[Now published in full after the initial publication as an Epub ahead of print on Dec 30, 2021 at www.fluorideresearch.online/epub/files/138.pdf]

CHRONIC FLUORIDE EXPOSURE INDUCED TESTICULAR MITOCHONDRIAL DYSFUNCTION ASSOCIATES WITH UPREGULATION OF ATP5B

Yanjia Tan,[a] Chen Liang,[b] Yan Gao,[a] Shuhui Wang,[a] Jie Yang,[a] Jundong Wang,[a] Jianhai Zhang[a,*]

Taigu, Shanxi, People's Republic of China

ABSTRACT: Excess fluoride can cause reproductive disorders but the exact mechanism is unclear. The purpose of the present study was to investigate mitochondrial dysfunction and the role of its subunit ATP5B in fluoride-induced male reproductive toxicity. Forty-eight healthy 8-week-old adult male C57BL-6 mice were exposed to 0, 0.6, 1.2, and 2.4 mM NaF via drinking water for 90 days. The ultrastructure of mitochondria in testis tissue was observed, and the localization of ATP5B was detected by immunofluorescence staining. The results showed that the ultrastructure of mitochondria in testis exposed to fluoride was seriously damaged, and ATP5B was mainly localized in testicular Leydig cells. Further, TM3 testicular Leydig cells were cultured and treated with 0, 0.125, 0.25, and 0.5 mM NaF for 24 hr. The expression level of ATP5B was then examined by real-time quantitative PCR and western blotting. The results indicated that fluoride significantly increased ATP5B expression in Leydig cells, both in transcription and protein levels. Taken together, fluoride exposure damaged mitochondrial structure and positively enhanced ATP5B expression in Leydig cells of testes. The study results provide a new explanation for the mechanism by which fluoride induced reproductive toxicity.

Key words: Fluoride; Testis; Mitochondria; Leydig cells; ATP5B.

INTRODUCTION

Fluoride can be found widely in air, soil, water, food, and other environmental media owing to strong electronegativity of fluoride ions.[1] For a long time, fluoride was considered necessary for the growth and development of teeth and bones of animals and human beings.[2,3] However, the toxicity caused by excessive fluoride has also been widely reported in recent years. Especially, animals and humans living in contaminated areas with high fluorine or industrial fluoride pollution suffer very serious health damage.[4,5] Fluoride can cause multiple toxicities including to teeth and bones[6], brain[7], kidney[8], liver[9], testis[10] and so on.

Among these injuries, male reproductive toxicity caused by fluoride cannot be ignored due to the global decline in sperm quality.[11,12] Previous results showed that fluoride can alter testosterone levels, resulting in lower sperm quality and increased sperm malformation rates.[13] Our previous studies indicated that the reproductive toxicological mechanism of fluoride was associated with swelling and vacuolization of the mitochondria in testicular cells, especially in testicular Leydig cells.[14,15] Therefore, it may be of great significance to explore the reproductive toxicity mechanism of fluoride from the mitochondrial perspective.

ATP synthase is the most important molecular machine in the mitochondria of the animal body. It is responsible for converting the energy in the food ingested by the organism into ATP molecules through oxidative phosphorylation in the body[1] which

[a]College of Veterinary Medicine, Shanxi Agricultural University, Taigu, Shanxi 030801, China; [b]College of Animal Science, Shanxi Agricultural University, Taigu, Shanxi 030801, China. *For correspondence: Professor Jianhai Zhang, College of Veterinary Medicine, Shanxi Agricultural University, Taigu, Shanxi 030801, China. E-mail: Jianhaiz@sxau.edu.cn, jianhaiz@163.com

is essential in spermatogenesis and sperm capacitation.[17] ATP synthase is composed of F_0 and F_1. ATP synthase subunit beta of F_1 (ATP5B) is the core subunit of mitochondrial ATP synthase, containing the catalytic sites required for ATP synthesis and hydrolysis. Normal expression of ATP5B plays a vital role in mitochondria function.[18] Testicular mitochondria are responsible for ATP synthesis related with male reproductive functions.[19] The disorders of energy metabolism and cell function occurred in the mitochondria of the testes, which can lead to cell death and impaired sperm function.[20] Abnormalities in mitochondria result in testicular dysfunction like failure of spermatogenesis.[18] Nevertheless, it is not clear whether mitochondrial damage and changes in ATP5B expression are the causes of reproductive disorders caused by fluorosis.

In present study, the structure of mitochondria and ATP5B expression in mRNA and protein levels in the testes exposed to fluoride were firstly evaluated. Further, the fluoride-treated TM3 Leydig cells model was used to verify the change of ATP5B expression in testicular mitochondria. This study results attempted to explain the potential mechanism of fluoride reproductive toxicity from a new perspective.

MATERIALS AND METHODS

Animal and treatment: Forty-eight healthy 8-week-old adult male C57BL-6 mice were purchased from the Experimental Animal Center of Shanxi Medical University (Taiyuan, China) and fed with the standard diet. All mice were raised at 25 ± 1ºC ambient temperature with 50 ± 5% relative humidity under a 12 hr–12 hr light-dark cycle. After one-week acclimatization, mice were randomly divided into four groups with different drinking treatments as following: distilled water, 0.6, 1.2, and 2.4 mM sodium fluoride solution. All protocols were in accordance with the rules of Institutional Animal Care and Use Committee of Shanxi Agricultural University.

