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FLUORIDE REDUCED CRISP2 EXPRESSION IN TESTIS AND EPIDIDYMAL SPERM OF RATS

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ABSTRACT: Fluorine, an active element which is widely distributed in the environment, has been demonstrated to have adverse effects on male reproductive systems at a high dose. The objective of the present study was to explore the key role of cysteine-rich secretory protein (CRISP)2 in fluoride-induced male reproductive toxicity by investigating, in rats, the effect of the administration of fluoride on the expression of CRISP2 in the testes, in the immature sperm in the initial segment of the epididymis, and in the mature sperm in the tail of the epididymis. In this study, after mating, female pregnant rats were randomly divided into four groups: control (distilled water) and NaF (25, 50, and 100 mg L^{-1}) groups. After weaning, the dosage was continued for 8 weeks for 36 puppies. After exposure, the histological structure of the testis was assessed using HE, and the localization and protein expression of CRISP2 in the rat testis and epididymal sperm were evaluated by indirect immunofluorescence. In addition, sperm survival and mitochondrial transmembrane potential were evaluated. The results showed that fluoride impaired the histological structure of the testis, decreased the level of CRISP2, and reduced sperm survival and mitochondrial transmembrane potential. Together, our present data suggest that the decreased fertility in rats affected by fluorosis might be, in part, through the low expression of CRISP2 in testis and epididymal sperm.

Key words: CRISP2; Fluoride; Sperm; Testis.

INTRODUCTION

Fluorine is widely distributed in the environment and is an active element existing only as compounds in combination with other elements.[1] It is well-known that high levels of fluoride, the ion of fluorine, have a considerable impact on humans and animals. Dental and skeletal fluorosis are the most typical clinical manifestations of fluorosis.[2] Moreover, it has been recognized that a high fluoride intake is not only detrimental to the skeletal system but also to the non-skeletal systems, such as nervous,[3,4] immune,[5,6] and reproductive systems.[7,8]

Since Schulz discovered the reproductive toxicity of fluoride in 1925,[9] there has been increasing evidence suggesting fluoride has a toxic effect on the male reproductive system, including the testis, epididymis, and sperm.[10-12] Mammalian fertilization is a complex process, consisting of male and female gametes recognizing, binding and eventually fusing with each other, which is mediated by multiple proteins on the surfaces of the gametes.[13] Our previous study found by the proteomic technique that the epididymal proteins of mice exposed to NaF were altered, such as several important proteins cysteine-rich cytochrome c, testis-specific (Cyct), sorbitol dehydrogenase (Sord), glutathione S-transferases (GSTs), cysteine-rich secretory protein (CRISP)1, and CRISP2.[14] The CRISP family is a group of cysteine-rich secretory proteins among mammalian species, in which CRISP2 is a testicular protein which has been shown to attach to the sperm in the testicular

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lumen.[15] The previous studies revealed that CRISP2 is a regulator of calcium influx through ryanodine receptors,[16] modulating sperm flagellar motility,[17] and implicating sperm-egg fusion.[18]

Therefore, the purpose of this study to see if new clue might be found for unraveling the toxic effects of fluoride on the male rat reproductive system by investigating the effects of fluoride on the expression of CRISP2 in the testis and epididymal sperm of rats exposed to NaF.

MATERIALS AND METHODS

Animals and treatments: Ten male and 20 female 8 week-old rats, weighing 200±10g, were obtained from the Experimental Animal Center of Shanxi Medical University (Taiyuan, Shanxi, China). After one-week of acclimation, 20 female rats mated with 10 normal male rats. Once the vaginal plug was observed in the morning, the female rats were divided into four groups, control (distilled water) and NaF (25, 50, and 100 mg L^{-1}) groups. After weaning, 9 offspring male rats of each group were selected and the fluoride intake, as given to their mothers, was continued for 8 weeks. During the treatment period, all rats had a standard diet and controlled conditions—temperature (22–25ºC), 12/12-hr light/dark cycle, good ventilation, and hygienic conditions. All the experimental procedures were approved by the Experimental Animal Ethics Committee of Shanxi Agricultural University (Taigu, Shanxi, China).

Histological analysis of testis: After the rats were sacrificed by cervical dislocation, samples of testis were immediately fixed in Bouin's solution for 24 hr and rinsed with running water. The samples were then transferred to different grades of alcohol, infiltrated with molten paraffin wax, and finally embedded in paraffin. The resulting paraffin block was cut into small sections and stained with hematoxylin and eosin (H&E). The histological structure of testes was observed with an Olympus BX51 microscope equipped with a CCD DP70 video camera (Olympus Optical, Tokyo, Japan).

Sperm isolation and evaluation: After 8 weeks of exposure, the control and treated rats were sacrificed by cervical dislocation. Sperm from the initial segment and the cauda epididymis were collected and dispersed into 5 mL normal saline solution at 37ºC for further experimentation. Then, 10μL of sperm suspension was placed on a red blood cell count plate to count sperm survival using an Olympus BX51 microscope (Olympus Optical, Tokyo, Japan).

