Biological Trace Element Research
https://doi.org/10.1007/s12011-022-03220-8

Exposure to Fluoride From in Utero to Puberty Alters Gonadal Structure and Steroid Hormone Expression in Offspring Rats

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Received: 27 December 2021 / Accepted: 28 March 2022

Abstract

The reproductive toxicity of fluoride has been proven by a large number of studies. While the underlying mechanism of reproductive toxicity during pregnancy is still unclear. Hence, in this study, we investigated the effects of fluoride exposure on ovarian and testicular steroid hormone synthesis in young and adult rat offspring. We established a model of fluoride-exposed rat pups from in utero to puberty to explore the mechanisms of fluoride impacts on reproductive toxicity in the offspring. The results showed that NaF exposure did not affect the 3 weeks of age offspring. Whereas the body weight in both sexes significantly decreased, and the ovarian and testicular tissue structures were damaged at 11 weeks of age. In females, the total number of secondary follicles and mature follicles were significantly reduced after NaF exposure. Moreover, estradiol (E2) and follicle-stimulating hormone (FSH) levels in the females were significantly reduced in the 100 mg/L NaF exposure group. In males, the sperm viability and testosterone (T) were significantly decreased in the NaF exposure groups. Additionally, during steroidogenesis in ovaries and testes, fluoride remarkably decreased the expression levels of genes and proteins, including acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3β-HSD), cytochrome P450 17a-hydroxylase (CYP17A1), and cholesterol side-chain cleavage enzyme (CYP11A1), while the mRNA levels of 17β-hydroxysteroid dehydrogenase (17β-HSD) decreased only in the testes. These results indicated that fluoride exposure disrupted the steroid hormone balance by changing several important steroidogenic-related genes associated with the development of the gonads, and damage the normal structure of the gonads in rat offspring.

Keywords Fluoride · Rat pups · Gonad · Sex steroid hormones · Steroidogenic-related genes

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Introduction

Fluorine, an abundant element in the Earth's crust, occurs extensively in air, water, food, and coal [1]. Notably, in various regions, especially Africa, Middle East, China, and southern Asia, with naturally high concentrations of fluoride (> 1.5 mg/L) in groundwater, more than 200 million people suffer from endemic fluorosis [2, 3]. Epidemiological investigations showed that low concentrations of fluoride were required for the development of teeth and bones in humans and animals [4–6]. However, long-term exposure to high concentrations of fluoride may damage a range of health problems, including skeletal and non-skeletal tissues, especially bones and teeth [7, 8], non-skeletal phases including ovaries [5], testes, liver, and brain [9–11].

Animal studies and epidemiological reports of sodium fluoride suggest that sodium fluoride negatively affects the function of both male and female reproductive systems [12–16]. Recent studies have shown that fluoride could negatively affect the male reproductive system by reducing sperm counts and

Published online: 21 April 2022

testosterone (T) levels, and increasing sperm deformity rates [17–19]. In addition, studies have demonstrated that exposure to high concentrations fluoride could cause ovarian damage in females, thereby affecting the release of mature oocytes for fertilization [20]. Furthermore, sodium fluoride (NaF) could impair normal female reproductive function by injuring the histological structure of the ovaries and uterus, reducing the levels of reproductive hormones, such as E2, progesterone (P), T, FSH, and luteinizing hormone (LH), and reducing steroid production, which in turn decreases overall female reproductive function [12, 21]. Research on the reproductive toxicity of fluoride has been extensively carried out. However, its specific mechanism is still unclear.

Approximately 80–90% of fluoride absorbed by infants and children accumulates in the body [22, 23]. It can enter into the umbilical cord blood of the child from the mother through the placenta [24]. In addition, significantly high fluoride content in breast milk is indicative of fluoride exposure to infants [25, 26]. Young children show less resistance to the toxic effects of fluoride than adults because of under-developed defense mechanisms and highly permeable blood-brain barrier. Fluoride can cross the blood-brain barrier, accumulate in the cortex, cerebellum, hippocampus, striatum, hypothalamus, midbrain, medulla oblongata, and other brain regions, and then pass through cell membranes into soft tissues, resulting in hypoxia. This situation resembles the effects of fluoride poisoning and increased NO levels due to hypoxia in the circulation. When fluoride accumulates in mouse brain and muscle, it causes stress and inhibits auto-oxidative mechanisms, leading to oxidative tissue damage. Increased oxidative stress has been shown to lead to hypothalamic dysfunction, which in turn reduces pituitary gonadotropin production [9, 27, 28]. Although some studies have reported the toxic effects of fluoride exposure in the early developmental stages (in utero and in juveniles), reproductive toxicity of fluoride on offspring, such as effects on the sex hormones, histological structure of gonads, and the transcriptional profiles of steroidogenic-related genes, and the underlying toxicity mechanisms are still not fully elucidated.

Therefore, in this study, we established a model in which offspring received high-dose fluoride chronic treatment from in utero to puberty, aiming to analyze the expression levels of genes and proteins related to the synthesis of sex steroid hormones in the ovary and testis. The toxic mechanism of fluoride exposure to the reproductive system of offspring was further evaluated from in utero to puberty model.

Materials and Methods

Animals

Forty-eight healthy adult SD rats (male:female = 1:2), weighting about 180 to 200 g, along with the standard diet, were purchased from the Laboratory Animal Center of Shanxi Medical University (Taiyuan, Shanxi, People's Republic of China). The standard pellet feed (according to GB 14924.3–2010) was purchased from the Laboratory Animal Center of Shanxi Medical University (Taiyuan, China), and the ingredients were 47% corn, 29% soybean, 9% bran, 8% fish meal, and 3% fish meal. Stone powder 1.5%, calcium bicarbonate 1.3%, edible oil 1%, salt 0.3%, trace elements (copper, iron, zinc, selenium, iodine, manganese) 0.02%, vitamins A, B, C, D, E contained in 0.02%. Before the experiment, rats were placed in a temperature and lighted animal facility room raised with shavings to acclimate to the environment. After 7 days of acclimation, the female rats were randomly divided into control group and three NaF (A500850, Sangon Biotech, Shanghai, China) groups. Female rats and male rats were put together for mating at the 2:1 (female to male ratio), until the vaginal plug was discovered, which was considered as a successful mating. And the day was regard as embryonic day 0. Pregnant rats were then housed separately and freely distributed to the following exposure conditions: 25 mg/L, 50 mg/L, and 100 mg/L NaF groups, and the control group was received deionized water during pregnancy, 8 rats in each group. Dose selection was performed according to the article by Zhao and Wu et al. [29, 30]. All the experimental procedures were permitted by the Institutional Animal Care and Use Committee of Shanxi Agricultural University.

Pregnant rats were given corresponding treated water for free from the first day of pregnancy to the 21st day postpartum (42 days in total). The pups were given fluoride through breastfeeding at 3 weeks of age, and then 8 pups from each group were randomly selected for testing after weaning. The remaining pups continued to receive the same NaF treatment as their mothers until the end of dosing after 11 weeks of age. The body weight of the pups was recorded every 7 days.

