EFFECTS OF SODIUM FLUORIDE ON ANDROGEN RECEPTOR EXPRESSION IN MALE MICE

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SUMMARY: To investigate the effects of different doses of sodium fluoride (NaF) on protein and gene expression of the androgen receptor (AR) in the testis of mice, sexually mature male Kunming mice in groups of 20 were exposed to 0 (control), 50, 100, 200, and 300 mg NaF/L in their drinking water for 8 weeks. At the end of this period, streptavidin peroxidase immunochemistry and computer imaging analysis were applied to examine the AR expression in the testis. Compared with the control group, expressions of AR protein decreased significantly in the 200 and 300 mg NaF/L groups ($p<0.05$). With quantitative real-time polymerase chain reaction (QRT-PCR), we also assessed the expression of AR mRNA by culturing primary Sertoli cells from immature mice with different concentrations of fluoride (10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} mol/L) for 48 hr, resulting in a significant F-induced decline in the AR mRNA level in the cells compared to controls. On the basis of these findings, we propose that decreased AR protein and gene expression in the testis is associated with impairment of reproductive functions by NaF.

Keywords: Androgen receptor; Computer imaging analysis; Immunochemistry; Mice testis; Real-time polymerase chain reaction (RT-PCR); Sertoli cells; Streptavidan peroxidase.

INTRODUCTION

In recent years, the toxic effects of fluoride (F) on spermatogenesis have become a matter of major concern. However, our understanding of the mechanism of these effects is still in its infancy. Various studies in men and animals, including our own, indicate that high F can lead to a marked reduction of testosterone, one of the most important androgens produced in the testis, that is vital for the initiation of spermatogenesis.[1-3] Although the action of testosterone is closely related to the androgen receptor (AR),[4] relatively little is known about the effects of F on AR.

In the present study, observations were made on morphological changes in the testis and the expression of androgen receptor on gene and protein levels in male mice exposed to various levels of NaF in their drinking water and by quantitative real time polymerase chain reaction (QRT-PCR) studies in Sertoli cells of immature male mice.

MATERIALS AND METHODS

Animals: One hundred adult male Kunming mice weighing 25–30 g were obtained from the Shanxi Medical University animal experiment center. All mice were maintained on a standard laboratory diet at 22–25°C under a 12/12-hr light/dark cycle with adequate ventilation and proper and hygienic conditions. The study design was approved by the Institutional Animal Care and Use Committee of China.

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Establishment of animal model: As in our recent reports,[5] 100 of the above mice were divided randomly into five groups of 20 animals each as follows: the control group was given double distilled water, and the animals in the four experimental groups received 50, 100, 200, and 300 mg NaF/L, respectively, in their drinking water. After 8 weeks, all mice were injected with 20% urethane solution for fatal anaesthesia. Testes were carefully removed, blotted free of blood, rinsed with distilled water, and used for further study.

Haematoxylin and eosin (HE) procedure: At the end of the experiment, one testis from each animal was fixed in Bouin's fluid for 14–18 hr and embedded in paraffin. From each testis, sections of 5-µm thickness were collected with a rotary microtome and stained with HE for observation under a lighted microscope.

Paraffin immunochemistry procedure: Fixed and paraffin-embedded testes were stained with AR immunohistochemistry kits (Maixin Bioengineering Co., Ltd, Fuzhou, China). In order to unmask the AR protein, sections in a 0.01 M citrate buffer solution (pH 6.0) were placed in a microwave oven for 20 min after deparaffination. The sections were then incubated with endogenous peroxidase blocking solution (0.3% H_2O_2) for 30 min at room temperature and with normal nonimmunone serum for the next 30 min. (The nonimmunone serum was taken from a control rabbit that was not immunized by goal or objective protein in order to eliminate a false positive reaction.). Subsequently, incubation with rabbit anti-rat AR polyclonal antibody at 1:150 diluted with phosphate-buffered saline (PBS, 0.1 mol/L, pH 7.4) was performed at 4°C overnight. After reaction with biotin-conjugated second antibody and streptavidin-peroxidase solution for 1 hr at room temperature, diaminobenzidine (DAB) solution was applied to color the AR protein.