After 90 days fluoride treatment, mice were sacrificed by cervical dislocation and testes were immediately isolated after heart perfusion. One part of the right testis was fixed in 2.5% glutaraldehyde phosphate for ultrastructural analysis and another part of the right testis was fixed in Bouin's fixative solution for immunofluorescence analysis. The left testis was frozen immediately in liquid nitrogen and stored at –80°C for RNA and protein extraction.

Transmission electron microscopy analysis (TEM): The fresh testes were cut into 1 mm^3 blocks and fixed in 2.5% glutaraldehyde for 2 hr at room temperature. Then the masses were transferred into 2.5% osmium tetroxide with phosphate buffer solution (PBS, pH=7.4). Ultrathin sections were obtained and stained with uranyl acetate and lead citrate. Observation and photography were performed by a transmission electron microscope (JEM-1011, JEOL, Tokyo, Japan).

Immunofluorescence analysis: The testes were fixed in Bouin's fixative solution for 24 hr. After washing by tap water overnight, the testes were cut into pieces and embedded with paraffin, and sectioned at 5 μm thickness. After dewaxing xylene, the slices were dehydrated with gradient concentration alcohol (100%, 95%, 90%, and 75%) and washed with double distilled water. Then antigen retrieval was implemented by boiling with citrate antigen retrieval solution and blocked with 5% (w/v) bovine serum albumin (BSA) for 1 hr at room temperature (RT). After incubation of primary antibodies (ATP5B 1:100) overnight in 4°C, PBST was

prepared for swilling three times. The slides were incubated with the fluorescent following antibodies diluted with PBS (1:200) for 2 hr in cassette. Finally, anti-fluorescence quenching tablets containing DAPI were prepared for sealing the tablets after being washed three times by PBST. All images were captured by the fluorescence microscope (Olympus, BX53F, Japan).

Cell culture and treatment: The mice Leydig TM3 cells (ZQ 0100) were purchased from the Shanghai Zhongqiao Xinzhou Biological Co., Ltd (Shanghai, China). Cells were cultured in DMEM/F-12 with 5% horse serum, 2.5% fetal bovine serum, and 1% penicillin-streptomycin in an incubator with 95% humidity and 5% CO_2 at 37°C. Cells were sub-cultured when reaching 80% confluence.

Quantitative real-time PCR: TM3 cells were incubated in the 6-well plates at a density of 3×10^5 cells per well with 0, 0.125, 0.25, and 0.5 mM NaF for 24 hr. The cells were harvested for total RNA and protein extraction.

RNAiso Plus was used to extract total RNA and A260/A280 absorbance ratio, measured by NanoDrop (2000) (Thermo Fisher, USA), was used to detect the concentration and purity of RNA. The RNA was reverse transcribed into cDNA by using a Reverse Transcriptase kit (Promega, Madison, WI, USA). cDNA was amplified by QuantStudio 7 Flex quantitative PCR (Life Technologies, USA) using SYBR® Premix Ex Taq™ II Kit (Takara, China). After that, $2^{-\Delta\Delta CT}$ method was used to calculate the relative expression level of ATP5B mRNA. The gene sequences of *ATP5B* and *β-actin* (Table 1) were obtained by NCBI, and the primers were designed by using the Primer 3 plus software and synthesized by Shanghai Yingweijie Trading co., LTD. (Shanghai, China).

Table 1. Primer sequences for qRT-PCR

Gene	Primer sequences	Accession No.	Product size (bp)
β-actin	F: CTGGGTATGGAATCCTGTGG	NM_031144.3	97
	R: GCACTGTGTTGGCATAGAGG		
ATP5B	F: CCAGGGAAGGAAGATACAG	NM_134364.1	150
	R: GTTCCGGGAGATGGTCATAG		

Western Blotting: The total protein of TM3 Leydig cell was extracted by RIPA lysis buffer (KeyGEN Biotech, China) containing 1% PMSF (Beyotime Biotechnology, China) and then centrifuged at 12,000×g for 5 min at 4°C. The protein concentration was tested by BCA protein assay kit (KeyGEN Biotech, China) and the protein was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose (NC) membrane. After blocking by 5% skim milk in TBST for 2 hr at RT, the membranes were incubated with primary antibodies: Mouse anti-Beta Actin monoclonal antibody (60008-1-Ig) and anti-ATP5B polyclonal antibody purchased from Proteintech Group (Wuhan, China). Then the membranes were incubated with secondary antibody (Proteintech Group, China), and an enhanced

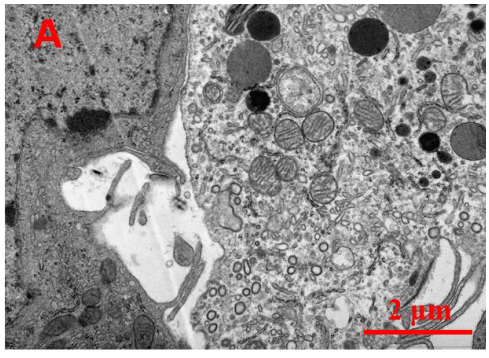
[Now published in full after the initial publication as an Epub ahead of print on Dec 30, 2021 at www.fluorideresearch.online/epub/files/138.pdf]