Eight rats in each group were sacrificed by cervical dislocation at 3 weeks of age and 11 weeks of age pups according to animal ethics standards, and testis and ovaries were collected. The cauda epididymis and vas deferens were collected and used for semen quality testing. The left ovary and testis were fixed in Bouin's solution for histological observation and the right one were stored in the refrigerator at − 80 °C for subsequent experiments.

Femur Fluoride Determination

The content of femur fluorine was conducted as Li et al. (2020) described previously. The fluoride content in femur was calculated ($n = 8$/group) as follows: sample fluoride concentration (μg/g) = $(A \times 12.5)/(w/a)$.

Sperm Counts and Viability

The epididymal cauda was isolated and was cut ($n = 8$/group). The sperm were flushed out with a syringe and

placed in 1 mL physiological saline for 10 min at 37 °C to allow the sperms to fully swim out. Sperm counts and sperm viability were calculated using the method of Wu et al. (2019), which the statistical of results were expressed as density as 10^6/L and percentage, respectively.

Histological Examination

After rinsing, the ovary and testis samples ($n = 8$/group) were dehydrated in a series of alcohol, and then cleared in xylene and embedded in paraffin. The ovaries and testes were histologically examined using the H&E staining method as described by Li et al. (2020).

Sex Steroid Hormone Assay

After weighing the ovary and testis tissues ($n = 6$/group), the samples were homogenized in PBS with 1% PMSF on ice, and centrifuged at 5000 rpm for 10 min. The supernatant was collected to evaluate sex steroid hormones levels, including T, E2, P, FSH, LH, and Inhibin-B (INH-B). The concrete procedures were conducted based on the manufacture's manual of ELISA kit (Shanghai Jianglai Biotechnology Co, Ltd, China). The absorbance (OD) value of each sex steroid hormone was all measured at a wavelength of 450 nm by the microplate reader.

RNA Extraction and Quantitative Real-Time PCR

Quantitative RT-PCR was performed in 10 μL samples based on a previously described protocol [1]. Total RNA of the ovary and testis tissues ($n = 6$/group) were extracted with TRIZOL reagent (Takara, Dalian, China) according to the manufacturer's protocol. RNA transcription and qRT-PCR was detected as described previous methods in our laboratory [20, 31]. All primers (Table 1) were sequence-synthesized according to previous methods [30]. All RT-PCR samples were run in duplicate. The data were calculated using $2^{-\Delta\Delta CT}$ method.

Western Blotting Analysis

Western blotting experimental analysis was performed as previously described [1], total protein (50 mg tissue; $n = 6$/group) was extracted from the ovaries and testes of pups using the BCA protein assay kit (KeyGEN Biotech, Nanjing, China) and assayed protein concentration. Protein samples (60 μg) were loaded on the 10% SDS-PAGE gel and transferred on the nitrocellulose (NC) membrane, which was blocked in solution containing 5% (w/v) nonfat dry milk for 2 h with primary antibodies against StAR (A16432, 1:500, AB clonal, Wuhan, Hubei, China), 3β-HSD (bs-3906R, 1:500, Bioss, Beijing), CYP11A1 (bs-10099R, 1:500, Bioss, Beijing), CYP17A1 (bs-3853R,

Table 1 Primer sequences for RT-PCR

Gene	Primer sequences	Accession no	Product sizes (bp)
GAPDH	F: ACTCACCCACGGCAAGTTC R: TACTCAGCACCAGCATCACC	NM_017008.43	133
PGR	F:GAGCCCACAATATGGCTTTG R:AGTTATGCTGCCCTTCCATC	NM_022847.1	143
AR	F:ATTCCTGGATGGGACTGATG R:AAACCAGGTCAGGTGCAAAG	NM_012502.1	93
3β-HSD	F:TTCAGACCAGAAACCAAGGAG R:CGGTGTGGATGACAACAGAG	NM_001007719.3	133
CYP11A1	F:GCTTTGCCTTTGAGTCCATC R:CACTGGTGTGGAACATCTGG	NM_017286.3	123
CYP19	F:ACGTGGAGACCTGACAAAGG R:ACAGAGTGACGGACATGGTG	NM_017085.2	86
FSHR	F:GCAGGGACTTCTTCATCCTG R:GGCATGGAAGTTGTGGGTAG	NM_199237.1	101
StAR	F:CTGGAAGTCCCTCAAAGACC R:AGTGGCTGGCGAACTCTACT	NM_031558.3	120
CYP17A1	F:TGCACAATCCTGAGGTGAAG R:GACCGGTCATTGAAAGTTGG	NM_026160.4	88
17β-HSD	F:GGGTTTCACAGTGTTTGCTG R:TTATCTGCTCTGGCTTGGTG	NM_024391.1	122

1:700, Bioss, Beijing), or GAPDH (10494–1-AP 1:2000; Proteintech Group, Wuhan, China), respectively, overnight at 4 °C. After washing, the membranes were incubated with goat anti-rabbit HRP-conjugated secondary antibody (SA00001-2, 1:8000, Proteintech Group, Wuhan, China) for 2 h. Finally, target protein bands were observed by ECL (Beyotime Biotechnology, Shanghai, China) on a FluorChem Q imaging system analyzer (ProteinSimple, San Jose, California, USA).

Immunohistochemistry Analysis

The tissues were examined by immunohistochemistry to analyze the expressions of StAR, 3β-HSD, CYP17A1, CYP11A1. Detection was performed using previously described methods [7]. Briefly, sections (4 μm) were deparaffinized and rehydrated, followed by antigen retrieval. Sections were incubated in 3% H_2O_2 for 20 min, then treated with sodium citrate buffer for 10 min to recover antigen, and blocked with 5% BSA at 37 °C for 30 min. Thereafter, anti-StAR, anti-CYP11A1, anti-3β-HSD, and anti-CYP17A1 antibodies (1:100) were incubated at 4 °C for 24 h. Sections were then incubated with biotinylated goat anti-rabbit antibody (Boster, Wuhan, China) and streptavidin–biotin complex (SABC). Finally, the slices were observed by DAB and counterstained with hematoxylin and examined under a light microscope (Olympus BX53, Tokyo, Japan).

Statistical Analysis

The value of the experimental data is expressed as the mean ± SEM and analyzed using Graph Pad Prism 5 software (San Diego, California, USA). The collected data was analyzed by one-way analysis of variance (ANOVA), followed by multiple comparisons by Tukey's test, and differences of $*p < 0.05$ were considered statistically significant.

Results

Weight Changes and Femur Fluoride Concentration in Rat Offspring

At 3 weeks of age, the dose of NaF did not change the weight of the offspring of both sexes (Fig. 1A–B). However, the body weights of male offspring and female offspring of 11 weeks of age in the 50 and 100 mg/L NaF exposure groups were significantly reduced, respectively ($*p < 0.05$, $**p < 0.01$, Fig. 1C–D). In addition, as shown in Fig. 1E, the fluoride content in femur bone significantly increased in females and males exposed to NaF than in the offspring in the control group ($*p < 0.05$, $**p < 0.01$).

