the differences in these parameters of the same organs of 23-month fluoride treated rabbits compared to their controls were significant (Table 1). It is likely that the fluoride induced changes in the epididymis and testis would be progressive. The different qualitative and quantitative changes due to fluoride may or may not commence at the same time and progress at the same rate. Chinoy and Sequeira (1989b) have reported no significant change in testicular cholesterol and serum testosterone levels but a significant decrease in succinic dehydrogenase levels in the testis and reduction in adenosine triphosphatase and sialic acid levels in the epididymis of mice at doses of 10 and 20 mg/kg body weight NaF given for 30 days. In the present investigation, however, different stages of spermatogenesis were observed in the testis of both the treated groups of rabbits with no significant change in the weight of the organs (Figures 2 and 3; Table 1).

Thus, it can be concluded that a significant decline in the fertility of rabbits exposed to fluoride toxicity (Chinoy *et al.* 1991) may ensue even before arrest of spermatogenesis and results from abnormalities in the structure and function of the post-testicular ducts (Susheela & Kumar 1991), particularly the caput and cauda ductus epididymis, as seen during the present investigation.

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