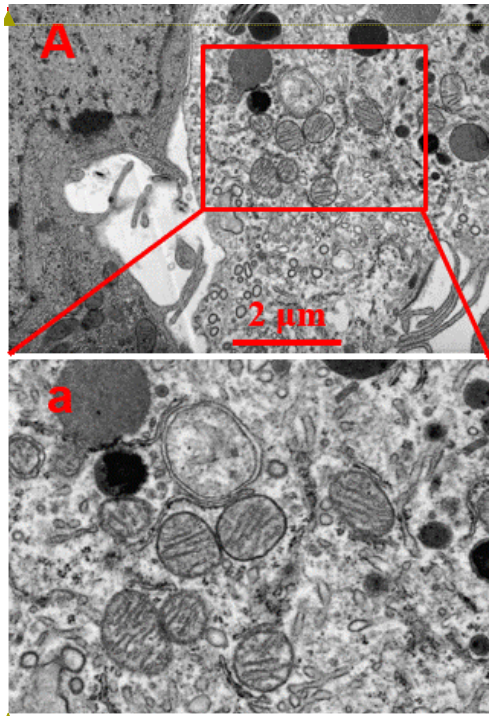
chemiluminescence (ECL) kit was applied to enhance chemiluminescence. The Fluor Chem Q System (Cell & Bioscience, USA) was employed to collect images and the target protein bands densitometry were detected with AlphaView SA software.

Statistical analysis: Experimental data were analyzed with GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA, USA) and were presented as mean ± SD. One-way analysis of variance (ANOVA) and Dunnett's multiple comparison were employed for the difference comparison. $p < 0.05$ and $p < 0.01$ were considered as statistically significant.

RESULTS

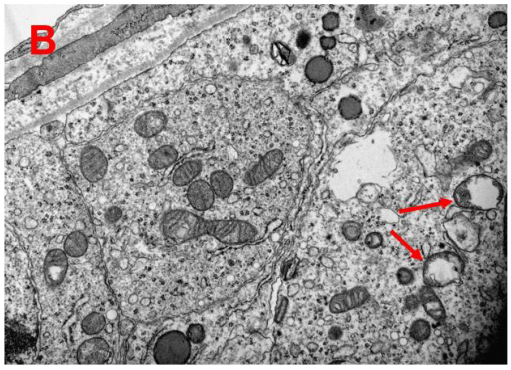
Fluoride induced the damage of mitochondrial ultrastructure in testicular tissue: TEM was performed to observe mitochondrial ultrastructural changes in testes. The ultrastructure of the mitochondria was complete and the nuclear membrane and cristae were clearly visible in control group (Figures 1A and 1a). In 0.6 mM NaF-treated group, bits of mitochondria were vacuolated compared with control group (Figures 1B and 1b). Mitochondria were swollen, and vacuolar lesions were increased in 1.2 mM NaF-treated group (Figures 1C and 1c). In the 2,4 mM NaF-treated group, mitochondria were enlarged and disorganized, with bilayer membrane structures and mitochondria cristae blurred (Figures 1D and 1d).

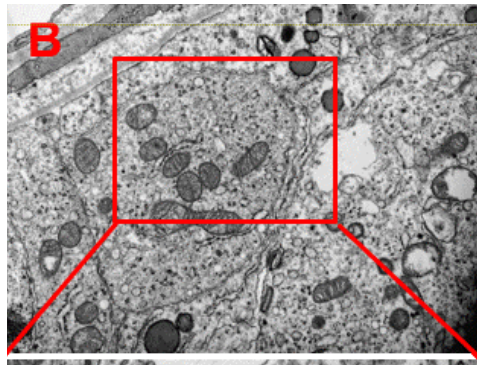


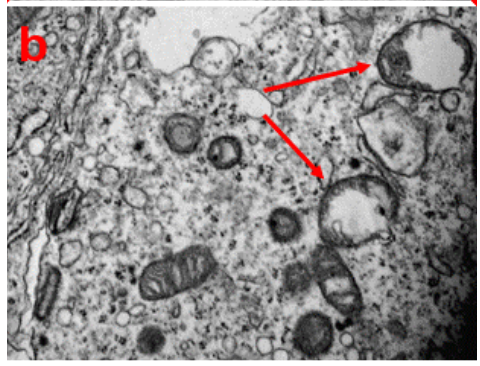


Figures 1A and 1a. Ultrastructural detail of the mitochondria of mice testes of the control group.The picture in 1A was taken at 20,000 × magnification by transmission electron microscopy. The marker in Figure 1A is 2 µm. 1a: Enlarged view of the rectangle shown in Figure 1A. The mitochondrial structure is normal.