Sperm Quality Analysis

Compared with the control group, the sperm viability rate of male offspring at 11 weeks of age was significantly reduced in the 100 mg/L NaF exposure group ($**p < 0.01$), but the differences in the sperm counts were not statistically significant (Fig. 2A–B).

Effects of NaF on the Histology of Gonad Tissues in Rat Offspring

The gonad tissue structure was intact and well-developed follicles were tightly arranged in female offspring of 3 weeks of age (Fig. 3A). Figure 3B shows the effects of NaF exposure on the ovarian histopathology in females of 11 weeks of age. Compared with the control group, the proportion of mature and secondary follicles in the females of NaF exposure groups was significantly reduced.

According to our statistical analysis, after 11 weeks of fluorine exposure, the proportion of ovarian secondary follicles in the 50 and 100 mg/L NaF groups was significantly decreased by 22% and 21% compare to the control group, respectively (Fig. 2C, $*p < 0.05$). Moreover, the proportion of mature ovarian follicles decreased significantly by 15% and 12% in the 50 and 100 mg/L NaF groups, respectively (Fig. 2C, $*p < 0.05$, $**p < 0.01$). However, no significant differences were observed in the number of primary follicles between the four groups.

Furthermore, no significant histological changes in the testes were observed in the offspring at 3 weeks of age of all groups (Fig. 3C). At 11 weeks of age, the seminiferous tubules and cells in the male offspring of the control group were arranged in a uniform pattern. However, vacuolation of the seminiferous epithelium and shedding of spermatogenic cells were observed in the male offspring at 11 weeks of age in the 50 and 100 mg/L NaF exposure groups (Fig. 3D).

Sex steroid Hormone Levels in Gonad of Rat Offspring

At 3 weeks of age, the levels of sex steroid hormones in all NaF-exposed male and female pups were not significantly different from those in the control group pups (Fig. 4A–F). Moreover, no significant differences were observed in the LH, P, and INH-B levels in the NaF-exposed female pups at 11 weeks of age. E2 and FSH levels were significantly reduced by NaF in female offspring at 11 weeks of age (Fig. 4b–c, $*p < 0.05$).

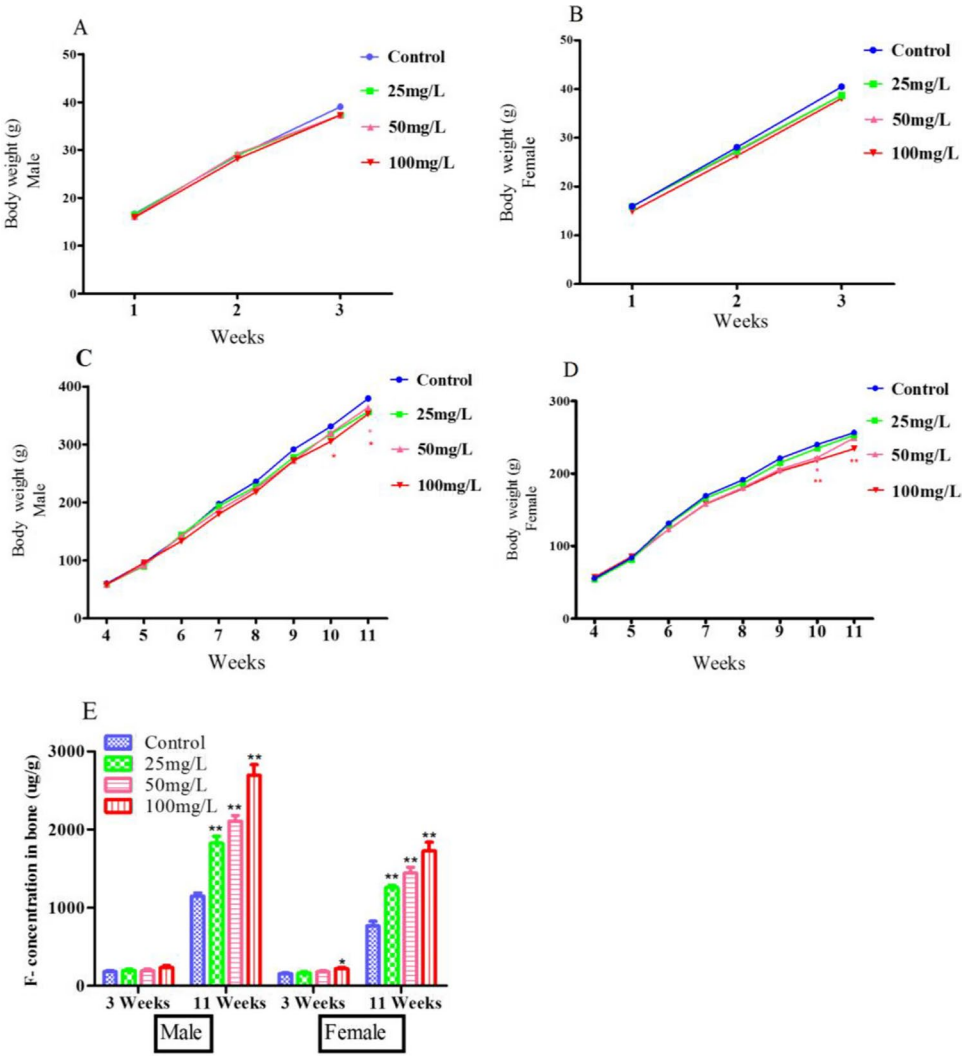

Fig. 1 Weight changes (**A**, **B**, **C**, **D**) and bone fluoride levels (**E**) in rat pups. The values are presented as mean ± SEM ($n = 8$). Asterisk indicates significant difference compared with the control group (*$p < 0.05$, **$p < 0.01$)

The T level significantly decreased in the testis of the male offspring exposed to 25 and 100 mg/L NaF at 11 weeks of age than in the offspring of the control groups (Fig. 4a, *$p < 0.05$, **$p < 0.01$). However, no significant effects of NaF on P, LH, E2, FSH, and INH-B levels were observed in the male offspring at 11 weeks of age.

mRNA Expression Levels of Steroidogenic-related Genes in Rat Offspring Ovaries and Testes

The relative mRNA expression of *StAR*, *CYP19*, *AR*, progesterone receptor (*PGR*), follicle-stimulating hormone receptor (*FSHR*), *CYP11A1*, *3β-HSD*, *CYP17A1*, and *17β-HSD* are shown in Figs. 5–6. At 3 weeks of age, no significant difference was observed in the offspring between the NaF exposure groups and the control group in both sexes. However, at 11 weeks of age, the mRNA levels of *3β-HSD*, *CYP11A1*, *17β-HSD*, *CYP17A1*, and *StAR* were evidently downregulated in the testes of male offspring exposed to 100 mg/L NaF (Fig. 5, *$p < 0.05$), however, mRNA levels of *CYP19*, *FSHR*, *PGR*, and *AR* remained unchanged in all NaF exposure groups.