Preparation of primary cultures of mice Sertoli cells: Primary cultures of mice Sertoli cells were prepared as described previously. [6] Under sterile conditions, testes were removed, decapsulated, and placed in 10 mL conical tubes containing 5 mL of 1×Hanks solution (calcium/magnesium-free, pH 7.4, adjusted with 7.5% sodium bicarbonate). The seminiferous tubules were dispersed in a collagenase IV solution (10 mL, 1 mg/mL in pH 7.4 Hanks solution at 34°C and shaken at 80 oscillations/ min for 15 min. Subsequently, the tubules were incubated in a trypsin solution (10 mL, 0.25 mg/mL trypsin in pH 7.4 Hanks solution at 37°C for 10 min without shaking. Dulbecco's Modified Eagle's Medium and Ham's F-12 nutrient medium (DMEM/F12), supplemented with 20% (v/v) fetal bovine serum, was added to terminate the digestion of trypsin. The isolation of single cells from the testis tissues was performed by consecutive filtration through a 100-µm mesh strainer, and the filtrate was centrifuged for 5 min at 2003 g. The cell pellet was suspended in DMEM/F12 supplemented with 20% (v/v) fetal bovine serum, then cultured at 37°C for 48 hr in a humidified atmosphere of 5% CO_2. Each culture flask was then subjected to hypotonic treatment with sterile 20 mmol/L Tris-HCI buffer for 3 min to detach contaminating germ cells from Sertoli cells. After washing with DMEM/F12, the culture flasks containing Sertoli cells were cultured in serum-free medium at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every second day, removing dead and nonattached cells. The

Sertoli cells were identified by their morphological characteristics, using an inverted phase-contrast microscope. After primary culture for 72 hr, the Sertoli cells were used for the F toxicity experiments.

Cell culture and fluoride exposure: After the hypotonic treatment, isolated Sertoli cells were cultured in serum-free DMEM/F12 at 37°C in a 5% CO_2 atmosphere for 24 hr until 70% confluence. Based on cell viability assay, different concentrations of NaF (10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} mol/L) were dissolved in fresh serum-free DMEM/F12, and the cells were cultured in medium containing NaF for a further 48 hr.

Total RNA extraction and QRT-PCR: The total cellular RNA was extracted and studied by the method of Blok et al. [7] Primers (Table 1) were designed as in the Genebank gene sequences using Primer 5.0 and Primer Express (Applied Biosystems, USA). All primers were tested for specificity with the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) against the mice genome. They were excluded if there was any potential for false-priming which could cause unwanted PCR amplification. The specificity of primer pairs was further verified by the production of a single peak in melting curve analysis (performed before Synergy Brands, Inc. (SYBR®) Green-based RT- PCR).

Table 1. Primer sequences with their corresponding PCR product size

Genes	Primers	Primer sequence	Production length (base pairs)	Genebank accession No.
AR	sense	5'- GCATGCACAAGTCTCGGATGT -3'	82	NM_013476
	anti-sense	5'- TCCAAACTCTTGAGACAGGTGC -3'		
GAPDH	sense	5'- GGCAAATTCAACGGCACAGT -3'	81	BC096440
	anti-sense	5'- TCGCTCCTGGAAGATGGTGAT -3'		

QRT-PCR was performed by using an ABI (Applied Biosystems) Prism® 7000 Sequence Detection System in sterile 96-well PCR plates (ABI). PCR reaction mixtures contained 10 µL SYBR® Green PCR Master Mix (ABI) and sense/anti-sense gene specific primers. A final reaction volume of 20 µL was made up with 5 µL of diluted cDNA and nuclease-free distilled water. The PCR reaction mixture was denatured at 95°C for 10 min before the first PCR cycle. Thermocycling involved: (1) denaturation at 95°C for 15 sec, and (2) annealing at 60°C for 60 sec. PCR efficiency, uniformity, and linear dynamic range of each QRT-PCR assay were assessed by standard curves.

Images and data analysis: Sections were observed and photographed by an upright microscope (model: BX51, OLYMPUS of Japan). Positive cells and average optical density were analyzed by Image-Pro® Plus Version 5.1 micrograph analysis software (made in Media Cybernetics Inc. of America).

The quantification of target gene expression was based on statistical analyses of an absolute standard curve method and a comparative cycle threshold (Ct) method. Differences in gene expression were evaluated by ANOVA followed by Tukey's test, using SPSS 11.0. Differences with $p < 0.05$ were considered to be significant.

RESULTS

Morphological observation of testis: The control mice testis showed neatly arranged Leydig and Sertoli cells in the seminiferous tubules (Figure 1). The mice with 50 and 100 mg NaF/L in their drinking water showed little difference in their Leydig and Sertoli cells compared to the control group (Figures 2 and 3). With 200 and 300 mg NaF/L, the Leydig and Sertoli cells became more disorganized (Figures 4 and 5).