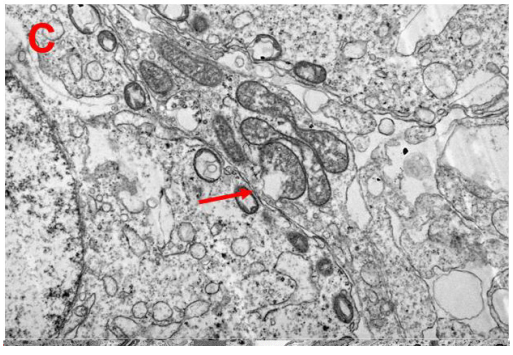
[Now published in full after the initial publication as an Epub ahead of print on Dec 30, 2021 at www.fluorideresearch.online/epub/files/138.pdf]

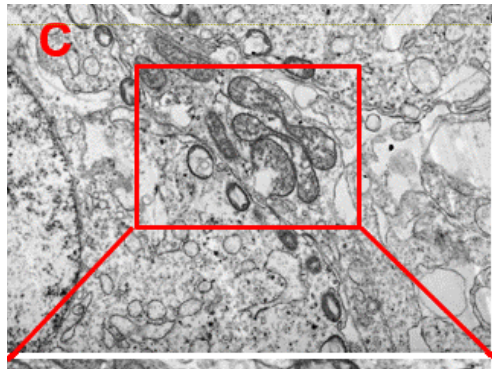


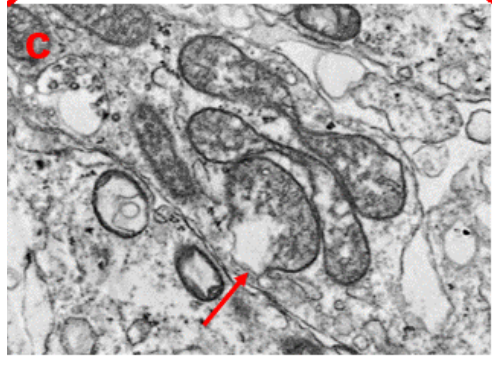




Figures 1B and 1b. Ultrastructural changes in mitochondria of mice testes of the group treated for 90 days with 0.6 mM NaF. The picture in 1B was taken at 20,000 × magnification by transmission electron microscopy. The marker in Figure 1B is 2 µm. 1b: Enlarged view of part of the area shown in the rectangle and an area to the right of and below the rectangle shown in Figure 1B. Red arrows indicate the significant changes in mitochondrial structure.

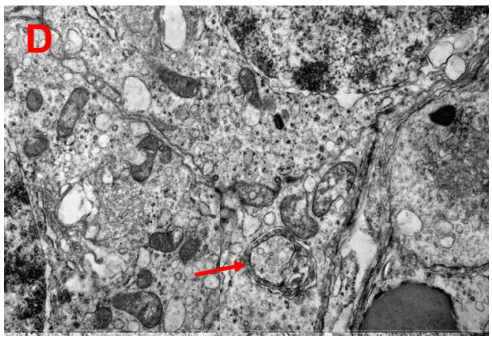


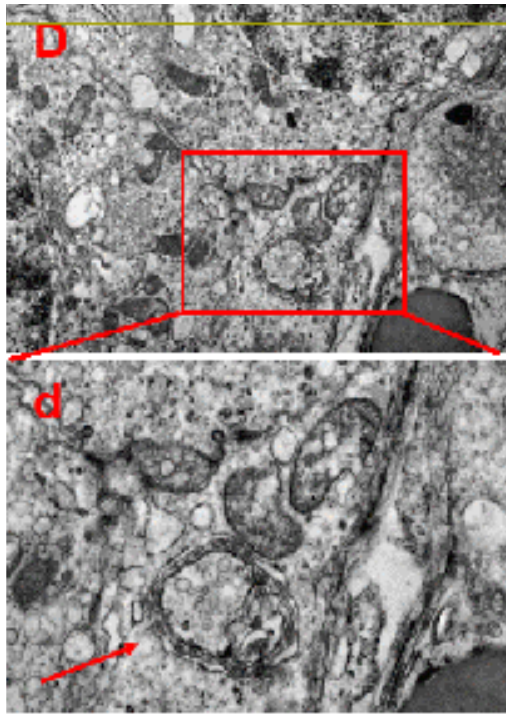




Figures 1C and 1c. Ultrastructural changes in mitochondria of mice testes of the group treated for 90 days with 1.2 mM NaF. The picture in 1C was taken at 20,000 × magnification by transmission electron microscopy.The marker in Figure 1C is 2 µm. 1c: Enlarged view of the area shown in the rectangle shown in Figure 1C. Red arrows indicate the significant changes in mitochondrial structure.

[Now published in full after the initial publication as an Epub ahead of print on Dec 30, 2021 at www.fluorideresearch.online/epub/files/138.pdf]