In female offspring at 11 weeks of age, mRNA expression levels of *3β-HSD*, *StAR*, *CYP17A1*, and *CYP11A1* significantly decreased in the 50 and 100 mg/L NaF exposure groups compared with the control group (Fig. 6, *$p < 0.05$), but no significant changes in the mRNA expressions of *CYP19*, *17β-HSD*, *FSHR*, *PGR*, and *AR* were observed.

Steroidogenic-related Proteins in the Ovaries and Testes of Rat Offspring

We evaluated the protein levels associated with steroid hormone synthesis in offspring at 11 weeks of age using IHC method and western blotting. In the testes, compared with the control group, the StAR, 3β-HSD and CYP17A1 protein

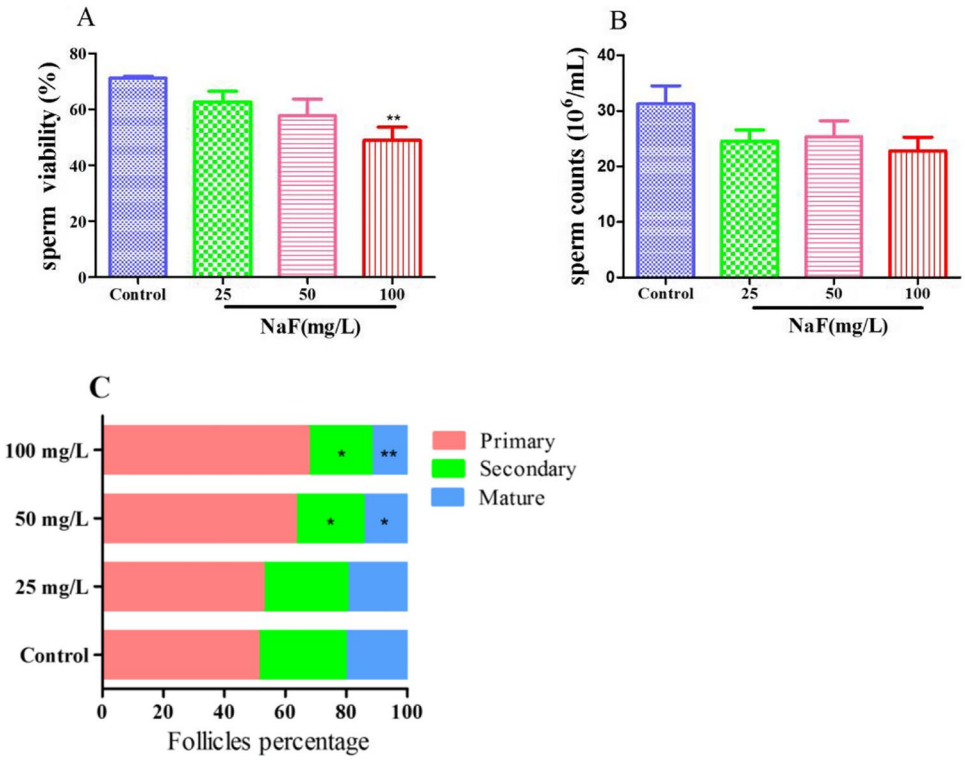

Fig. 2 Effects of NaF exposure on sperm quality (A and B), and follicle counts (C) at 11 weeks of age (n=8, mean ± SEM). Asterisk indicates significant difference compared with the control group (*$p < 0.05$, **$p < 0.01$)

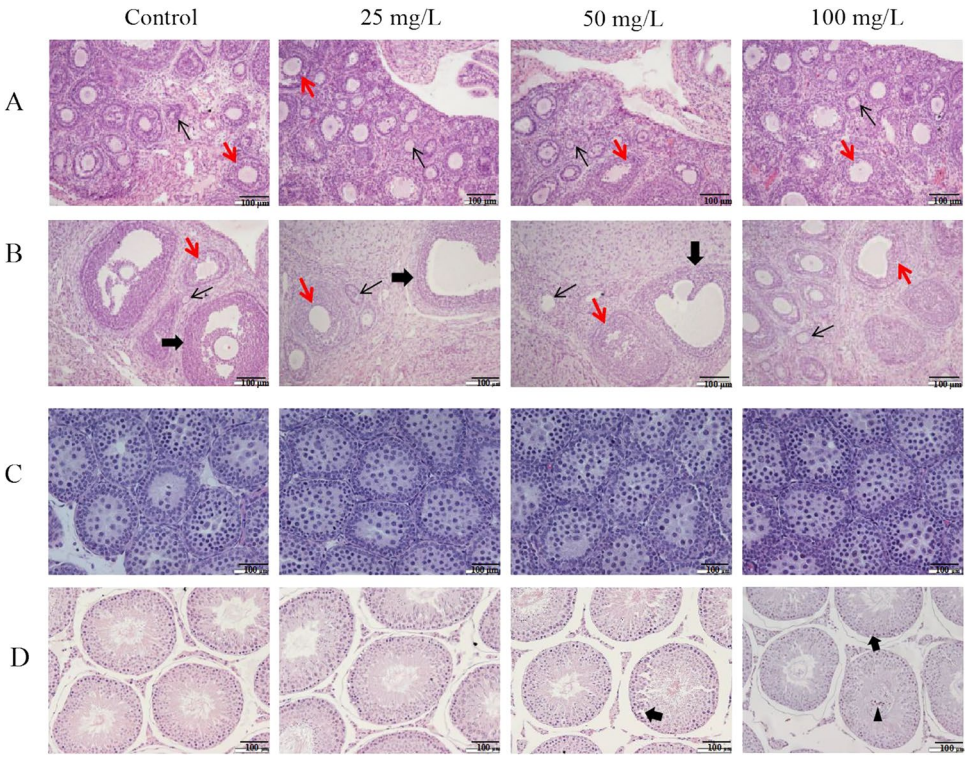

Fig. 3 Effects of NaF on ovarian and testicular histology at 3 and 11 weeks of age (n=8). Effects on female offspring at 3 weeks of age (**A**) and 11 weeks of age (**B**). Effects on male offspring at three weeks of age (**C**) and 11 weeks of age (**D**). A and B: The thick arrowhead points to the primary follicle; Red arrowhead points to the secondary follicle; The fine arrowhead points to the mature follicle. C and D: The thick arrowhead indicates vacuolation of spermatogenic epithelium; Triangle arrowhead indicates cell shedding

expression levels of 100 mg/L NaF were significantly reduced, and the CYP11A1 protein expression levels of 50 and 100 mg/L NaF were still markedly reduced (Fig. 7B, *$p < 0.05$, *$p < 0.01$). The results of the IHC showed that StAR, 3β-HSD, CYP11A1, and CYP17A1 proteins were co-localized in rat Leydig cells (Fig. 7A). In the ovaries, compared with the