The mitochondrial membrane potential (ΔΨm) of sperm was evaluated by JC-1 (Beyotime Biotechnology, Beijing, China) under a BX53F fluorescent microscope (Olympus Optical, Tokyo, Japan), and the quantitative analysis of ΔΨm was carried out through the ratio of red fluorescence and green fluorescence, which were calculated by Image-Pro Plus (Version 5.1, Media Cybernetics, MD, USA).

Indirect immunofluorescence: Indirect immunofluorescence was used to determine the localization of CRISP2 in the testis and sperm. The sperm samples were smeared onto slides and fixed with cold methanol for 5 minutes. Sperm smears and testis sections were blocked with 5% bovine serum albumin at 37ºC for 1 hr. After blocking, the slides (1:200) and testis sections (1:400) were treated with the primary antibody (anti-CRISP2, Bioss, Beijing, China) overnight at 4ºC. After washing with PBS three times, the slides and testis sections were incubated with the IgG-FITC

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secondary antibody (1:1000, Bioss, Beijing, China) for 2 hr at room temperature in the dark and then washed again three times with PBS. Finally, the slides and testis sections were sealed with a sealant containing DAPI and images were obtained with a BX53F fluorescent microscope (Olympus Optical, Tokyo, Japan).

Statistical analysis: The experimental data between the groups were analyzed using one-way ANOVA with GraphPad Prism 8 software (GraphPad Software Inc., San Diego, USA). The differences were considered statistically significant if $P<0.05$. Results were represented as mean ± SEM.

RESULTS

Representative images of testis are shown in Figure 1. The testis of the control group exhibited normal features with a large number of sperm found in the seminiferous tubules, and well-arranged spermatogenic cells. In comparison to the control group, increased intertubular space and irregularly arranged spermatogenic cells were found in the NaF group.

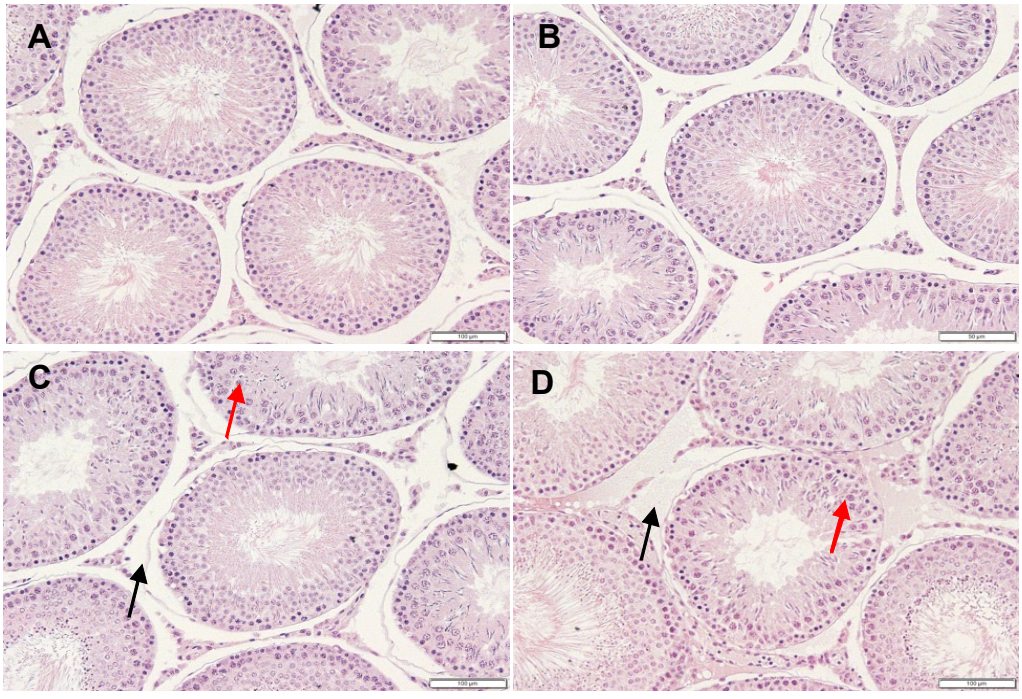

Figure 1. Testis histological changes in rats treated with different doses of NaF by H&E staining (200×); A: control; B: 25 mg L^{-1}; C: 50 mg L^{-1}; D: 100 mg L^{-1}.). Black arrows: increased intertubular space; Red arrows: the irregularly arranged spermatogenic cells. Length of marker bar in lower right corners=100 μm.