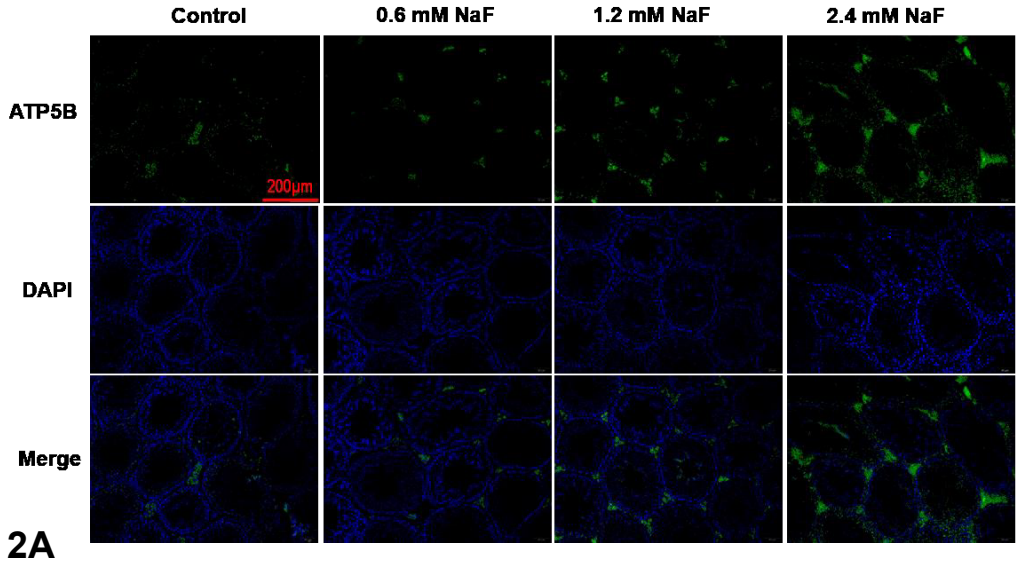


Figures 1D and 1d. Ultrastructural changes in mitochondria of mice testes of the group treated for 90 days with 2.4 mM NaF. The picture in 1D was taken at 20,000 × magnification by transmission electron microscopy. The marker in Figure 1D is 2 μm. 1d: Enlarged view of part of the area shown in the rectangle in Figure 1D. Red arrows indicate the significant changes in mitochondrial structure.

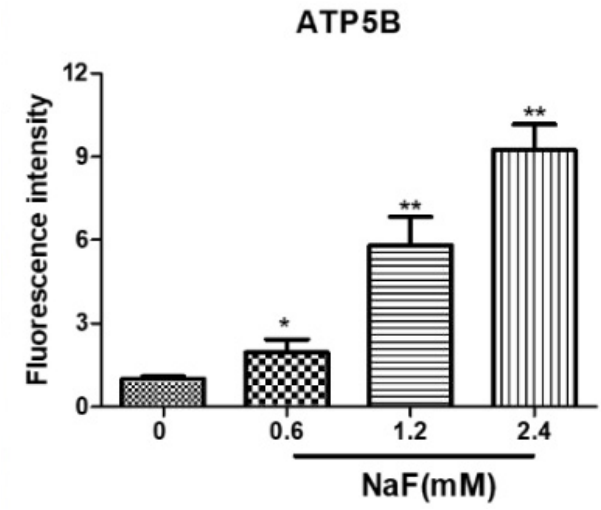
Fluoride changed the expression level of ATP5B in testis of mice: Immunofluorescence analysis was implemented to detect the distribution and expression level of ATP5B. We found that ATP5B assembled in the Leydig cells of the testis (Figure 2A). The enhancement of green fluorescence indicated that the expression level of ATP5B went up with the increase of fluoride concentration. The protein expression level of ATP5B increased significantly in 0.6 mM NaF group ($p < 0.05$), 1.2 mM NaF group ($p < 0.01$), and 2.4 mM NaF group ($p < 0.01$) with a dose-dependent manner (Figure 2B). The result showed that ATP5B probably should be one of the targets of fluorosis in the testis.

Fluoride upregulated the mRNA and protein expression levels of ATP5B in TM3 Leydig cells: To verify the quantification of ATP5B *in vitro*, the mRNA and protein expression levels were also detected in the TM3 Leydig cells (Figure 3). Compared with the control group, both relative mRNA and protein expression level of ATP5B had no significant change in 0.125 mM NaF group. In the other two NaF treatment groups, ATP5B expressions showed a marked increase compared to the controls. Interestingly, the mRNA expression of ATP5B increased with fluoride exposure (Figure 3A), while the protein expression level did not have a dose-effect relationship (Figure 3B).

[Now published in full after the initial publication as an Epub ahead of print on Dec 30, 2021 at www.fluorideresearch.online/epub/files/138.pdf]