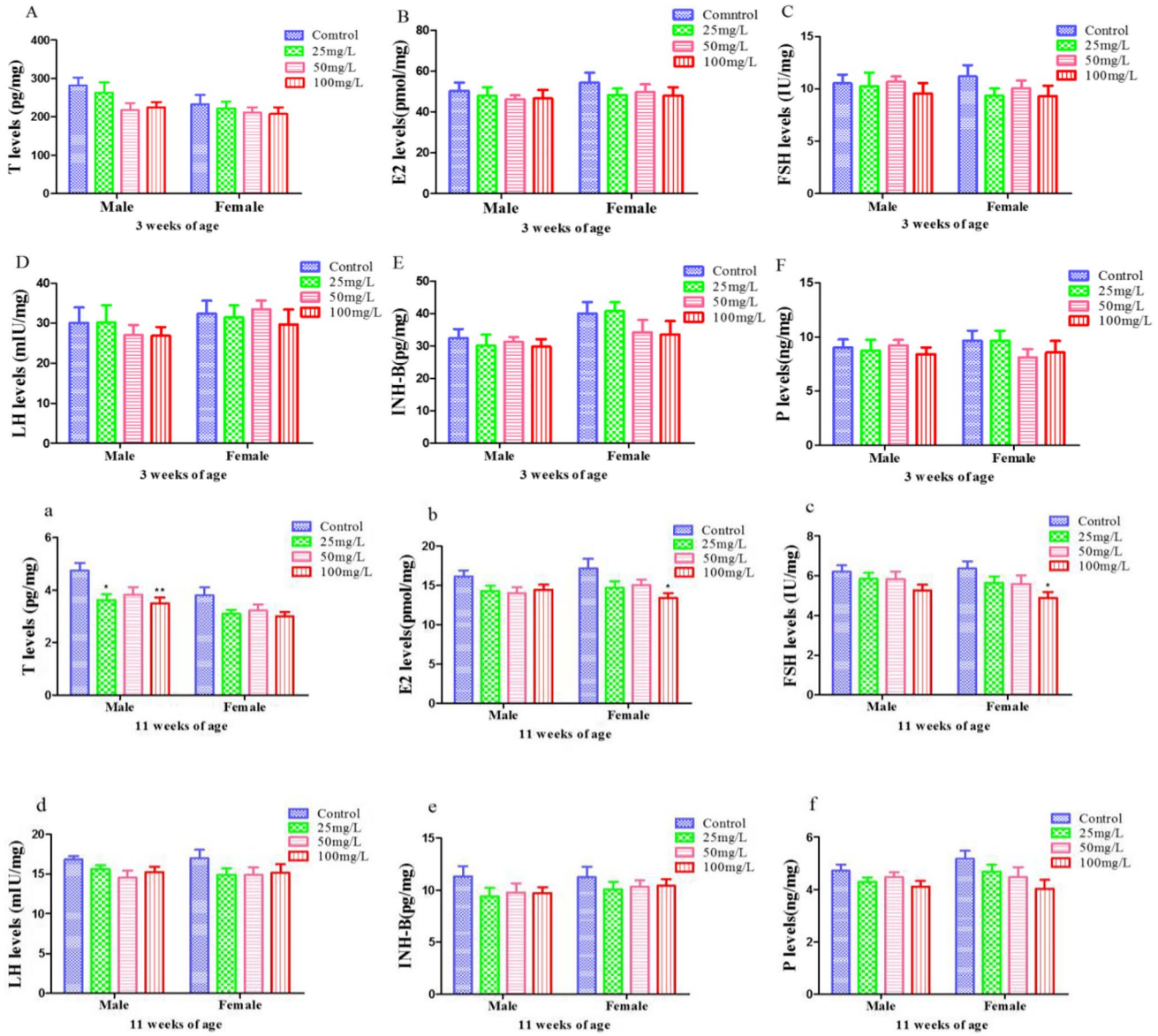

Fig. 4 Effect of fluoride exposure on the concentration of T, E2, LH, FSH, P and INH-B in the testis and ovary of female and male offspring at 3 weeks of age (A-F) and 11 weeks of age (a-f). The values are presented as mean ± SEM (n=6). Asterisk indicates significant difference compared with the control group (*$p < 0.05$, **$p < 0.01$)

control group, the StAR protein expression levels of 50 and 100 mg/L NaF were significantly reduced, and the protein expression levels of 3β-HSD and CYP17A1 of 100 mg/L NaF were significantly reduced. The expression level of CYP11A1 protein in the 25 and 100 mg/L NaF was significantly reduced (Fig. 8B, *$p<0.05$, **$p<0.01$). The results of immunohistochemistry showed that CYP17A1 and 3β-HSD were mainly located in follicular membrane cells and granulosa cells. StAR was localized in corpus luteum and granular cells, and CYP11A1 was localized in granular cells (Fig. 8A).

Discussion

Multiple investigations have shown that exposure to high concentrations fluoride during pregnancy and childhood can hinder the growth and physical health of offspring and young children [6, 25, 32, 33, 34]. In this context, we established a fluoride exposure model from in utero to puberty stages in rats. Previous studies demonstrated that excess fluoride mainly gets deposited on bones [35]. Additionally, other studies indicated that the fluoride concentration in bones can be used as an indicator of environmental fluoride exposure [36]. The toxicity of