The results of sperm survival and the ΔΨm are shown in Figures 2 and 3. In a dose-dependent manner, sperm survival in fluoride treatment groups was lower compared to the control group. One hundred mg L^{-1} NaF significantly reduced sperm survival ($P < 0.01$), while no significant changes were observed in the 25 and 50 mg L^{-1} NaF groups ($P > 0.05$). A significant decrease in the ΔΨm of sperm from the epididymal initial segment was found with different concentrations (25, 50, and 100 mg L^{-1}) of NaF compared to the control ($P < 0.05$). Furthermore, the ΔΨm of sperm from the epididymal cauda in 100 mg L^{-1} NaF was decreased significantly ($P < 0.01$), in

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comparison to the control group, while no significant changes were found in the other two fluoride exposed groups ($P > 0.05$).

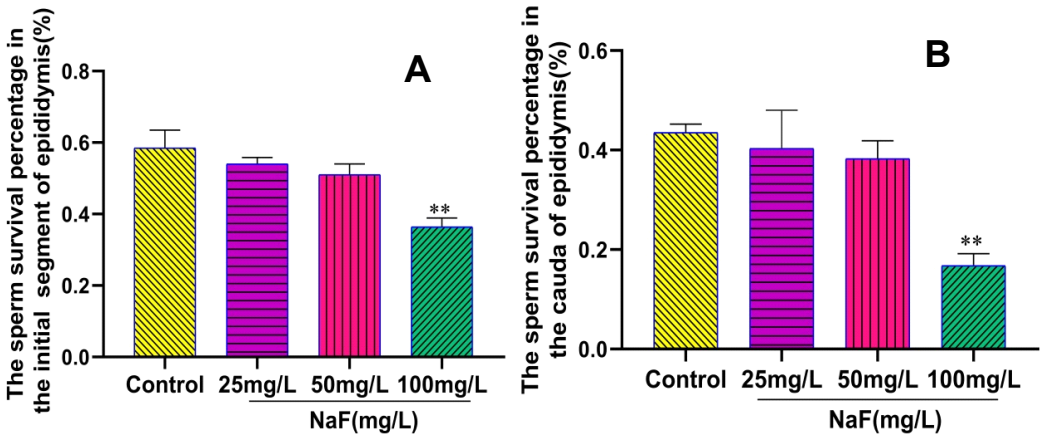

Figure 2. Sperm survival in the initial segment (A) and the cauda (B) of the epididymis of rats after exposure to different doses of NaF. Each bar represents the mean ± SEM of six rats per group. $^{**}P < 0.01$, compared to the control group.

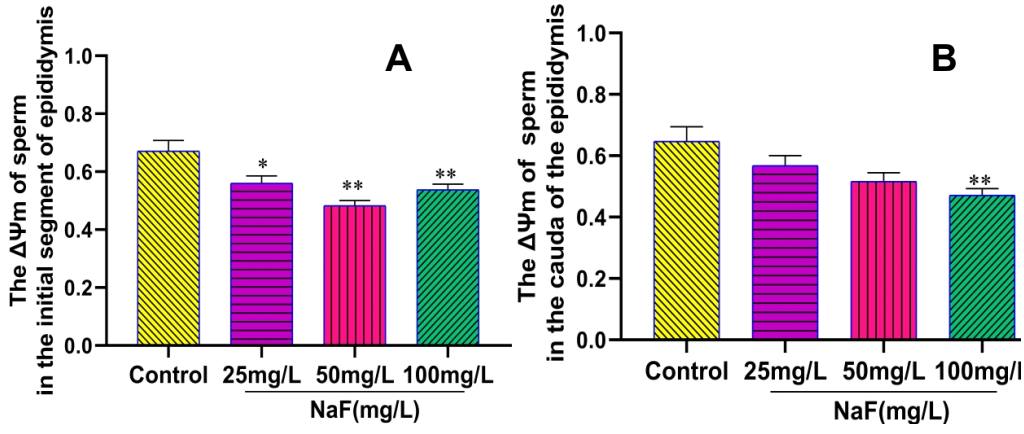

Figure 3. Sperm mitochondrial transmembrane potential (ΔΨm) in the initial segment (A) and the cauda (B) of the epididymis of rats after exposure to different doses of NaF. Each bar represents the mean ± SEM of six rats per group. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to the control group.

The localization and expression of CRISP2 in the rat testis were detected by immunofluorescence and are displayed in Figure 4. CRISP2 is a cysteine-rich secretory protein specifically expressed in male haploid germ cells. The results showed that, compared to the control group, the expression of CRISP2 in the testis of the groups of rats exposed to 50 and 100 mg L^{-1} NaF was significantly decreased ($P < 0.05$), while no remarkable changes were found in the 25 mg L^{-1} NaF group ($P > 0.05$).

Figures 5 and 6 show that CRISP2 expression was located in the surface of sperm. The fluorescence intensity of CRISP2 in the sperm from the initial segment and the cauda epididymidis of the rats exposed to 50 and 100 mg L^{-1} NaF was significantly reduced ($P < 0.01$). Meanwhile, there was no change in CRISP2 expression between the 25 mg L^{-1} NaF group and the control group ($P > 0.05$).

