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Chronic fluoride exposure-induced testicular toxicity is associated with inflammatory response in mice

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HIGHLIGHTS

- Chronic fluoride exposure affected sperm quality.
- Chronic fluoride exposure injured testicular histomophology.
- Chronic fluoride exposure elevated expressions of testicular inflammatory mediators.
- Chronic fluoride exposure increased testicular NO concentration.

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ABSTRACT

Previous studies have indicated that fluoride (F) can affect testicular toxicity in humans and rodents. However, the mechanism underlying F-induced testicular toxicity is not well understood. This study was conducted to evaluate the sperm quality, testicular histomorphology and inflammatory response in mice followed F exposure. Healthy male mice were randomly divided into four groups with sodium fluoride (NaF) at 0, 25, 50, 100 mg/L in the drinking water for 180 days. At the end of the exposure, significantly increased percentage of spermatozoa abnormality was found in mice exposed to 50 and 100 mg/L NaF. Disorganized spermatogenic cells, vacuoles in seminiferous tubules and loss and shedding of sperm cells were also observed in the NaF treated group. In addition, chronic F exposure increased testicular interleukin-17(*IL-17*), interleukin-17 receptor C (*IL-17RC*), tumor necrosis factor-α (*TNF-α*) and interleukin-6 (*IL-6*) in transcriptional levels, as well as IL-17 and TNF-α levels in translational levels. Interestingly, we observed that F treated group elevated testicular inducible nitric oxide synthase (*iNOS*) mRNA level and nitric oxide (NO) concentration. Taken together, these results indicated that testicular inflammatory response could contribute to chronic F exposure induced testicular toxicity in mice.
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1. Introduction

Long-term excessive fluoride (F) intake is known to be toxic and can damage reproductive tissues (Huang et al., 2007; Wang et al., 2009; Li et al., 2015; Han et al., 2015). The importance of reproductive health to offspring development has prompted epidemiological investigations into the apparent connection between excessive F exposure to male infertility and low birth rates (Freni, 1994; Ortiz-Pérez et al., 2003). *In vivo* and *in vitro* studies further demonstrated a negative impact of F ingestion on testis, spermatogenesis and leydig cells (Spittle, 2008; Sun et al., 2010; Song et al., 2014). Previously, our study indicated that F adversely affected sperm motility, hyperactivation and sperm chemotaxis (Sun et al., 2011; Zhang et al., 2006; Lu et al., 2014). However, the molecular mechanisms underlying fluoride-induced testicular toxicity are not well understood.

The role of cytokines in male reproductive function has been widely reported (Diemer et al., 2003; Guazzone et al., 2009). Lampiao and du Plessis (2009) has found that tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) have an effect on sperm function, and the effect is possibly mediated via an increase in nitric oxide (NO) production. A good deal of evidences have suggested that

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interleukin-17 (IL-17) plays an important role in orchitis especially in experimental autoimmune orchitis (EAO) (Jacobo et al., 2011; Komiyama et al., 2006; Garret-Sinha et al., 2008; Jandus et al., 2008). Although a number of studies have indicated F exposure disrupts testicular development (Dvoráková-Hortová et al., 2008; Sun et al., 2010), it is not clear whether F alters the levels of testicular cytokines, such as IL-17, TNF-α, and IL-6. Interestingly, a recent study has confirmed F also up-regulates the expression of inducible nitric oxide synthase (iNOS), which is key enzyme for NO synthesis (Zhang et al., 2013). Given on the role of cytokines and NO in male reproductive, we propose a hypothesis that F-induced testicular toxicity is ascribed to reproductive inflammatory response.

To prove this hypothesis, we used a mice model to simulate the situations where a human being may be exposed to chronic fluoride and evaluated testicular histology and sperm quality. Subsequently, we determined levels of cytokines and NO in testis.

2. Materials and methods

2.1. Materials and chemicals

Trizol was obtained from Invitrogen (Carlsbad, CA, USA). SYBR® Premix Ex Taq™ II QRT-PCR kit was purchased from Takara Biotechnology Company (Dalian, China). All the real-time PCR primers were provided by Beijing AuGCT biotechnology Co., Ltd (Beijing, China). Bicinchoninic acid (BCA) kit was obtained by Beyotime Institute of Biotechnology (Shanghai, China). ELISA kit was supplied by Shanghai westang bio-tech co., LTD (Shanghai, China). NO kit was prepared by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other reagents used were of analytical grade.