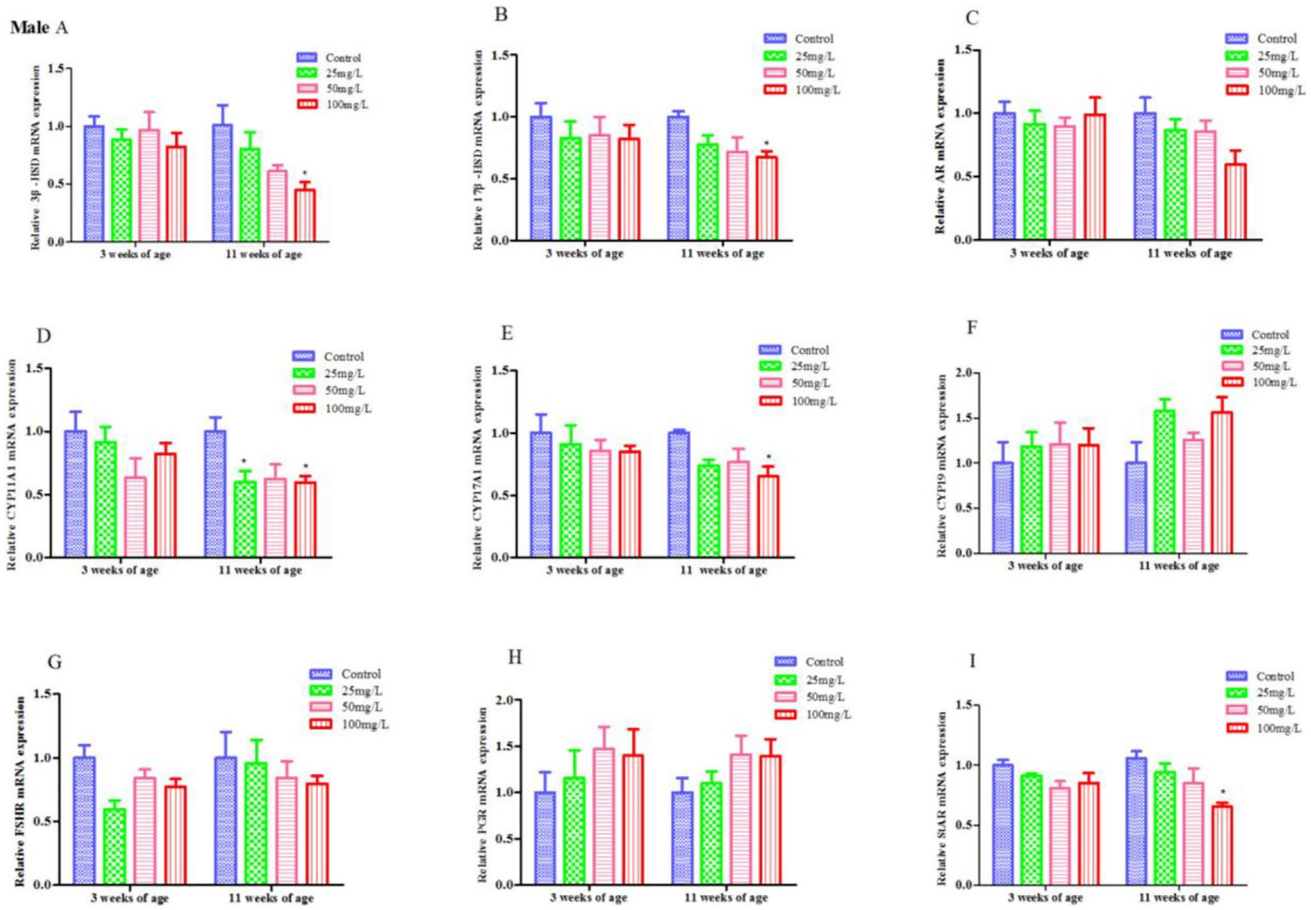

Fig. 5 Effects of NaF on 3β-HSD, 17β-HSD, AR, CYP11A1, CYP17A1, CYP19, FSHR, PGR, StAR mRNA expression of steroidogenic-related genes in male offspring. The values are presented as mean ± SEM (n=6). Asterisk indicates significant difference compared with the control group ($*p < 0.05$)

various environmental pollutants changes with the growth and developmental stages of organisms [31, 37]. Studies have shown that the body weight of male mice offspring was significantly reduced after fluoride exposure from the pregnancy to post-puberty stages [38]. This was consistent with the findings of the present study, which reported that fluoride exposure from in utero to puberty stages significantly reduced the body weight of male and female offspring at 10 and 11 weeks of age. Remarkably, bone fluoride concentration increased after fluoride exposure, confirming the successful establishment of prenatal and postnatal fluoride exposure animal models.

Histopathological observations can be used to analyze the morphological changes in the ovarian and testicular tissues to some extent. Recently, some researches have demonstrated that fluoride exposure can induce toxicity in male and female reproductive systems [12, 39]. Zhou et al. (2013a) showed that NaF exposure for 6 months damaged the ovarian structure and reduced the follicle number in rats and decreased number of mature follicles suppresses ovulation and reduces fertility [40, 41]. Consistent with these reports, in the present study, we observed the structure of ovaries and testis were significantly. These results were consistent with those reported in previous studies [42–44], suggesting fluoride significantly inhibited the development and maturation of follicles. In male, we speculate that the low sperm motility caused by sodium fluoride may be due to the destruction of the structure and function of sperm flagella, and the disordered arrangement of spermatogenic cells leads to fertilization failure.

Sex steroid hormones are secreted mainly by the gonads, and are one of the most integrative and functional endpoints for reproduction [45, 46]. In this study, the levels of sex hormones were significantly reduced in both sexes exposed to NaF. Specifically, the levels of E2 were significantly reduced in the female offspring. These results were consistent with the findings of Zhou et al. (2013a), who demonstrated that prenatal and postnatal fluoride exposure could impair the endocrine function of rat offspring by inhibiting the sex steroids hormone levels, which could be due to damages in the

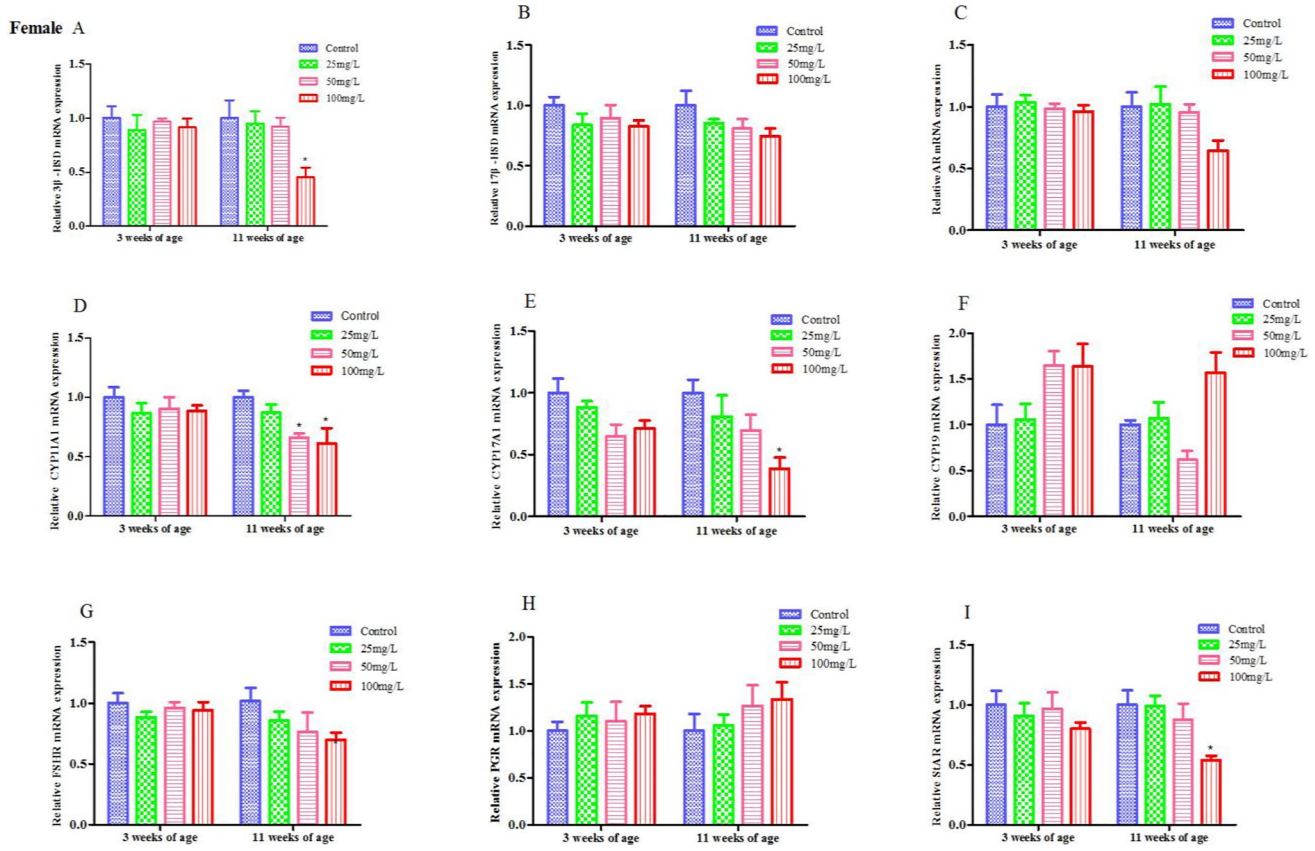

Fig. 6 Effects of NaF on 3β-HSD, 17β-HSD, AR, CYP11A1, CYP17A1, CYP19, FSHR, PGR, StAR mRNA expression of steroidogenic-related genes in female offspring. The values are presented as mean ± SEM (n=6). Asterisk indicates significant difference compared with the control group (*$p < 0.05$)