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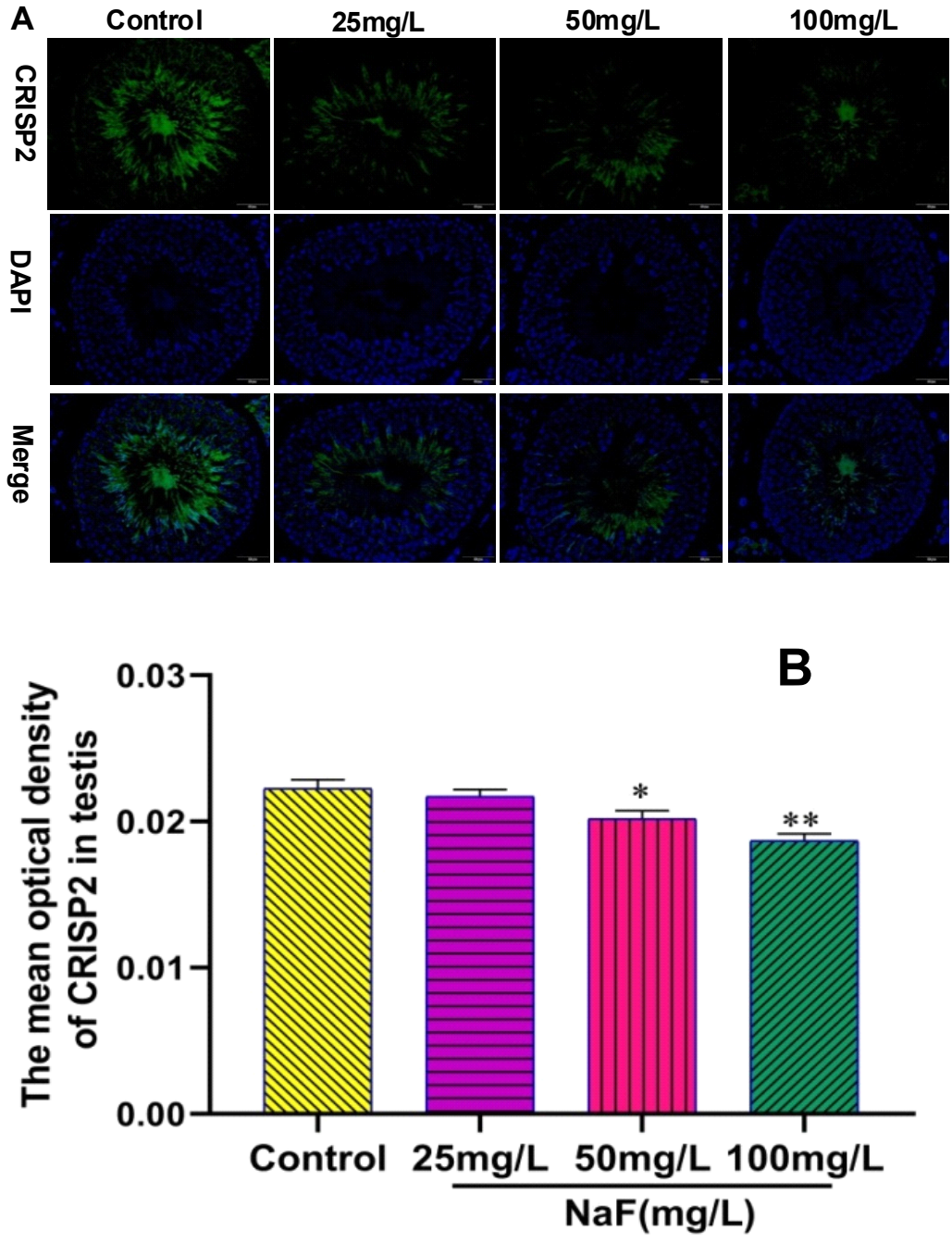

Figure 4. Indirect immunofluorescent staining of CRISP2 protein in rat testes. (A) The representative images of CRISP2 expression localizations in the testis of rats (400×). DAPI for nuclei staining (blue); CRISP2 protein (green). (B) Analysis results of average optical density of CRISP2 expression in the testis of rats. Each bar represents mean ± SEM of three rats per group. *$P < 0.05$, **$P < 0.01$, compared with the control group.

