FLUORIDE EXPOSURE CHANGED THE STRUCTURE AND THE FUNCTION OF SPERM IN THE TESTIS AND EPIDIDYMIS OF MALE RATS

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ABSTRACT: This study systematically investigated the effects of fluoride ion (F) exposure on the male reproductive organ and on spermatogenesis. Twenty male rats were divided into two groups of 10 and treated with 0 or 150 mg F/L in drinking water. After 70 days, the body weight, organ coefficients of the testis and epididymis, sex hormone levels in serum, and sperm quality were measured. The histology of the testis and epididymis was observed via light microscopy, and the ultrastructure of the epididymis and sperm was studied via transmission electron microscopy. Fluoride treatment significantly decreased ($p<0.01$) the body weight and organ coefficients, as well as seriously damaging the histological structure of the testis and epididymis of the rats. The fluoride-exposed rats exhibited obvious ultrastructural changes of the sperm and epididymis, such as shedding sperm heads and acrosome, as well as mitochondrial swelling and vacuolization. In addition, fluoride treatment significantly decreased the serum levels of sex hormones, including testosterone, luteinizing hormone and estradiol ($p<0.01$) while also decreasing the serum level of follicle-stimulating hormone ($p<0.05$). Furthermore, fluoride treatment significantly decreased sperm density and motility ($p<0.01$), and significantly increased the rate of teratospermia ($p<0.01$). These results indicate that fluoride exerts toxic effects on the testis and epididymis by decreasing the organ coefficients of the testis and epididymis, damaging the structure of the testis and epididymis, disturbing the secretion of sex hormones, and affecting sperm quality, with the ultimate result of diminishing sperm function.

Keywords: Epididymis; Fluoride; Rat; Sex hormones; Sperm; Testis.

INTRODUCTION

The production and differentiation of sperm depend on the close coordination among spermatogenic Sertoli and Leydig cells in the testis.[1,2] However, the sperm produced by the testis is not fully mature and cannot fertilize.[3] The sperm should be transported to the epididymis through the contraction of the peripheral myoid cells in the seminiferous tubules and the movement of lumen fluid to mature fully and acquire the capability to fertilize.[4,5] Damage of the structure and function of the testis and epididymis seriously affects spermatogenesis and maturation.[6,7] Noxious substances, particularly fluoride, [8,9] can readily damage the structure of the testis and epididymis; thus, exposure to this element could seriously affect spermatogenesis and maturation, as well as sperm fertilization.[10,11] In addition, sex hormones participate in spermatogenesis and sperm maturation. Specifically, testosterone (Ts) plays a crucial role in spermatogenesis, maturation, and maintaining various physiological functions; luteinizing hormone (LH) promotes the secretion of Ts by acting on Leydig cells; follicle-stimulating hormone (FSH) promotes the formation of seminiferous tubules and spermatogenesis by coordinating with Ts, and estradiol (E2) affects the secretion of gonadotropin and Ts and thus regulates spermatogenesis and

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sperm maturation.[12,13] These hormones mutually interact to regulate spermatogenesis and sperm maturation. Previous studies have indicated that the secretion levels of androgen and estrogen, particularly in infertile male adults, are significantly decreased in fluorosis endemic areas.[14,15] Epidemiological surveys have also indicated that maternal exposure to excessive fluoride significantly changes serum Ts, FSH, and LH levels in neonatal male rats.[16,17] Thus, changes in the structure of the testis and epididymis and imbalance in the secretion of sex hormones may affect spermatogenesis and sperm maturation.

As an environmental pollutant, the fluoride ion (F) causes dental and skeletal fluorosis; however, in recent years, reproductive toxicity induced by fluoride has emerged.[18,19] Fluoride has been associated with decreased capacitation, acrosome reaction, and chemotaxis, which result in impaired fertility.[20,21] Moreover, long-term excessive fluoride exposure suppresses spermatogenesis, decreases the counts and mobility of sperm, and disturbs the secretion of Ts and LH.[14,21] In addition to its effects on spermatogenesis and sperm quality, fluoride may affect the structure and function of the testis and epididymis. As an anion with strong penetrability, fluoride can easily penetrate cell membranes through free diffusion and cause adverse effects on cell structure and function.[22] Long-time exposure to excessive fluoride can cause pyknosis and karyolysis, as well as mitochondrial vacuolization in the liver, myocardium, and uterus.[22-24] Therefore, systematic studies on the effects of excessive fluoride exposure on the structure of the testis and epididymis, the secretion of sex hormones, and the function of sperm are warranted.

In the present study, a rat model was established to investigate the effect of systemic fluoride exposure on male rats. The histological structure of the testis and epididymis, and the ultrastructure of the epididymis and sperm were observed, the serum levels of Ts, FSH, LH, and E2 were measured, and sperm quality was analyzed.

MATERIALS AND METHODS

Animals and treatment: Twenty 30-day-old healthy male Sprague-Dawley rats weighing 50±1.5 g were obtained from the Experimental Animal Center of Zhengzhou University and kept in a standard animal house with air conditioning at 22–25ºC under hygienic conditions.

The experimental rats were randomly divided into two groups of 10 after a week of balanced feeding. The control group was fed a standard diet and given distilled water, whereas the treatment group was fed a standard diet and given drinking water containing 150 mg F/L. During the treatment period, the weight of all rats was measured each week. After treatment with fluoride for 70 days, the rats were anesthetized with 20% urethane solution and then killed by decapitation. The testicular and epididymal tissues were removed and rapidly fixed in 10% formaldehyde and 2.5% glutaraldehyde for histological and ultrastructure observation, respectively. The study design was approved by the Institutional Animal Care and Use Committee of China.

Analysis of the body weight, organ coefficients of the testis and epididymis, and sperm quality: Sperm quality was evaluated as previously described.[25] The sperm suspension of the epididymis was collected in 0.9% physiological saline at 37ºC.