reproductive system as confirmed by the histopathological observations of this study. The T concentrations significantly decreased in the male offspring exposed to NaF, while the E2 levels in the male offspring did not significantly differ between the NaF exposure and control groups. This result was consistent with previous reports of fluoride exposure in mice [9]. Decreased testosterone levels in offspring male rats may be due to fluoride-induced male aromatase, which promotes the conversion of testosterone to estrogen, which in turn interferes with T synthesis.

In the feedback loop of the hypothalamic-pituitary-gonad (HPG) axis, FSH and LH are two important gonadotropin hormones secreted by the pituitary gland. These hormones induce the production of sex steroid hormones, T and E2 [47, 48]. In this study, the FSH level in the female offspring was significantly reduced at 11 weeks of age, which is consistent with the findings by Zhou et al. (2013a), thus, indicating that fluoride weakened the promotion activity of FSH on ovarian particulate cells. The FSH and LH concentrations in males were not affected by fluoride, similar to the results of gene expression, we speculate from these results that the mechanism by which fluoride affects low testosterone levels in male offspring rats does not appear to be related to the effects of LH and FSH, and may be physiological differences between the females and males. We speculate that men are less sensitive to fluoride than women [9, 12, 13].

Several studies suggested that altered steroid hormones as a result of fluoride exposure were associated with the expressions of steroidogenic pathway genes, including *StAR*, *CYP11A1*, *3β-HSD*, *17β-HSD*, *CYP17A1*, and *CYP19* [9, 49–51]. StAR is the most significant mediator and rate-limiting factor in the steroid production pathway, which is necessary for the passage of cholesterol through the mitochondrial membrane (an important step during steroid production) [50, 51]. After cholesterol is transported to the inner mitochondrial membrane, it is converted into pregnenolone though CYP11A1, which is the first and slowest step in steroid production. Owing to the catalytic action of 3β-HSD, pregnenolone is immediately converted to progesterone, and progesterone is converted to androstenedione under the action of CYP17A1. Finally, T is transformed from androstenedione by the action of 17β-HSD [52]. In this study, the protein levels of StAR, 3β-HSD, CYP11A1, and CYP17A1 were significantly reduced, which is consistent with the protein

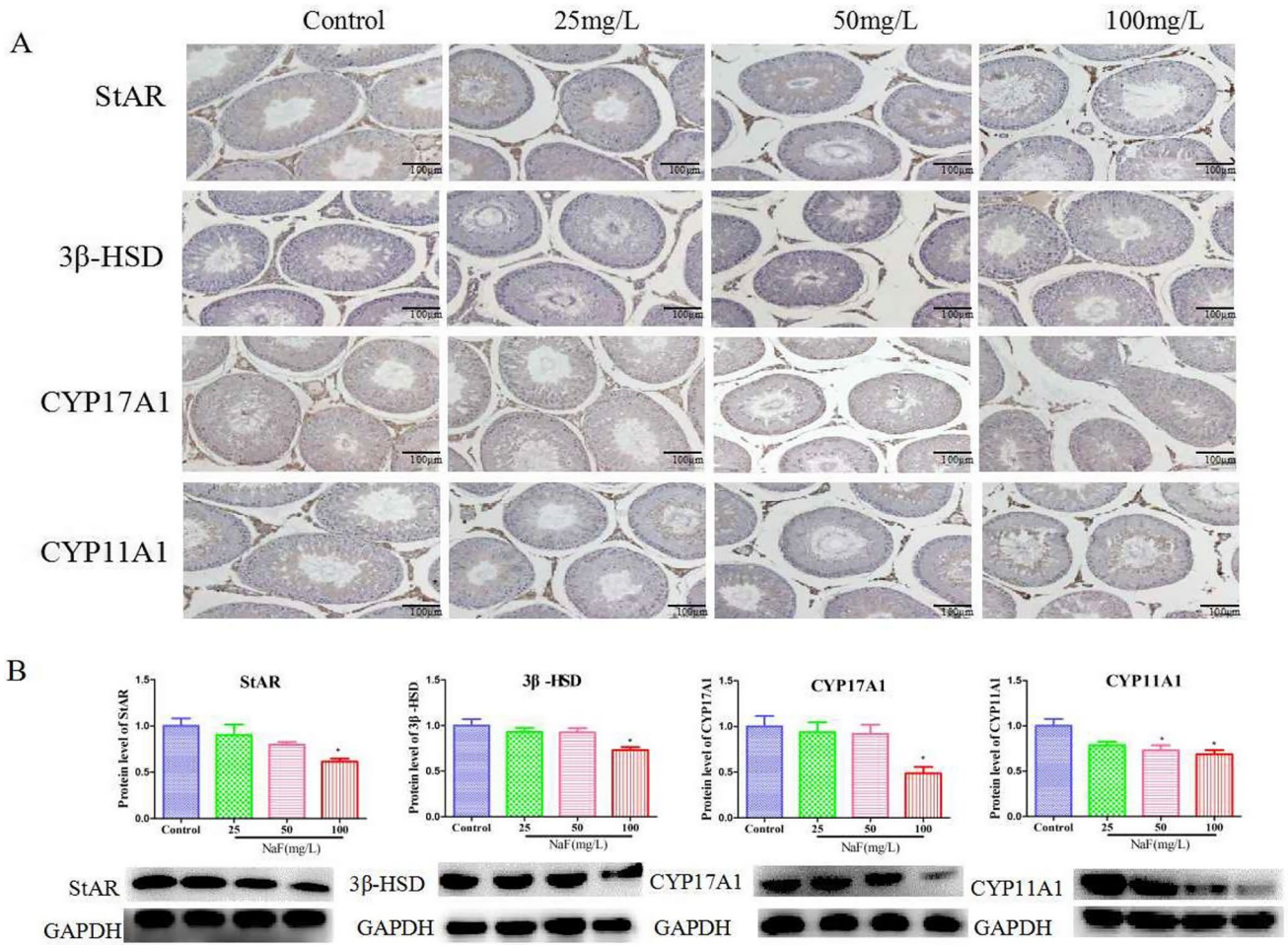

Fig. 7 Immunohistochemistry (**A**) and western blotting analysis (**B**) for steroidogenic-related genes in male offspring at 11 weeks of age. Immunohistochemistry reflects the identification of the expression sites of StAR, 3β-HSD, CYP17A1 and CYP11A1. The protein levels of StAR, 3β-HSD, CYP17A1 and CYP11A1 of testis in male rats. The values are presented as mean ± SEM (n=6). Asterisk indicates significant difference compared with the control group (*$p < 0.05$, **$p < 0.01$)