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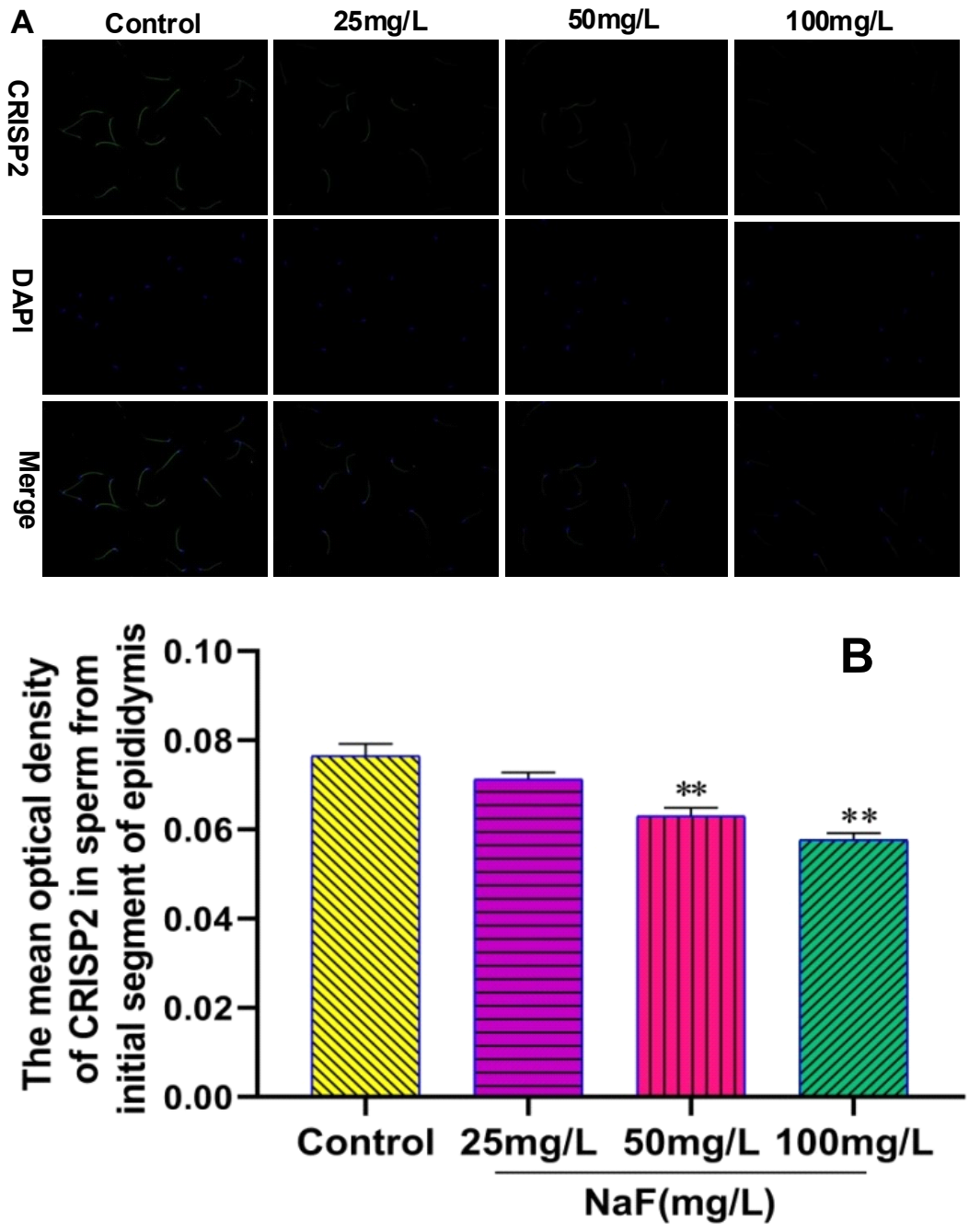

Figure 5. Indirect immunofluorescent staining of CRISP2 protein in rat sperm. (A) The representative images of CRISP2 expression localizations in the sperm of the initial segment of the epididymis of rats (200×). (B) Analysis results of average optical density of CRISP2 expression in the sperm of the initial segment if the epididymis of rats. Each bar represents mean ± SEM of three rats per group. ***P* < 0.01, compared with the control group.