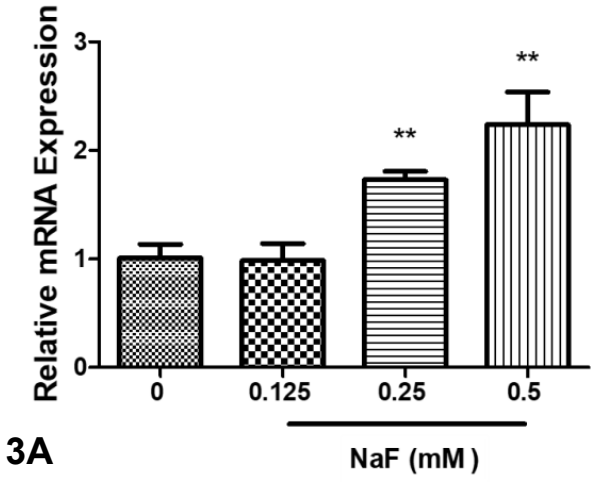


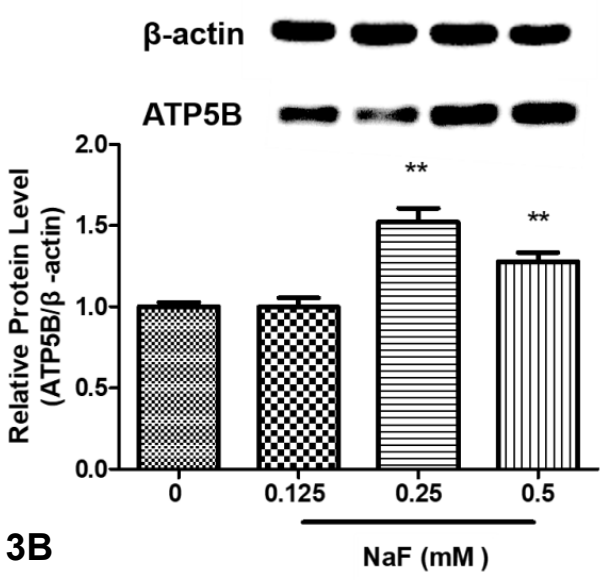
2A



2B

Figures 2A and 2B. Location and quantification of ATP5B in mouse testis treated with fluoride for 90 days. 2A: Immunofluorescent staining (IF) images of ATP5B in mouse testis treated with 0, 0.6, 1.2, 2.4 mM NaF(20 ×). The images were selected randomly from ten different areas of testes sections. The nucleus was stained by DAPI (blue). Green color fluorescence indicates ATP5B expression. 2B: The quantitative analysis results of fluorescence intensity to ATP5B expressions. *p<0.05, **p<0.01 indicate significant differences compared to the controls.





Figures 3A and 3B. Effects of fluoride on mRNA and protein expression levels of ATP5B in TM3 Leydig cells. 3A: mRNA expression levels of ATP5B in TM3 Leydig cells detected by qPCR. The results were presented as mean ± SEM (n = 6). ***p*<0.05 shows significant differences compared to the control group. 3B: Western blot analysis results of ATP5B expression in TM3 Leydig cells treated by different does of NaF. β-actin was used as a control. Data were presented as means ± SEM (n = 6). ** *p*<0.05 shows significant differences compared to the control group.

DISCUSSION

Fluorine is widely distributed in the earth's crust and the content is about 300–900 ppm.[21] After volcanic eruption and other crustal activities, fluorine erupts through magma and magma gas. Under the action of rain, fluorine precipitates into nearby soil and water sources in the form of volcanic ash. According to the survey, Sicily, New Zealand, Iceland, the East African Rift, China, and South India all have high-fluorine-rich volcanoes,[22] so they have become the most frequent areas of fluorosis.

[Now published in full after the initial publication as an Epub ahead of print on Dec 30, 2021 at www.fluorideresearch.online/epub/files/138.pdf]

In addition, more than 300 minerals contain fluoride ions.[23] These minerals are mined in large quantities and used as raw materials by various industries, such as smelters and fertilizer plants.[24] Fluorine invades the organism through the respiratory tract, digestive tract, and skin in the form of various compounds. Fluorosis has now become a public health disease that cannot be ignored in humans and animals.[25]

For this experiment *in vivo*, fluoride in drinking water was used to construct a model of fluorosis in mice, which is safer compared with other treatments by gavage or intraperitoneal injection and is closest to the real situation of fluoride exposure. Exposure dose in mice is based on the concentration of fluoride ions (0.5–48 mg/L, between 0.01 to 1.14 mM) in groundwater, in China, India an other countries, and 1.2 mM as a medium dose is chosen. Content of fluoride in bones was measured immediately after the death of the mice to verify the success of the model.[26] Compared with the control group, the bone fluoride content of the 0.6, 1.2, and 2.4 mM NaF group all increased significantly (41.27%, 65.13%, and 90.83%, respectively). The NaF concentrations used in the present *in vitro* experiment were chosen based on the treatment concentrations used in previous experiments in our laboratory.[15]

Our laboratory has been committed to the study of the mechanism on fluorosis in the reproductive system. Our results showed that fluoride exposure can cause abnormal spermatogenesis by affecting meiosis.[26,27] Autoimmune orchitis caused by fluorosis has an important relationship with IL-17A.[28,29] The ultrastructural results showed that the cell membrane and cytoskeleton of Leydig cells were damaged.[30]

The damage of fluoride on different organs' mitochondria has also been widely studied. Cardiomyocytes and ameloblasts may produce reactive oxygen species under the condition of fluorosis causing mitochondrial damage and inducing cell apoptosis and autophagy.[31,32] In addition, fluoride can also cause mitochondrial damage in nervous system.[33] In severe cases, fluoride caused abnormal expression of mitochondrial complex, lead to fission, fusion disorder, and liver dysfunction.[34] Similar effects may occur in the kidney,[35] ovary,[36] and other organs. In the current experiment, the changes in mitochondria in fluoride-induced testicular toxicity were investigated, and the results showed that mitochondria in testis are also one of the targets in fluorosis.