expression of immunohistochemistry. We also found that in the testis, they are mainly co-localized in mesenchymal cells. On the other hand, in females, they mainly locate granular cells, membrane cells, and corpus luteum cells. Therefore, we speculated that these cells, such as mesenchymal cells, granular cells, membrane cells, and corpus luteum cells, are the main sites for the production of T and E2. The decreased expression of *StAR* in this study could be attributed to the disruption of cholesterol transport due to high NaF concentration. The mRNA and protein expressions of *CYP11A1*, *3β-HSD*, and *CYP17A1* were downregulated in the NaF exposure groups after 11 weeks of age, implying that the E2 production was influenced by NaF in the ovaries of the female offspring. In males, the expressions of *CYP11A1*, *3β-HSD*, *17β-HSD*, and *CYP17A1* mRNA were significantly inhibited, which may have further decreased T. The enzyme CYP19 is important as it catalyzes the conversion of androgens to estrogen. Although changes were observed in the *CYP19* mRNA results, these changes were not statistically significant. This result differed from that of another study, in which *CYP19* mRNA levels significantly increased in NaF-treated rats [9, 46]. This indicates that the steroid-dependent mechanism of fluoride exposure in offspring rats regulates T-induced E2 release in the body, but this does not necessarily involve a significant increase or decrease in the transcription level of CYP19, and there may be unproven regulation. Overall, the downregulated steroidogenic-related genes indicated that long-term excessive intake of fluoride could disturb steroidogenesis in female and male offspring.

Taken together, our findings suggested that long-term fluoride exposure from in utero to puberty inhibited the growth and development of rat offspring, caused ovaries

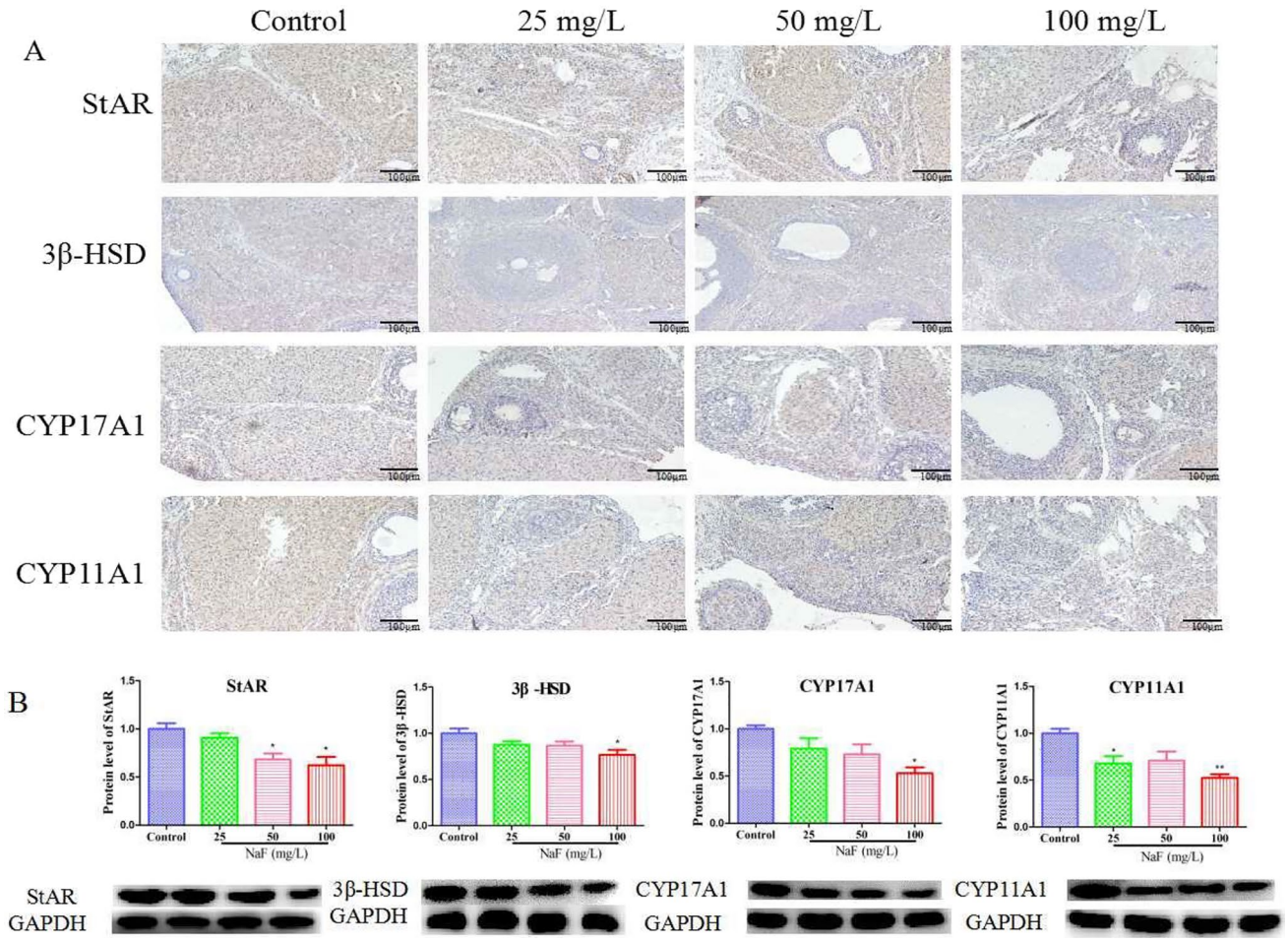

Fig. 8 Immunohistochemistry (**A**) and western blotting analysis (**B**) for steroidogenic-related genes in female offspring at 11 weeks of age. Immunohistochemistry reflects the identification of the expression sites of StAR, 3β-HSD, CYP17A1 and CYP11A1. The protein levels of StAR, 3β-HSD, CYP17A1 and CYP11A1 of ovary in female rats. The values are presented as mean ± SEM (n=6). Asterisk indicates significant difference compared with the control group (*p < 0.05, **p < 0.01)

and testes structural damage, reduced follicular maturation and sperm motility, and decreased T, E2, and FSH levels. In addition, fluoride exposure altered the expression of several important steroid genes, thereby disrupting the balance of steroid hormones. Our study compared the effects of fluoride exposure from in utero to puberty on the female and male reproductive systems and explored the underlying mechanisms, which provided a theoretical basis for the reproductive toxicity study of fluoride.

Funding This work was supported by the National Natural Science Foundation of China (Grant no. 31672623, 31372497) and Veterinary Environmental Lab (Shanxi Key Lab).

Declarations

Competing Interest The authors declare no competing interests.

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