Sperm cells were collected and counted using an improved Neubauer counting chamber. The rates of teratospermia and sperm motility were measured as previously described by Sun et al.[26] After the weight of the testis and epididymis was measured, the organ coefficient was calculated according to the following equation:

$$\text{Organ coefficient (\%)} = \frac{\text{Wet weight of organ (g)}}{\text{Body weight (g)}} \times 100$$

Histology: Samples of the testis and epididymis were immersed in 10% formaldehyde for 24 hr, washed three times with phosphate buffered saline, dehydrated in ethyl alcohol series, rendered transparent using xylene, and then embedded in paraffin. The sections were cut into 5 μm slices, stained with hematoxylin and eosin, and then observed under a light microscope.

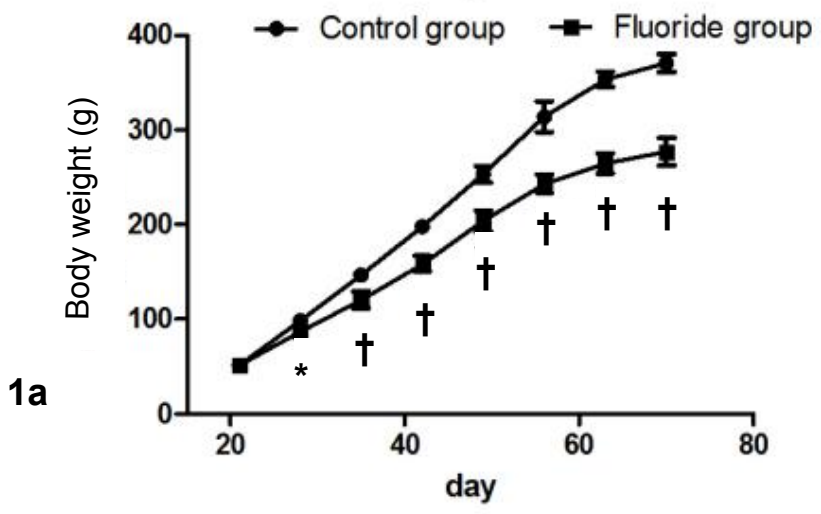
Transmission electron microscope (TEM) observation: Epididymal tissues were fixed in 2.5% glutaraldehyde (pH 7.4) at 4ºC, and then washed three times with phosphate buffer before immersing in 1% osmic acid. The samples were dehydrated in ethyl alcohol and finally embedded in Araldite resin. Ultrathin sections of 50 nm were cut, stained with uranyl acetate and lead citrate, and then examined under a transmission electron microscope (H-7500).

Detection of the serum levels sex hormones: The serum levels of FSH, E2, LH and Ts in male rats were measured with commercially available diagnostic Kits (Bidi Biological Technology Co. Ltd. Nanjing, China) according to the standard procedures. The serum samples and standard products were added into the microplate, followed by biotin labeled second antibody and enzyme labeled reagents at 37ºC for 60 minutes. The color liquids A and B were added and the samples and standard products were colored for 10 minutes. Then, after washing 5 times, the TMB termination solution was added. The wavelength of 450 nm was used to analyze spectrophotometrically with a microplate reader.

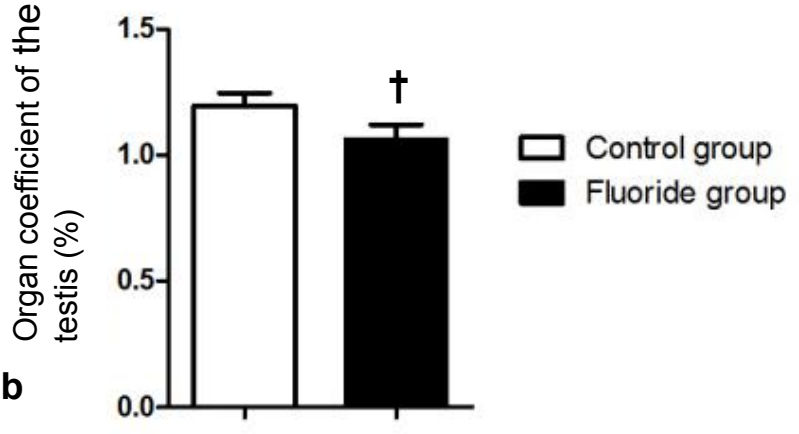
Statistical analysis: All data were expressed as the mean±standard deviation. Statistical and data analyses were measured by the Software of SPSS13.0 version and Student's *t*-test was employed to analyze parametric data. Statistical significance was considered to be present at $p<0.05$.

RESULTS

Effects of fluoride on the body weight, organ coefficients of the testis and epididymis, and sperm quality in male rats: After treatment with fluoride for 70 days, the body weight of the treatment group significantly decreased ($p<0.01$) compared with that of the control group (Figure 1a). The organ coefficients of the testis and epididymis in the treatment group were significantly lower ($p<0.01$) than those in the control group (Figures 1b and 1c). In addition, the sperm density and motility significantly decreased ($p<0.01$) (Figures 1d and 1e) whereas the rate of teratospermia significantly increased ($p<0.01$) (Figure 1f) in the treatment group compared with the control group.