In addition to energy production, mitochondria also play an important role in the execution of cell death.[37] ATP synthase works by generating ROS, and a large amount of ROS can trigger oxidative stress and cause damage to cells and mitochondria.[38] As a receptor for various ligands, ATP5B is related to the activity of transmembrane transporters and is involved in various biological processes.[39,40] The low expression of ATP5B has been found in various human cancers.[41] Under the action of cytotoxic agents, high expression of ATP5B has been shown to cause cell death, whereas decreased or loss of mitochondrial activity can reduce the damage caused by ROS signaling.[42] In the mitochondria of Leydig cells treated with fluoride, the expression of ATP5B increased, which caused ATP synthase to produce ROS, resulting in mitochondrial damage.

[Now published in full after the initial publication as an Epub ahead of print on Dec 30, 2021 at www.fluorideresearch.online/epub/files/138.pdf]

CONCLUSIONS

In conclusion, fluoride exposure damaged mitochondrial structure and led to a compensatory enhancement of ATP5B expression in Leydig cells of testes. The proactive ATP5B expression is closely related to the fluoride-induced mitochondrial dysfunction in testicular Leydig cells, and provides a potential mechanism for the reproductive toxicity induced by fluoride.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (Grant No.32072934), Shanxi Youth Top Talent Plan, and The Key Research and Development Project of Shanxi Province (201903D211008) and Graduate Education Innovation Project of Shanxi Province in 2020 (2020SY200).

REFERENCES

1 Liu Y, Liang C, Gao Y, Jiang S, He Y, Han Y, et al. Fluoride interferes with the sperm fertilizing ability via downregulated SPAM1, ACR, and PRSS21 expression in rat epididymis. J Agric Food Chem 2019;67(18):5240-9.

2 Dhar V, Bhatnagar M. Physiology and toxicity of fluoride. Indian J Dent Res 2009;20(3):350-5.

3 Zuo H, Chen L, Kong M, Qiu L, Lü P, Wu P, et al. Toxic effects of fluoride on organisms. Life Sci 2018;198:18-24.

4 Choubisa SL, Choubisa D. Status of industrial fluoride pollution and its diverse adverse health effects in man and domestic animals in India. Environ Sci Pollut Res Int 2016;23(8):7244-54.

5 Death C, Coulson G, Kierdorf U, Kierdorf H, Ploeg R, Firestone SM, et al. Skeletal fluorosis in marsupials: A comparison of bone lesions in six species from an Australian industrial site. Sci Total Environ 2017;584-5:1198-211.

6 Li J, Shi Y, Fan H, Li Y, Zhu Y, Lin X, et al. Effects of fluoride on surface structure of primary culture Leydig cells in mouse. Biol Trace Elem Res 2018;183(1):123-7.

7 Cao K, Xiang J, Dong Y, Xu Y, Li Y, Song H, et al. Exposure to fluoride aggravates the impairment in learning and memory and neuropathological lesions in mice carrying the APP/PS1 double-transgenic mutation. Alzheimers Res Ther 2019;11(1):35.

8 Jiang S, Liang C, Gao Y, Liu Y, Han Y, Wang J, et al. Fluoride exposure arrests the acrosome formation during spermatogenesis via down-regulated Zpbp1, Spaca1 and Dpy19l2 expression in rat testes. Chemosphere 2019;226:874-82.

9 Sharma D, Singh A, Verma K, Paliwal S, Sharma S, Dwivedi J. Fluoride: A review of pre-clinical and clinical studies. Environ Toxicol Pharmacol 2017;56:297-313.

10 Liang C, He Y, Liu Y, Gao Y, Han Y, Li X, et al. Fluoride exposure alters the ultra-structure of sperm flagellum via reducing key protein expressions in testis. Chemosphere 2020;246:125772.

11 Zhao Y, Zhao J, Wang J, Wang J. Fluoride exposure changed the structure and the expressions of HSP related genes in testes of pubertal rats. Chemosphere 2017;184:1080-8.

12 Han H, Sun Z, Luo G, Wang C, Wei R, Wang J. Fluoride exposure changed the structure and the expressions of reproductive related genes in the hypothalamus-pituitary-testicular axis of male mice. Chemosphere 2015;135:297-303.

13 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.

14 Zhao W, Wang H, Liu J, Tan P, Luo X, Zhu S, et al. 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.

15 Liang C, Gao Y, He Y, Han Y, Manthari RK, Tikka C, et al. Fluoride induced mitochondrial impairment and PINK1-mediated mitophagy in Leydig cells of mice: *In vivo* and *in vitro* studies. Environ Pollut 2020;256:113438.

16 Geyik E, Igci YZ, Pala E, Suner A, Borazan E, Bozgeyik I, et al. Investigation of the association between ATP2B4 and ATP5B genes with colorectal cancer. Gene 2014; 540(2):178-82.

17 Vertika S, Singh KK, Rajender S. Mitochondria, spermatogenesis, and male infertility – An update. Mitochondrion 2020;54:26-40.

18 Huang S, Wang J, Cui Y. 2,2',4,4'-Tetrabromodiphenyl ether injures cell viability and mitochondrial function of mouse spermatocytes by decreasing mitochondrial proteins Atp5b and Uqcrc1. Environ Toxicol Pharmacol 2016;46:301-10.

19 Huang W, Cao Z, Yao Q, Ji Q, Zhang J, Li Y. Mitochondrial damage are involved in Aflatoxin B1-induced testicular damage and spermatogenesis disorder in mice. Sci Total Environ 2020;701:135077.