2.2. Animals

Eighty adult male Kunming mice (aged 8 weeks, 25–26 g b.w.) were purchased from the Experimental Animal Center of Shanxi Medical University (Taiyuan, China), along with supplies of their standard diet. After one-week acclimation, these mice were divided randomly into four groups of 20 each: a control group, which was drank distilled water, and other three treatment groups, which were got 25, 50, and 100 mg NaF/L in their drinking water. The doses were chosen on the basis of the previous studies associated with reproductive and other toxicities of fluoride and modified (Barbier et al., 2010; Long et al., 2009; Zhang et al., 2013). To make sure more than four spermatogenic cycle in mice (approximately 35 days) (Amann, 1986; Sun et al., 2010), animals were kept for 180 days. All mice were provided with standard laboratory diets and ultrapure water under standard temperature (22–25 °C), a 12-h light/dark cycle, ventilation, and hygienic conditions. The approval of the Institutional Animal Care and Use Committee was followed for this study.

2.3. Testis and sperm sample preparation

All mice were killed by cervical dislocation on the 180th day. Sperm from the cauda epididymis and vas deferens were allowed to disperse into 1 ml PBS medium at 37 °C.Testes were immediately isolated and weighted. One part of the right testis was fixed in Bouin's solution for histological analysis while the left testis was frozen immediately in liquid nitrogen and stored at −80 °C for RNA isolation and protein extraction.

Table 2
Primer sequences with their corresponding PCR product size.

Gene	Sequences (5′-3′)
18s	F: TACCACATCCAAGAAGGCAG R: TGCCCTCCAATGGATCCTC
IL-17	F: AAAGCTCAGCGTGTCCAAAC R: ACGTGGAACGGTTGAGGTAG
IL-17RC	F: CACATCAGTCTGTGCCTTGG R: TGTGATCGGAAGTCTTGCAG
IL-6	F: CTGATGCTGGTGACAACCAC R: TCCACGATTTCCCAGAGAAC
TNFa	F: TATGGCTCAGGGTCCAACTC R: GCTCCAGTGAATTCGGAAAG
iNOS	F: TGGTGGTGACAAGCACATTT R: AAGGCCAAACACAGCATACC
IL-21	F: TTGCACAGCAGTCTTGAACC R: AGCTTACACCAGTGGCAACC
IL-1β	F: GCTGCTTCCAAACCTTTGAC R: TTCTCCACAGCCACAATGAG
TGF-β	F: TGGAGCAACATGTGGAACTC R: TGCCGTACAACTCCAGTGAC

2.4. Morphological examination

Briefly, the testes were fixed in Bouin's solution, embedded in paraffin, deparaffinized in dimethylbenzene, hydrated in gradient ethanol and rinsed with distilled water, and sectioned perpendicular to the longest axis of the testis at a 5 μm thickness. The consecutive sections were stained with hematoxylin–eosin (HE) for light microscopic examination.

2.5. RNA extraction and real-time PCR

Total RNA in the testis was isolated according to the TRizol reagent manufacturer's instructions. The A260/280 ratio was got to be in the range of 1.8–2.0 by the Nanodrop ND-1000 spectrophotometer (Nano drop Technologies Inc., DE, USA). The total RNA was also electrophoresed on a 0.8% agarose gel (Sigma, St. Louis, MO) to visually assess RNA quality. The total RNA was converted into cDNA with 300 μg total RNA. *IL-17*, *IL-6*, *TNF-a*, *IL-21*, *TGF-β*, *IL-1β* and *iNOS* primers (Table 2) were designed with Primer 3.0 plus. QRT-PCR was used the Mx3000P™ QRT-PCR system (Stratagene, USA) and SYBR® Premix Ex Taq™ II QRT-PCR kit. The conditions for real-time PCR were as follows: after initial denaturation at 95 °C for 15 s, 55 PCR cycles were started with thermocycling conditions at 95 °C for 5 s, 61 °C for 15 s, and 72 °C for 6 s, and then a melting curve analysis was performed to verify the specificity of the PCR product. Every sample was analyzed in triplicate. System software and the $2^{-\Delta\Delta Ct}$ method were applied to quantitative calculation.

Table 1
Effects of NaF exposure on body weight gain, sperm count and morphology in mice.

Dose (mg/L)	Control	25	50	100
body weight gain (n = 15)	11.290 ± 0.568	11.160 ± 0.489	9.640 ± 0.448	10.490 ± 0.699
sperm count (10^6/ml, n = 6)	4.986 ± 0.440	4.483 ± 0.533	3.392 ± 0.277*	3.920 ± 0.453
sperm morphology (n = 9)	40.51 ± 0.85	44.82 ± 2.55	57.68 ± 3.68**	52.26 ± 3.17*

*$P < 0.05$, **$P < 0.01$ (compared with the control group).

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