1a



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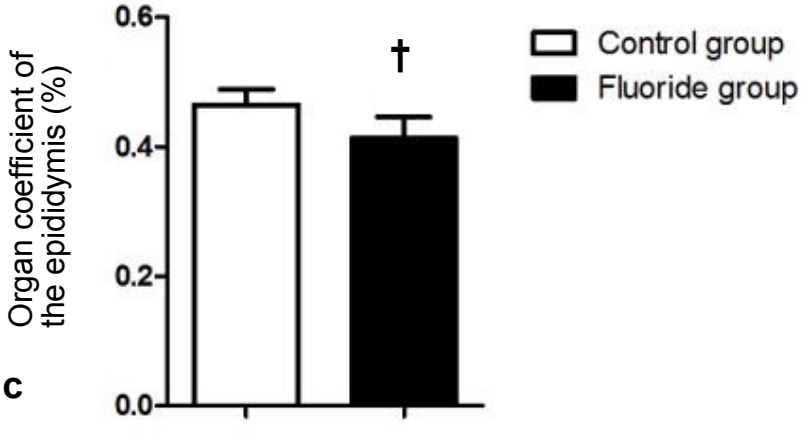
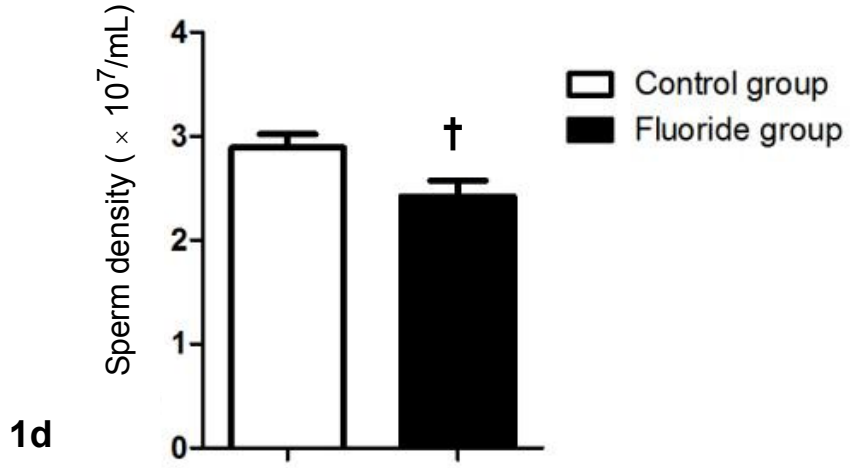


1c

Figures 1a, 1b, and 1c. Effects of fluoride on the body weight and the organ coefficients of the testis and epididymis. 1a: body weight; 1b: organ coefficient of the testis; and 1c: organ coefficient of the epididymis. *$p<0.05$, †$p<0.01$.



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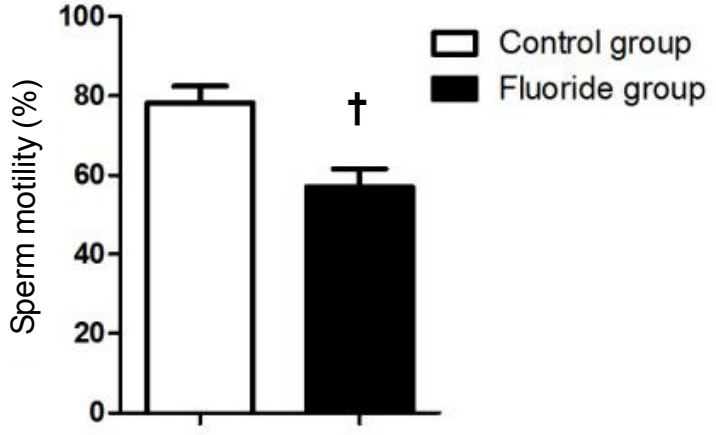

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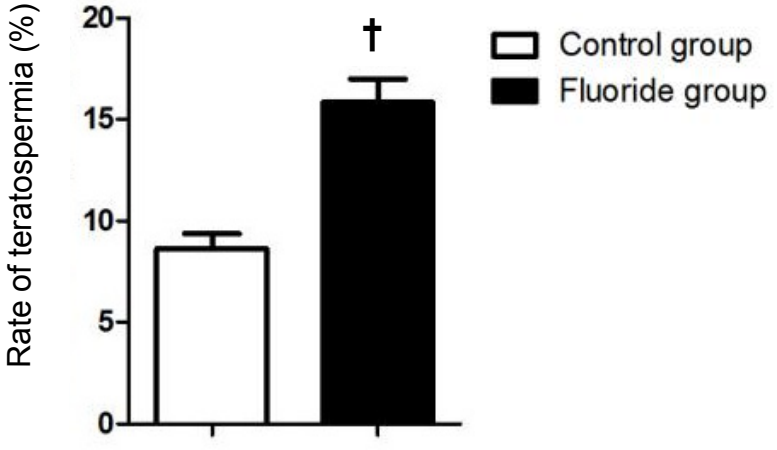
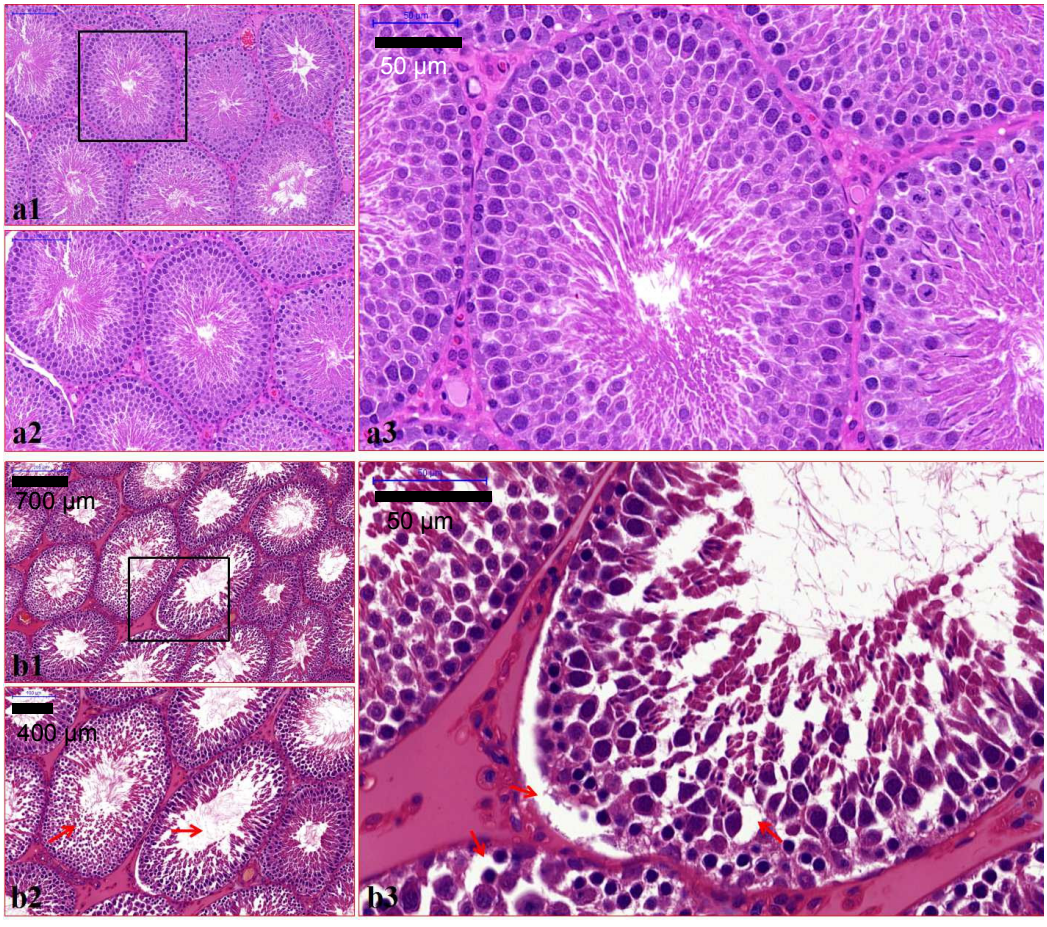