20 Zhang J, Bao X, Zhang M, Zhu Z, Zhou L, Chen Q, et al. MitoQ ameliorates testis injury from oxidative attack by repairing mitochondria and promoting the Keap1-Nrf2 pathway. Toxicol Appl Pharmacol 2019;370:78-92.

21 Koga K, Rose-Koga E. Fluorine in the earth and the solar system, where does it come from and can it be found? CR Chim 2018;21:749 -56.

22 Johnston NR, Strobel SA. Principles of fluoride toxicity and the cellular response: a review. Arch Toxicol 2020;94(4):1051-69.

23 Garcia MG, Borgnino L. Fluorine: chemistry, analysis, function, and effects. Cambridge: RSC Publishing; 2015.

24 Yadav KK, Kumar S, Pham QB, Gupta N, Rezania S, Kamyab H, et al. Fluoride contamination, health problems and remediation methods in Asian groundwater: A comprehensive review. Ecotoxicol Environ Saf 2019;182:109362.

25 Lacson CF, Lu M, Huang Y. Fluoride network and circular economy as potential model for sustainable development-A review. Chemosphere 2020;239:124662.

26 Linhares D, Camarinho R, Garcia PV, Rodrigues ADS. Mus musculus bone fluoride concentration as a useful biomarker for risk assessment of skeletal fluorosis in volcanic areas. Chemosphere. 2018;205:540-4.

27 Han Y, Yu Y, Liang C, Shi Y, Zhu Y, Zheng H, et al. Fluoride-induced unrestored arrest during haploid period of spermatogenesis via the regulation of DDX25 in rats. Environ Pollut 2019;253:538-51.

28 Wang C, Chen Y, Manthari RK, Wang J. Abnormal spermatogenesis following sodium fluoride exposure is associated with the downregulation of CREM and ACT in the mouse testis. Toxicol Ind Health 2018;34(4):219-27.

29 Wu P, Sun Z, Lv X, Pei X, Manthari RK, Wang J. Fluoride induces autoimmune orchitis involved with enhanced IL-17A secretion in mice testis. J Agric Food Chem 2019; 67(48):13333-43.

30 Huo M, Han H, Sun Z, Lu Z, Yao X, Wang S, et al. Role of IL-17 pathways in immune privilege: a RNA deep sequencing analysis of the mice testis exposure to fluoride. Sci Rep 2016;6:32173.

31 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.

32 Yan X, Wang L, Yang X, Qiu Y, Tian X, Lv Y, et al. Fluoride induces apoptosis in H9c2 cardiomyocytes via the mitochondrial pathway. Chemosphere 2017;182:159-65.

33 Suzuki M, Bandoski C, Bartlett JD. Fluoride induces oxidative damage and SIRT1/autophagy through ROS-mediated JNK signaling. Free Radic Biol Med 2015;89:369-78.

34 Zhao Q, Niu Q, Chen J, Xia T, Zhou G, Li P, et al. Roles of mitochondrial fission inhibition in developmental fluoride neurotoxicity: mechanisms of action *in vitro* and associations with cognition in rats and children. Arch Toxicol 2019;93(3):709-26.

35 Zhou B, Wei S, Jia L, Zhang Y, Miao C, Wang H. et al. Drp1/Mff signaling pathway is involved in fluoride-induced abnormal fission of hepatocyte mitochondria in mice. Sci Total Environ 2020;725:138192.

36 Wang H, Zhu S, Liu J, Miao C, Zhang Y, Zhou B. Fluoride-induced renal dysfunction via respiratory chain complex abnormal expression and fusion elevation in mice. Chemosphere 2020;238:124607.

37 Zhao W, Wang H, Liu J, Zhang Z, Zhu S, Zhou B. Mitochondrial respiratory chain complex abnormal expressions and fusion disorder are involved in fluoride-induced mitochondrial dysfunction in ovarian granulosa cells. Chemosphere 2019;215:619-25.

38 Wang X. The expanding role of mitochondria in apoptosis. Genes Dev 2001;15:2922-33.

39 Santamaria G, Martinez-Diez M, Fabregat I, Cuezva JM. Efficient execution of cell death in non-glycolytic cells requires the generation of ROS controlled by the activity of mitochondrial H^+ATP synthase. Carcinogenesis 2006;27:925-35.

40 Zhao Q, Yang Y, Wang C, Hou Y, Chen H. ATP5B and ETFB metabolic markers in children with congenital hydronephrosis. Mol Med Rep 2016;14(6):5111-5.

41 Zuo J, Lei M, Wen M, Chen Y, Liu Z. Overexpression of ATP5b promotes cell proliferation in asthma. Mol Med Rep 2017;16(5):6946-52.

42 Willers IM, Cuezva JM. Post-transcriptional regulation of the mitochondrial H (+)-ATP synthase: a key regulator of the metabolic phenotype in cancer. Biochim Biophys Acta 2011;1807:543-51.

43 Sun J, Yang Z, Miao X, Zou Q, Li J, Liang L, et al. ATP5b and β2-microglobulin are predictive markers for the prognosis of patients with gallbladder cancer. J Mol Histol 2015;46(1):57-65.