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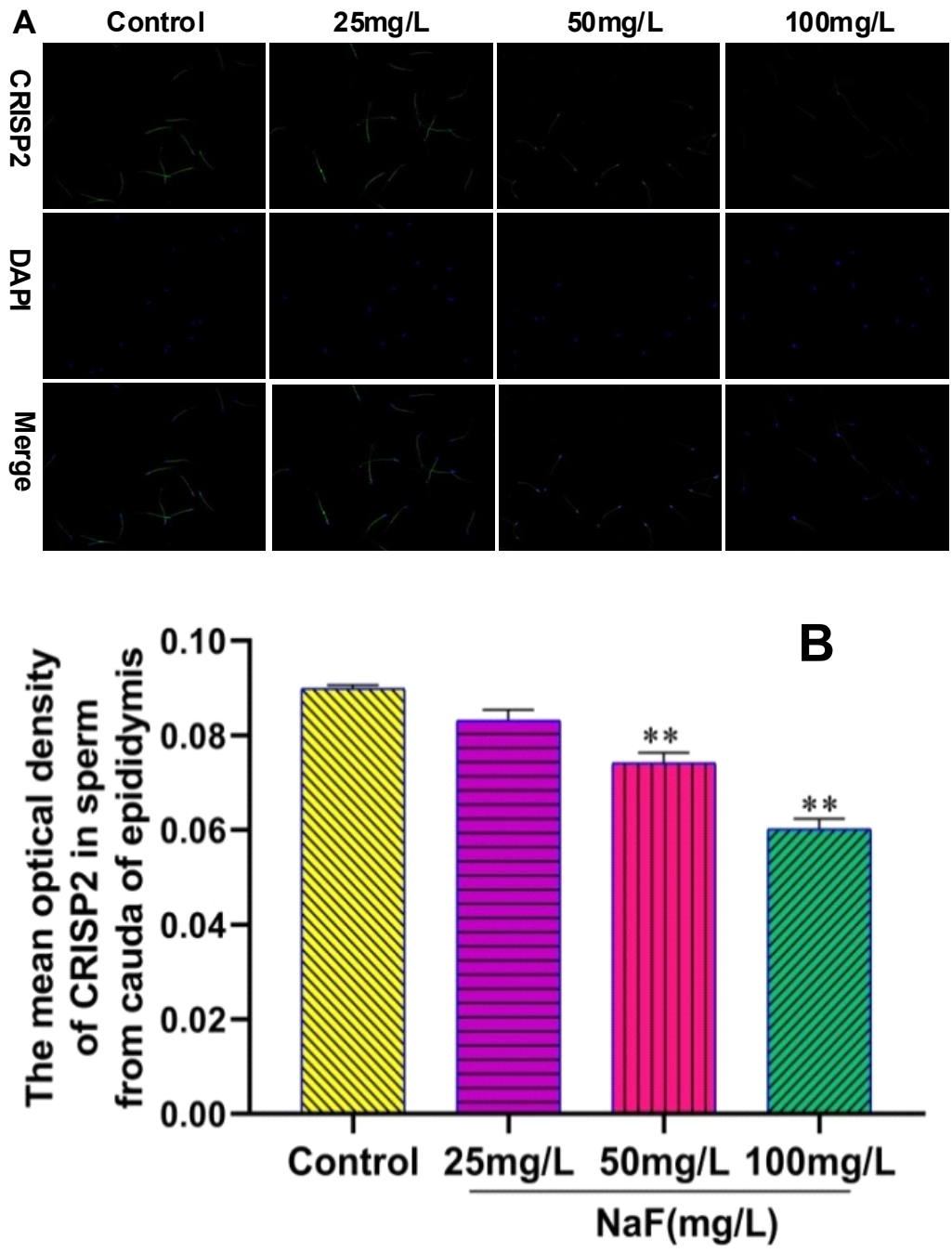

Figure 6. Indirect immunofluorescent staining of CRISP2 protein in rat sperm. (A) The representative images of CRISP2 express localizations in the sperm of the cauda of the epididymis of rats (200×). (B) Analysis of the results of average optical density of CRISP2 expression in the sperm of the cauda of the epididymis of rats. Each bar represents mean ± SEM of three rats per group. $^{**}P < 0.01$, compared with the control group.

DISCUSSION

The testis is the site of spermatogenesis which is a complex process, whereby spermatozoa are produced by mitosis and meiosis of male spermatogenic cells.[19] Fluoride-induced testicular toxicity is reflected in many aspects. It has been proven that fluoride may damage the structure of the blood-testis barrier, enhance the testicular inflammatory response, reduce the activity of antioxidant enzymes in the testis, and induce testicular oxidative stress, as well as spermatogenic cell apoptosis and autophagy.[20-24] These mechanisms of fluoride-induced testicular damage are likely to reduce the fertility of fluoride-exposed animals. In this study, we also found that fluoride altered the histological structure of the testis, which is in agreement with findings of previous studies.[25,26]

In addition to observing the histological structure of the testes in rats exposed to fluoride, the expression of CRISP2 in the testis was also evaluated. CRISP2, also known as Tpx-1, is highly enriched in the testis and is specifically expressed in the male haploid germ cells.[27] Furthermore, several reports have shown that CRISP2 is involved in the acrosome reaction and sperm-egg fusion.[18,28] It has been demonstrated that male mice lacking a functional CRISP2 gene exhibited clear fertilization deficiencies, suggesting that CRISP2 is essential for male fertility.[29] In agreement with this, a recent study reported a reduction in the expression of CRISP2 in spermatozoa was related to decreased pregnancy rates in Holstein bulls.[30] In this experiment, the results indicated that fluoride did not alter the localization of CRISP2 in the testes, but the expression of CRISP2 in the testis was decreased in a dose-dependent manner. To some extent, the decline of CRISP2 expression may be responsible for testicular toxicity induced by fluoride.