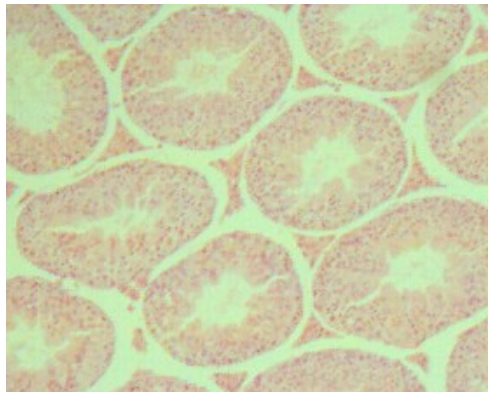

Figure 1. Structure of testis in male mice in the control group drinking distilled water; HE staining (× 100).

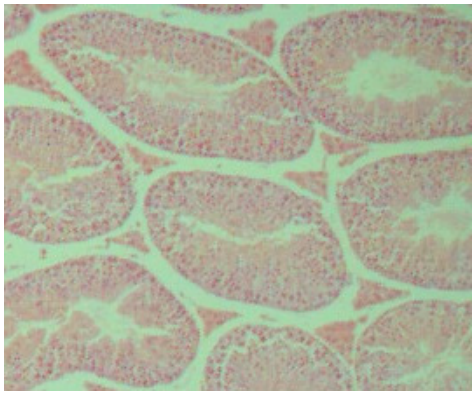

Figure 2. Histopathological changes in testis of male mice with 50 mg NaF/L in their drinking water; HE staining (× 100).

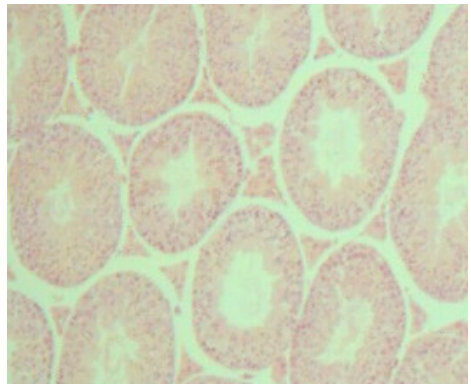

Figure 3. Histopathological changes in testis of male mice with 100 mg NaF/L in their drinking water; HE staining (× 100).

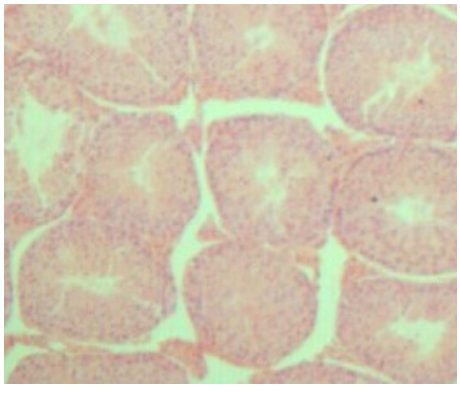

Figure 4. Histopathological changes in testis of male mice with 200 mg NaF/L in their drinking water; HE staining (× 100).

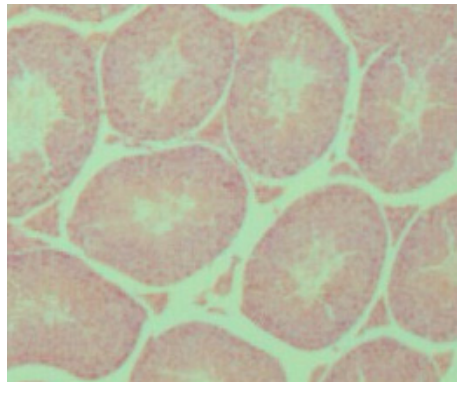

Figure 5. Histopathological changes in testis of male mice with 300 mg NaF/L in their drinking water; HE staining (× 100).

Protein Expression of AR: The results of AR protein expression in the testis in mice are shown in Table 2 and Figures 6–10. Although positive cell numbers were marginally higher with increasing concentrations of NaF in the drinking water in treatment groups, they are not statistically significant. The average gray scale of reflected visible wavelength light for the mice treated with the 200 and 300 mg NaF/L was significantly lower than that of the control ($p<0.05$).

Table 2. The average cell number and average gray scale in the different groups of mice (n=8; mean±SD)

		Groups (mg/L)				
		Control	50	100	200	300
Average optical density of AR positive cells	Positive cell number	23.64±5.16	22.48±7.50	19.21±5.31	18.68±4.39	18.35±4.61
	Average gray scale	78.37±5.34	82.09±5.62	81.08±6.51	97.58±6.92*	118.64±10.65*

*P<0.05 (compared with the control group).