1f

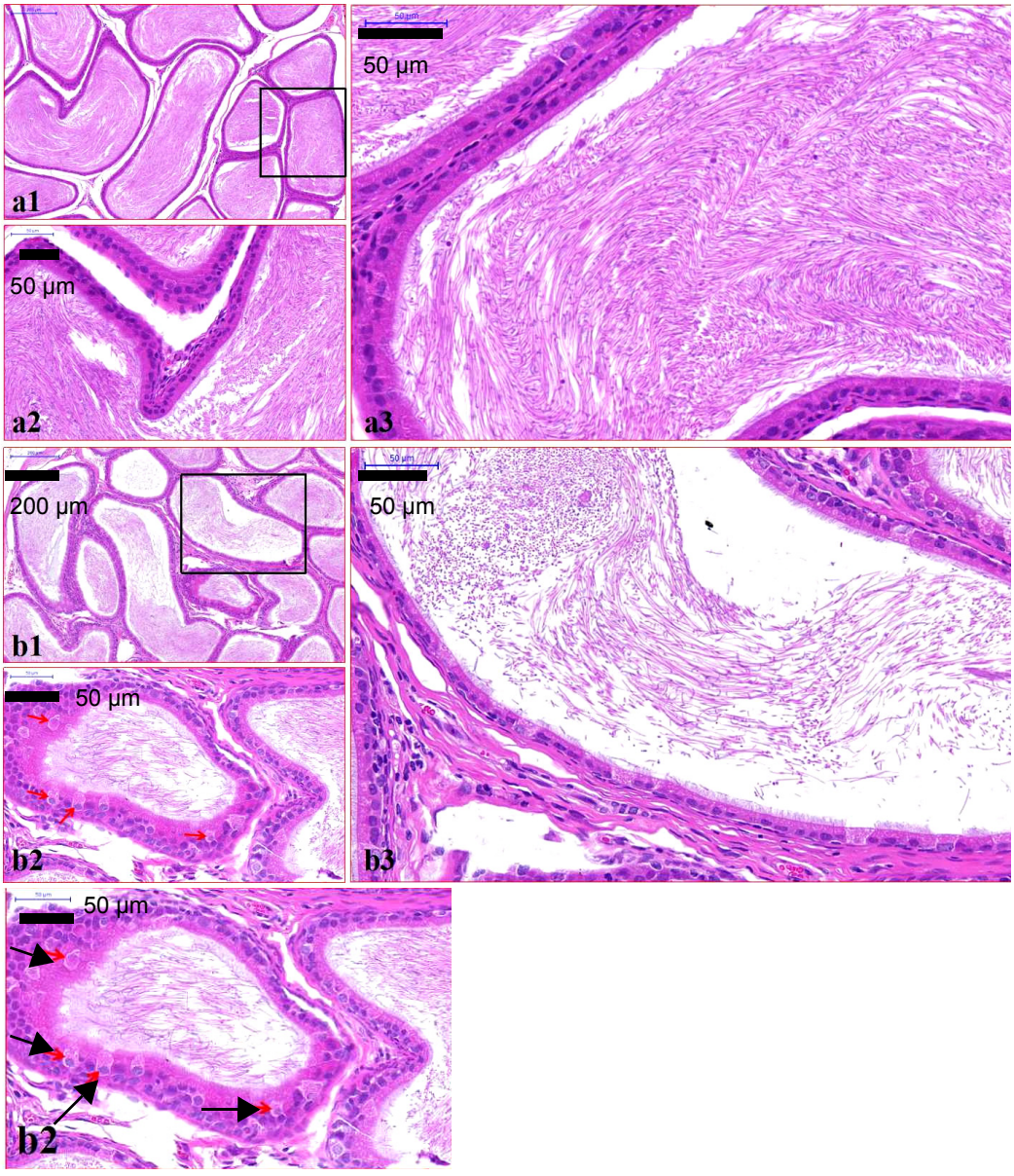
Figures 1d, 1e, and 1f. Effects of fluoride on the sperm density, the sperm motility, and the rate of teratospermia. 1d: sperm density; 1e: sperm motility; and 1f: rate of teratospermia. †$p<0.01$.

Effects of fluoride on the histological structure of testes in male rats: The histological changes of the testis are shown in Figure 2. In the control group, the normal structure of the testis was observed, and the arrangement of seminiferous tubules and spermatogenic cells was regular and compact (Figures 2a1 and 2a2. Simultaneously, the well-organized distribution of seminiferous epithelial cells and a large number of sperm in seminiferous tubules are presented in Figure 2a3. However, after fluoride treatment, the testicular tissues and the interstitial tissues of the testis and cytoplasm were stained with red dye. In the treatment group, the seminiferous tubules were irregularly arranged and the content of spermatogenic cells was reduced with a disordered organization (Figures 2b1 and 2b2. Meanwhile, the layers of spermatogenic cells in the seminiferous tubules were significantly decreased, and a few vacuoles were also observed in the seminiferous tubules (Figure 2b3.