The sperm produced in the testis is immature sperm. In order to observe whether the low expression of CRISP2 in sperm of testis persisted to fertilization, we selected immature sperm in the initial segment and mature sperm in the tail (cauda) of the epididymis. In the current study, through immunofluorescence, CRISP2 was observed to locate around the neck and the outer dense fibers of the tail in mature sperm, which is similar to the description in a previous study. In this study, the level of CRISP2 in both the immature sperm in the initial stage and the mature sperm in the epididymis of rats exposed to NaF, showed a significant decrease in a dose-dependent manner, which indicated that lower levels of CRISP2 in the sperm of rats exposed to NaF may be a potential cause of poor sperm quality. To the best of our knowledge, fluoride can significantly decrease sperm quality by, for example, decreasing sperm count, sperm survival, sperm vitality, sperm hyperactivated motility, sperm chemotaxis, sperm capacitation and the acrosome reaction, and fertilization ability.[31-35] The decreased sperm survival and $\Delta\Psi m$ induced by fluoride found in this study show that fluoride affected sperm quality, thus supporting the above earlier investigations.

CONCLUSIONS

Taken together, the results of the present study confirm that fluoride impaired the morphology of the testis, reduced sperm survival and mitochondrial transmembrane potential, and decreased the CRISP2 expression from the testis to the tail of the epididymis. This indicates that CRISP2 should be taken into account as a potential biomarker for fluoride reproductive toxicity.

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REFERENCES

1 Zhou Z, Wang H, Zheng B, Han Z, Chen Y, Ma Y. A rat experimental study of the relationship between fluoride exposure and sensitive biomarkers. Biol Trace Elem Res 2017;180(1):100-9.
2 Yan X, Hao X, Nie Q, Feng C, Wang H, Sun Z, et al. Effects of fluoride on the ultrastructure and expression of Type I collagen in rat hard tissue. Chemosphere 2015;128:36-41.
3 Yuan J, Li Q, Niu R, Wang J. Fluoride exposure decreased learning ability and the expressions of the insulin receptor in male mouse hippocampus and olfactory bulb. Chemosphere 2019;224:71-6.
4 Yan N, Liu Y, Liu S, Cao S, Wang F, Wang Z, et al. Fluoride-induced neuron apoptosis and expressions of inflammatory factors by activating microglia in rat brain. Mol Neurobiol 2016;53(7):4449-60.
5 Kuang P, Guo H, Deng H, Cui H, Fang J, Zuo Z, et al. Sodium fluoride impairs splenic innate immunity via inactivation of TLR2/MyD88 signaling pathway in mice. Chemosphere 2019;237:124437.
6 Shi Z, Zhan Y, Zhao J, Wang J, Ma H. Effects of fluoride on the expression of p38MAPK signaling pathway-related genes and proteins in spleen lymphocytes of mice. Biol Trace Elem Res 2016;173(2):333-8.
7 Zhang J, Li Z, Qie M, Zheng R, Shetty J, Wang J. Sodium fluoride and sulfur dioxide affected male reproduction by disturbing blood-testis barrier in mice. Food Chem Toxicol 2016;94:103-11.
8 Sun Z, Wei R, Luo G, Niu R, Wang J. Proteomic identification of sperm from mice exposed to sodium fluoride. Chemosphere 2018;207:676-81.
9 Schulz JA, Lamb AR. The effect of fluorine as sodium fluoride on the growth and reproduction of albino rats. Science 1925;61(1569):93-104.
10 Jagtap S, Yenkie MK, Labhsetwar N, Rayalu S. Fluoride in drinking water and defluoridation of water. Chem Rev 2012;112(4):2454-66.
11 Doull J, Boekelheide K, Farishian BG, Isaacson RL, Klotz JB, Kumar JV, et al. Fluoride in drinking water: a scientific review of EPA's standards. Washington, DC, USA: National Academies Press; 2006.
12 Long H, Jin Y, Lin M, Sun Y, Zhang L, Clinch C. Fluoride toxicity in the male reproductive system. Fluoride 2009;42:260-76.
13 Antalikova J, Jankovicova J, Simon M, Cupperova P, Michalkova K, Horovska L. Localization of CD9 Molecule on Bull Spermatozoa: its Involvement in the Sperm-Egg Interaction. Reprod Domest Anim 2015;50(3):423-30.
14 Sun Z, Li S, Yu Y, Chen H, Ommati MM, Manthari RK, et al. Alterations in epididymal proteomics and antioxidant activity of mice exposed to fluoride. Arch Toxicol 2018;92(1):169-80.
15 Busso D, Cohen DJ, Hayashi M, Kasahara M, Cuasnicu PS. Human testicular protein TPX1/CRISP-2: localization in spermatozoa, fate after capacitation and relevance for gamete interaction. Mol Hum Reprod 2005;11(4):299-305.
16 Gibbs GM, Scanlon MJ, Swarbrick J, Curtis S, Gallant E, Dulhunty AF, et al. The cysteine-rich secretory protein domain of Tpx-1 is related to ion channel toxins and regulates ryanodine receptor Ca2+ signaling. J Biol Chem 2006;281(7):4156-63.