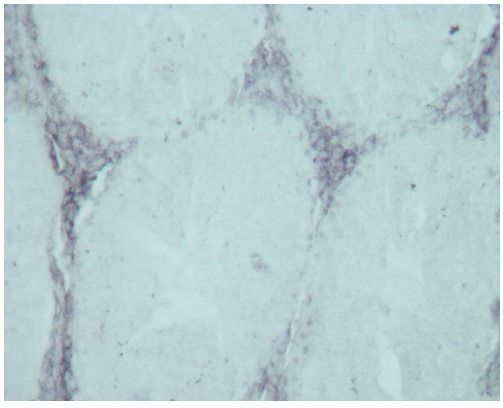

Figure 6. AR expression in testis of male mice in the control group drinking distilled water (×400).

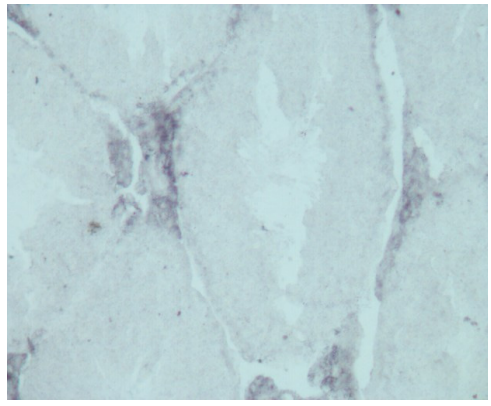

Figure 7. AR expression in testis of male mice with 50 mg NaF/L in their drinking water (×400).

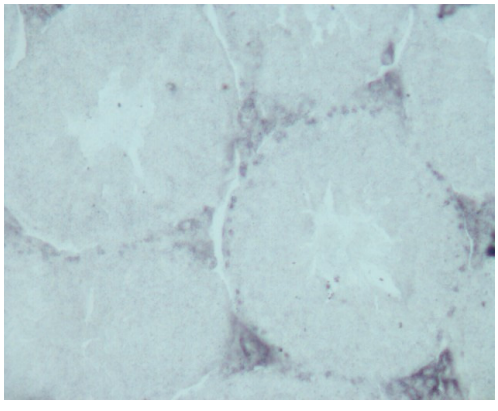

Figure 8. AR expression in testis of male mice with 100 mg NaF/L in their drinking water (×400).

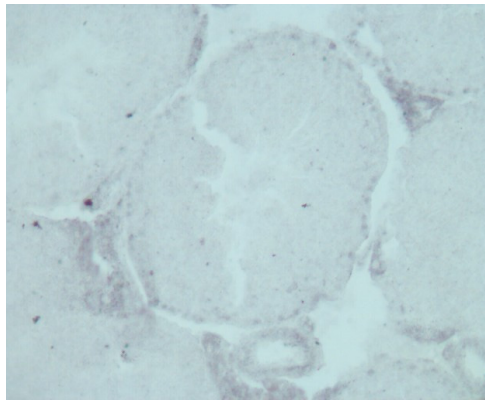

Figure 9. AR expression in testis of male mice with 200 mg NaF/ in their drinking water (×400).

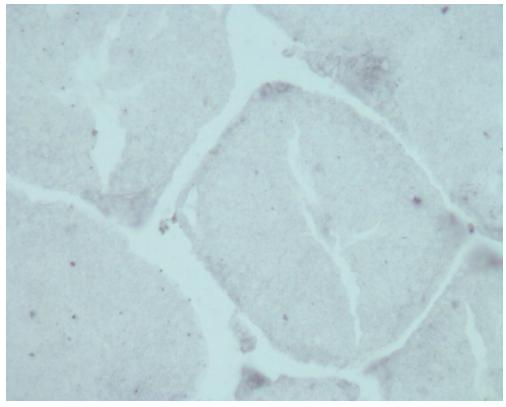

Figure 10. AR expression in testis of male mice with 300 mg NaF/L in their drinking water (×400).

Quantification of AR gene expression: As seen in Figure 11, the standard curve obtained by correlation of the Ct values (threshold cycles) with the dilution series of the housekeeping gene exhibited a relatively low intra-assay variation. The linear regression equation with y = –0.2667x+10.262 showed a very high correlation coefficient of 0.999.