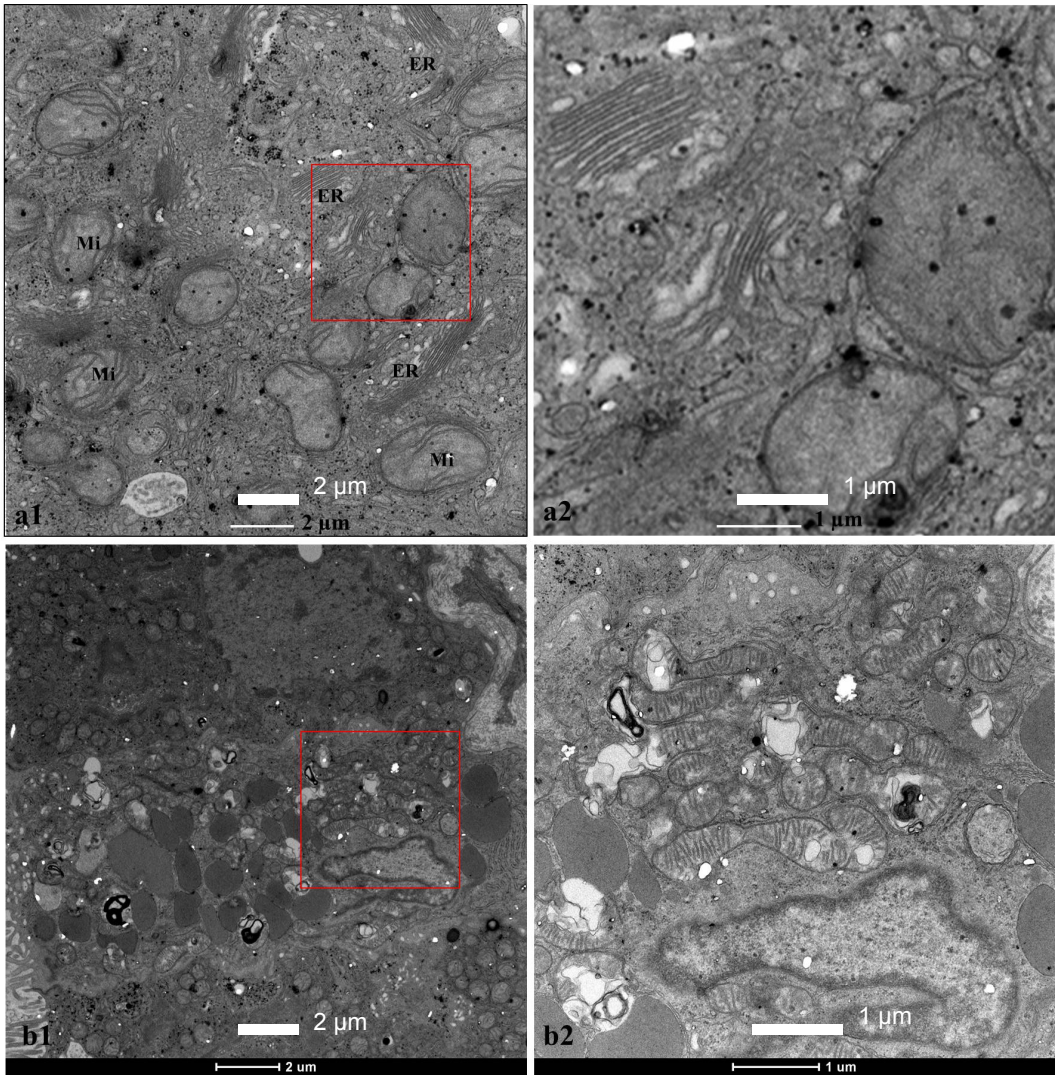
Figures 2a1, 2a2, 2a3, 2b1, 2b2, and 2b3. Effects of fluoride on the histological structure of testes in male rats. (2a1), (2a2), and (2a3) sections from the control group; (2b1), (2b2), and (2b3) sections from the fluoride group. The red arrows indicate decreased sperm numbers and damaged seminiferous tubules.

Effects of fluoride on the epididymal histopathology in male rats: The histological changes of the epididymal tissue are depicted in Figure 3. In the control group, the morphology of the epithelial cells in the epididymal tubules was normal and the epididymal tubules were filled with a large number of sperm (Figures 3a1 and 3a2). Denser sperm is illustrated in Figure 3a3. In the treatment group, the numbers of sperm in the epididymal tubules were significantly decreased and swelling and lysis of epithelial cells in the epididymal tubules occurred (Figures 3b1 and 3b2). Meanwhile, the interstice between epididymis tubules and smooth muscle layer was widened and the sperm density was less dense (Figure 3b3).