17 Harper CV, Barratt CL, Publicover SJ. Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. Induction of [Ca(2+)](i) oscillations and cyclical transitions in flagellar beating. J Biol Chem 2004;279(44):46315-25.

18 Busso D, Goldweic NM, Hayashi M, Kasahara M, Cuasnicu PS. Evidence for the involvement of testicular protein CRISP2 in mouse sperm-egg fusion. Biol Reprod 2007;76(4):701-8.

19 Zhang J, Zhu Y, Liang C, Qie M, Niu R, Sun Z, et al. Effects of fluoride on expression of P450, CREM and ACT Proteins in rat testes. Biol Trace Elem Res 2017;175(1):156-60.

20 He X, Sun Z, Manthari RK, Wu P, Wang J. Fluoride altered rat's blood testis barrier by affecting the F-actin via IL-1alpha. Chemosphere 2018;211:826-33.

21 Zhang S, Jiang C, Liu H, Guan Z, Zeng Q, Zhang C, et al. Fluoride-elicited developmental testicular toxicity in rats: roles of endoplasmic reticulum stress and inflammatory response. Toxicol Appl Pharmacol 2013;271(2):206-15.

22 Sun Z, Li S, Guo Z, Li R, Wang J, Niu R, et al. Effects of fluoride on SOD and CAT in Testis and Epididymis of Mice. Biol Trace Elem Res 2018;184(1):148-53.

23 Zhang S, Niu Q, Gao H, Ma R, Lei R, Zhang C, et al. Excessive apoptosis and defective autophagy contribute to developmental testicular toxicity induced by fluoride. Environ Pollut 2016;212:97-104.

24 Zhao WP, Wang HW, Liu J, Tan PP, Luo XL, Zhu SQ, Chen XL, Zhou BH. Positive PCNA and Ki-67 expression in the testis correlates with spermatogenesis dysfunction in fluoride-treated rats. Biol Trace Elem Res 2018;186(2):489-97.

25 Cao J, Chen Y, Chen J, Yan H, Li M, Wang J. Fluoride exposure changed the structure and the expressions of Y chromosome related genes in testes of mice. Chemosphere 2016;161:292-9.

26 Gupta RS, Khan TI, Agrawal D, Kachhawa JB. The toxic effects of sodium fluoride on the reproductive system of male rats. Toxicol Ind Health 2007;23(9):507-13.

27 O'Bryan MK, Sebire K, Meinhardt A, Edgar K, Keah HH, Hearn MT, et al. Tpx-1 is a component of the outer dense fibers and acrosome of rat spermatozoa. Mol Reprod Dev 2001;58(1):116-25.

28 Nimlamool W, Bean BS, Lowe-Krentz LJ. Human sperm CRISP2 is released from the acrosome during the acrosome reaction and re-associates at the equatorial segment. Mol Reprod Dev 2013;80(6):488-502.

29 Brukman NG, Miyata H, Torres P, Lombardo D, Caramelo JJ, Ikawa M, et al. Fertilization defects in sperm from cysteine-rich secretory protein 2 (Crisp2) knockout mice: implications for fertility disorders. Mol Hum Reprod 2016;22(4):240-51.

30 Arangasamy A, Kasimanickam VR, DeJarnette JM, Kasimanickam RK. Association of CRISP2, CCT8, PEBP1 mRNA abundance in sperm and sire conception rate in Holstein bulls. Theriogenology 2011;76(3):570-7.

31 Sun Z, Niu R, Su K, Wang B, Wang J, Zhang J, et al. Effects of sodium fluoride on hyperactivation and Ca2+ signaling pathway in sperm from mice: an *in vivo* study. Arch Toxicol 2010;84(5):353-61.

32 Lu Z, Wang S, Sun Z, Niu R, Wang J. In vivo influence of sodium fluoride on sperm chemotaxis in male mice. Arch Toxicol 2014;88(2):533-9.

33 Dvorakova-Hortova K, Sandera M, Jursova M, Vasinova J, Peknicova J. The influence of fluorides on mouse sperm capacitation. Anim Reprod Sci 2008;108(1-2):157-70.

34 Izquierdo-Vega JA, Sanchez-Gutierrez M, Del Razo LM. Decreased in vitro fertility in male rats exposed to fluoride-induced oxidative stress damage and mitochondrial transmembrane potential loss. Toxicol Appl Pharmacol 2008;230(3):352-7.

35 Kim J, Kwon WS, Rahman MS, Lee JS, Yoon SJ, Park YJ, et al. Effect of sodium fluoride on male mouse fertility. Andrology 2015;3(3):544-51.