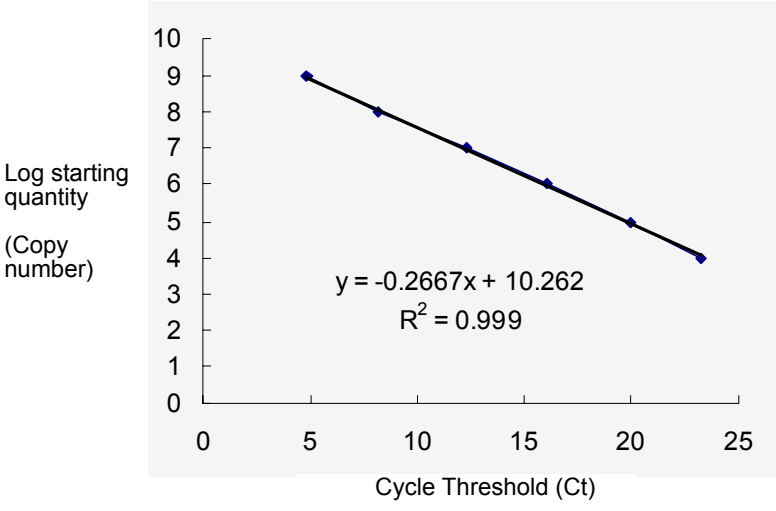

Figure 11. Standard curve for GAPDH gene obtained by the correlation of the Ct values with log starting quantity (Copy number) of the GAPDH gene.

Figure 12 shows the mRNA expression levels of AR in Sertoli cells exposed to different doses of NaF (10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3}mol/L) for 48 hr. The AR mRNA expression level from treatment with different concentrations of NaF was significantly lower ($p<0.05$) than that in the control group and presented an obvious dose-effect relationship.

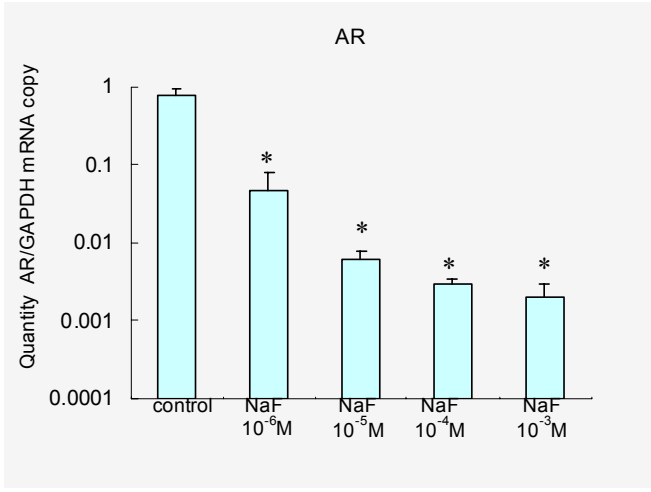

Figure 12. AR gene expression in Sertoli cells of immature mice exposed to NaF for 48 hr by real-time PCR, SYBR Green detection (*p <0.05 compared with the control group).

DISCUSSION

AR in Sertoli cells is considered to play an important role in male fertility.[8,9] The investigation by Zhou et al. has demonstrated that the expression of AR was significantly increased during period in which the initial germ cell population is expanding and maturing.[10] Another investigation from Chang et al. provided *in vivo* evidence of the need for functional AR in Sertoli cells to maintain normal spermatogenesis and testosterone production and to ensure normal male fertility, by applying Sertoli cell-specific AR to mice lacking the AR.[11]

The effects of androgens, primarily testosterone, within the testis, are affected by the androgen receptor (AR) that functions as a ligand-activated transcription factor to mediate the effects of testosterone on spermatogenesis.[12] Moreover, quantitative immunohistochemical studies indicate that androgens stimulate AR expression levels in Sertoli cells,[4,13,14] and some *in vitro* studies have shown that androgens also increase AR protein levels in cultured Sertoli cells.[15-17] Therefore, there is a close relationship between androgens and AR.

It is well known that toxic effects of fluoride are seen in diminished sperm quality and concentrations of testosterone in various animals.[1,2,5,18,19] To our knowledge, few studies have addressed the expression of AR exposed to fluoride. In the present work, the average AR level in testis, compared to controls, was significantly lowered in mice with 200 and 300 mg NaF/L in their drinking water. Moreover, *in vitro* examination by QRT-PCR suggested that NaF at concentrations ranging from 10^{-6} to 10^{-3}mol/L inhibited AR mRNA expression in Sertoli cells. These results support the view that fluoride adversely affects AR function, resulting in changes of androgen concentrations in serum and testis and weakening of male fertility.

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