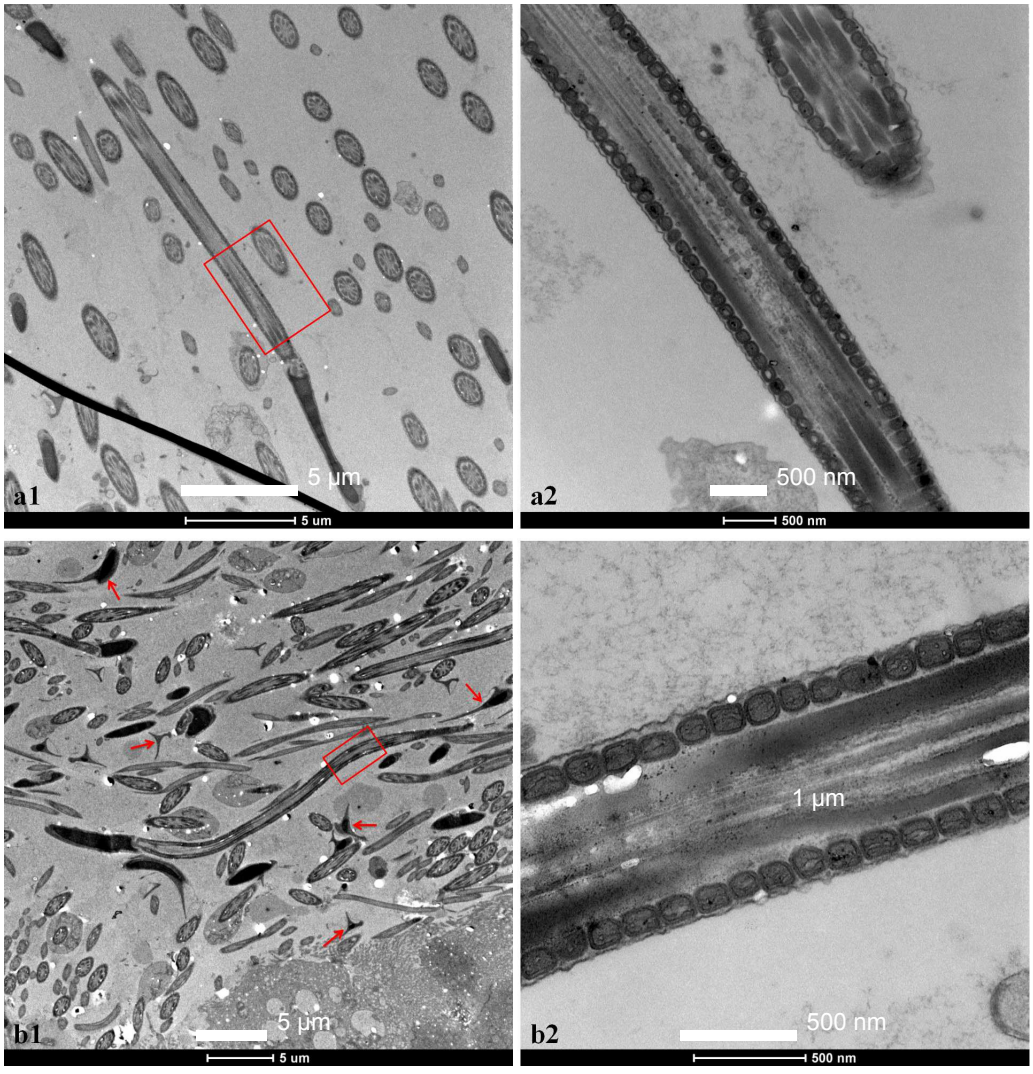
Figures 3a1, 3a2, 3a3, 3b1, 3b2, and 3b3. Effects of fluoride on the structure of epididymal tissue in male rats. (3a1), (3a2), and (3a3) sections of epididymal tissue from the control group; (3b1), (3b2), and (3b3) sections from the fluoride group. The red arrows in 3b2 indicate swelling and lysis of cells in epididymis tubule. Figure 3b2 is repeated with black arrows to indicate the position of the red arrows.

Ultrastructural changes of epididymal tissue in male rats: As shown in Figure 4a1, the control group depicted a normal structure, including abundant mitochondria and endoplasmic reticulum. Concurrently, a clear mitochondrial ridge was observed (Figure 4a2). By contrast, an unclear nucleus, an indistinct nuclear membrane, and a decreased number of mitochondria were observed in the epididymal tissue of the fluoride-exposed rats (Figure 4b1). Indications of mitochondria ridge dissolution, and slight vacuolization were also observed (Figure 4b2).



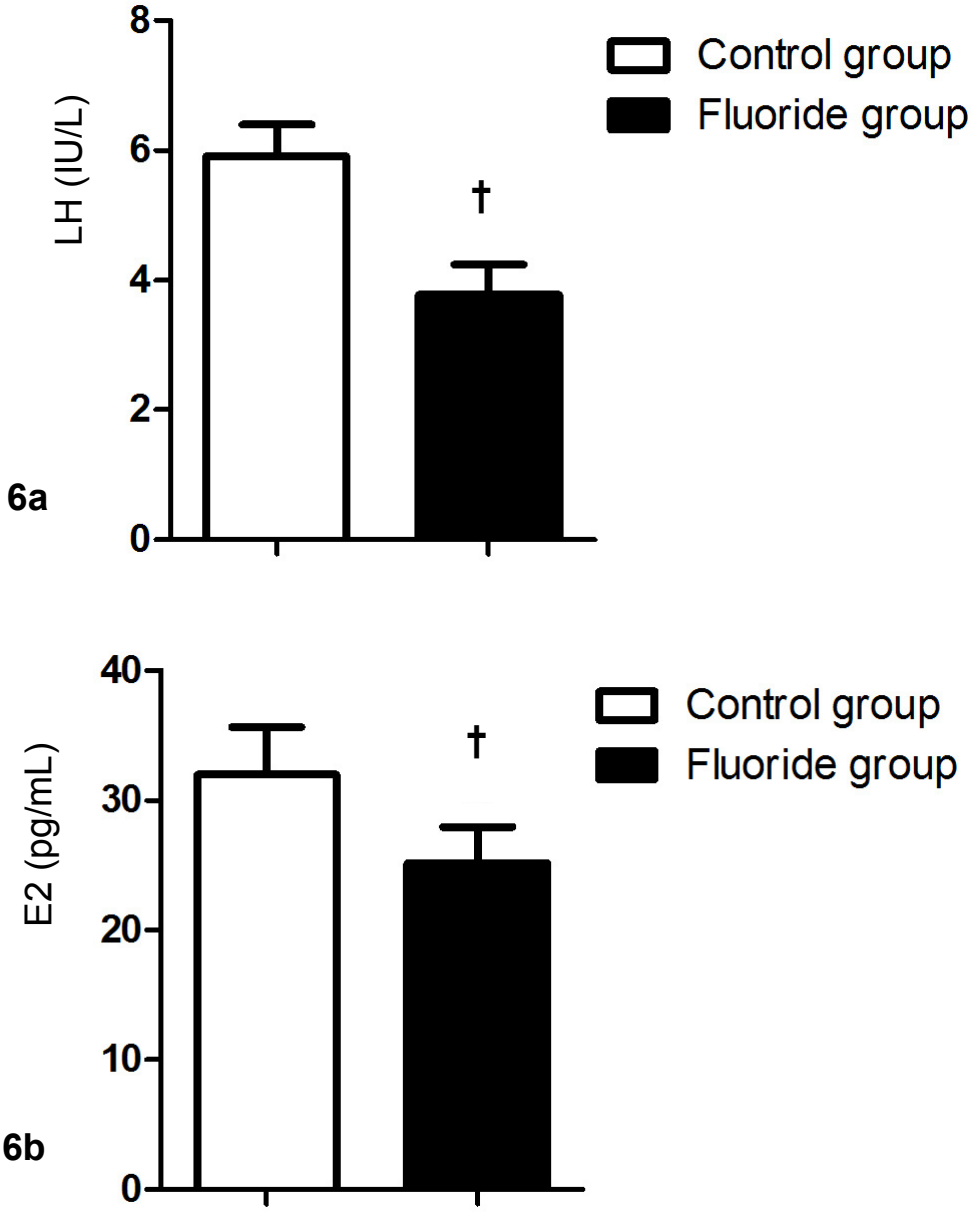
Figures 4a1, 4a2, 4b1, and 4b2. Effects of fluoride on the ultrastructure of epididymal tissue in male rats. (4a1) and (4a2) control group; (4b1) and (4b2) fluoride group. Mi: mitochondrion; NM: nuclear membrane; Nu: nucleus; and ER: endoplasmic reticulum.

Effects of fluoride on the ultrastructural alterations in sperm: Figure 5 shows the ultrastructural alterations in sperm. The sperm in the control group showed a normal morphology, i.e., a straight line with distinct acrosome was observed (Figure 5a1). Figure 5a2 presents the orderly arrangement, uniform size, and morphological rules of mitochondria and the clear membrane of sperm. The curvilinear shape of sperm and the number of shed sperm heads after fluoride treatment are shown in Figure 5b1. Mitochondrial swelling and vacuolization in sperm were also observed (Figure 5b2).

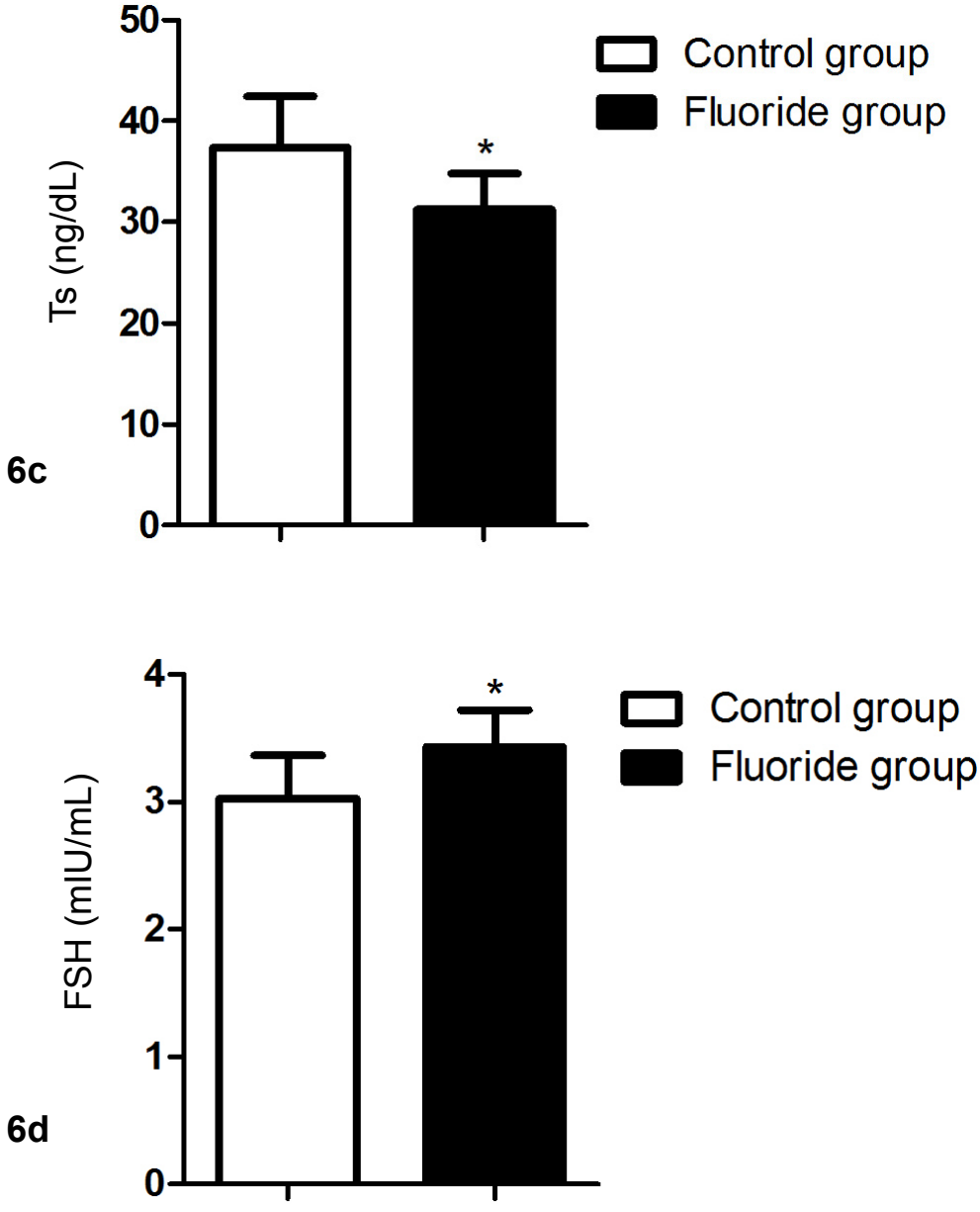


Figures 5a1, 5a2, 5b1, and 5b2. Effects of fluoride on the ultrastructure of sperm in male rats. (5a1) and (5a2) control group; (5b1) and (5b2) fluoride group. The red arrows indicate shedding of sperm heads.

Effects of fluoride on the sex hormones in the serum of male rats: As shown in Figure 6, the levels of estrogen, including LH and E2, significantly decreased ($p<0.01$) in the treatment group compared with the control group (Figures 6a and 6b). The level of FSH also decreased ($p<0.05$), but not significantly in the treatment group compared with the control group (Figure 6d). Although the level of Ts decreased ($p<0.05$), no significant differences were observed between the treatment and control groups (Figure 6c).



Figures 6a and 6b. Hormonal changes in the serum of male rats in the control group and treatment groups. Concentrations of (6a) luteinizing hormone (LH), and (6b) estradiol (E2). *$p<0.05$ and †$p<0.01$.



Figures 6c and 6d. Hormonal changes in the serum of male rats in the control group and treatment groups. Concentrations of (6c) testosterone (Ts) and (6d) follicle-stimulating hormone (FSH). *$p<0.05$ and †$p<0.01$.

DISCUSSION

In the male reproductive system, the normal structure of the testis and epididymis, and the balance between the secretion of androgen and estrogen are crucial for spermatogenesis and sperm maturation. The abnormal morphology, structure, and function of sperm not only affect the quality of semen but also cause male infertility.

The testis is the only organ that generates sperm in the male reproductive system. Thus, the normal structure of the testis is significant for spermatogenesis and in maintaining the various physiological functions. A large number of epidemiological investigations have indicated that excessive exposure to fluoride can cause obvious damage in the testis by causing degeneration and necrosis of the seminiferous tubules;[27,28] destroying the structure and function of Sertoli, Leydig, and spermatogenic cells, and suppressing the differentiation and maturation of spermatocytes.[21,29,30] In this study, fluoride treatment for 70 days significantly damaged the histological structure of the testis by causing the deformation of seminiferous tubules, decreasing the layers of spermatogenic cells, and inducing the swelling of Sertoli cells with light vacuoles, The changes in the structure of the Sertoli and spermatogensis cells implied that spermatogensis was impaired to a certain degree, which can be attributed to testicular damage induced by excessive fluoride exposure.

The smooth progress of spermatogenesis not only requires a suitable environment in the testis, but also involves various sex hormones.[31,32] Ts produced by Leydig cells in the testis regulate spermatogenesis in the initial stage; a decrease in the content of Ts can lead to failure of spermatogenesis and infertility.[14,31] Previous studies have shown that fluoride can inhibit the production of Ts in Leydig cells, thereby affecting spermatogenesis.[16,31] In the present study, the level of Ts decreased in the treatment group possibly because of the structural damage to Leydig cells. Ma et al. reported that fluoride can affect the synthesis of Ts mainly by destroying the structure of Leydig cells.[33] In addition, estrogen, including FSH, LH, and E2 is involved in spermatogenesis and maturation. Testicular Sertoli cells synthesize E2 and estrone under the action of FSH. Meanwhile, estrogen can influence the secretion of gonadotropin and androgen, thereby regulating spermatogenesis and sperm maturation. However, previous studies have indicated that fluoride can inhibit the secretion of E2, the circulation of FSH, and the distribution of LH and Ts in the hypothalamus-pituitary-testis axis.[15,34] The present results indicate that the levels of Ts, LH, and E2 decreased in the treatment group. These changes indicate that fluoride can disturb the secretion of sex hormones, which inhibits sperm production and blocks maturation, and finally causes reproductive dysfunction.

In addition, the sperm produced by the testis needs further processing in the epididymis to mature fully and acquire the capability to fertilize.[6,35] The TEM examination showed serious damage in the nucleus and mitochondria of epithelial cells in the epididymis, such as the loss of distinct nuclei and nuclear membranes, as well as mitochondrial swelling and vacuolization. The epididymis is an important organ for sperm to mature fully and acquire the capability to fertilize; thus, fluoride-induced damage in the structure of the epididymis may further affect sperm quality.[9] In the present study, fluoride treatment significantly decreased sperm density and motility while obviously increasing the rate of teratospermia. Although the epididymis protects the sperm, fluoride can break through the blood-testis barrier and the blood-epididymis barriers and produce adverse effects on sperm in the epididymis.[24,36] In the present study, the ultrastructure of sperm showed that the morphology of sperm was changed, with curved mitochondria, mild swelling of the tail, shedding of the acrosome of sperm heads, and damaged outer dense fiber with

light lysis and vacuolization. Previous studies indicated that shedding of the plasma membrane can affect the acrosome reaction,[37,38] mitochondrial damage can reduce sperm motility,[26] and acrosome abnormality of the sperm can directly affect sperm function.[39] Any of these conditions could lead to a low fertilization rate or fertilization failure. Furthermore, the tail of mature sperm is rich in mitochondria that provide the power for motility; moreover, the mammalian sperm tail consists of several outer dense fibers that play a crucial role in sperm morphology and function.[40,41] Thus, these changes confirmed that fluoride can damage the structure and function of the epididymis, and further affect sperm quality.

CONCLUSION

In this study, fluoride treatment for 70 days decreased the body weight and the organ coefficients of the testis and epididymis in male rats indicating that fluoride is a systemic toxicant that can damage the testis and epididymis. The structural damage of the testis and epididymis, the disorder in the secretion of sex hormones, and the changes in sperm quality indicate that fluoride exerted reproductive toxicity in the male reproductive system. Fluoride can induce structural damage of the testis and epididymis, disturb the secretion of sex hormones, and affect sperm quality, thereby altering sperm function.

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DATA AVAILABILITY

Readers who want to access the raw data should contact HW Wang, E-mail: sxwhw@126.com

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