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RESEARCH

The Footprints of Mitochondrial Fission and Apoptosis in Fluoride-Induced Renal Dysfunction

Qiyong Zuo[1] · Lin Lin[1] · Yuling Zhang[1] · Mohammad Mehdi Ommati[1] · Hongwei Wang[1] · Jing Zhao[1]

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Abstract

Fluoride (F) is widely distributed in the environment and poses serious health risks to humans and animals. Although a good body of literature demonstrates a close relationship between F content and renal system performance, there is no satisfactory information on the involved intracellular routes. Hence, this study used histopathology and mitochondrial fission to explore fluorine-induced nephrotoxicity further. For this purpose, mice were exposed to the F ion (0, 25, 50, 100 mg/L) for 90 days. The effects of different F levels on renal pathomorphology and ion metabolism were assessed using hematoxylin and eosin (H&E), periodic acid-Schiff stain (PAS), periodic acid-silver methenamine (PASM), Prussian blue (PB), and alkaline phosphatase (ALP) staining. The results showed that F could lead to glomerular atrophy, tubular degeneration, and vacuolization. Meanwhile, F also could increase glomerular and tubular glycoproteins; made thickening of the renal capsule membrane and thickening of the tubular basement membrane; led to the accumulation of iron ions in the tubules; and increased in glomerular alp and decreased tubular alp. Concomitantly, IHC results showed that F significantly upregulated the expression levels of mitochondrial fission-related proteins, including mitochondrial fission factor (Mff), fission 1 (Fis1), and mitochondrial dynamics proteins of 49 kDa (MiD49) and 51 kDa (MiD51), ultimately caused apoptosis. To sum up, excessive fluorine has a strong nephrotoxicity effect, disrupting the balance of mitochondrial fission and fusion, interfering with the process of mitochondrial fission, and then causing damage to renal tissue structure and apoptosis.

Keywords Apoptosis · Fluorosis · Mitochondrion · Nephrotoxicity

Introduction

Fluorine is a kind of active polar small molecule compound, widely distributed in drinking water, air, and soil [1]. As a vital metabolic organ, the kidney plays a crucial role in the body's waste elimination process, helping to remove waste products, toxins, and drugs [2]. With the escalating levels of environmental pollution, the global incidence of kidney disease has increased by over 5% annually [3]. It is well-known that F could cause severe nephrotoxicity [4]. Elevated levels of F have been linked to the prevalence of chronic kidney disease [5]. Epidemiological surveys showed that 12.5% of the population in areas with high F exposure suffers from chronic kidney disease, much higher than in areas with low F exposure [6, 7]. The fluorosis population has pain in the kidney area, polyuria, proteinuria, renal dysfunction, and significantly higher than normal concentrations of fluorine in urine and serum [8–10]. Undoubtedly, humans and animals are highly susceptible to F in regions with elevated F levels

✉ Jing Zhao
hkdzhao1230@haust.edu.cn

Qiyong Zuo
zuoqy0730@163.com

Lin Lin
linlin403@163.com

Yuling Zhang
zhangyulings@haust.edu.cn

Mohammad Mehdi Ommati
mehdi_ommati@outlook.com

Hongwei Wang
wanghw@haust.edu.cn

[1] Henan Key Laboratory of Environmental and Animal Product Safety, Henan University of Science and Technology, Kaiyuan Avenue 263, Luoyang 471000, Henan, People's Republic of China

[11–13]; hence, the impact of F on the renal system requires further in-depth studies.

It is a well-known fact that there is a strong connection between F level in epithelial cells with increasing F intake and prolonged exposure time [14]. Renal failure prevents the efficient removal of F and consequently makes the body susceptible to fluorosis, even with normal F levels [15]. In the same vein, excessive F can adversely affect renal tubular reabsorption in the ascending medulla of the loop of Henle, alter the degree of ion exchange, and ultimately affect the functionality of the kidney [16]. In addition, progress in the field has revealed that F exposure could significantly decrease calcium, phosphorus, and creatinine levels and increase uric acid concentration, leading to impaired kidney function [17]. Accumulating evidence reveals that oxidative stress and mitochondrial impairment are the main mechanisms of F-induced kidney damage [18–21]. In this regard, our previous studies have shown that F can cause kidney damage by disrupting the expression of subunits of the mitochondrial complex and increasing mitochondrial synthesis [17]. Mitochondrial fission is a highly regulated process that, when disrupted, can alter metabolism, proliferation, and apoptosis [22]. Proteins involved in mitochondrial fission include mitochondrial fission factor (Mff), fission 1 (Fis1), and mitochondrial dynamics proteins of 49 kDa (MiD49) and 51 kDa (MiD51) [23]. Mitochondrial damage due to mitochondrial fission–fusion imbalance is a key cause of renal tubular injury [24]. However, to the best of the authors' knowledge, there is a paucity of information to explain the nephrotoxicity of F through the possible intracellular events, including oxidative stress and mitochondrial-related routes. Hence, the main aim of the current study was, therefore, undertaken to investigate the deleterious role of F on the renal system by assessing the histopathology and mitochondrial fission.

Materials and Methods

Animals and Grouping

Forty-eight sexually mature healthy female Kunming mice (35 days old) were obtained from the Experimental Center of Zhengzhou University, Henan, China. The animals were kept in a standard animal house under 12/12-h light/dark cycle, 22–25 ℃, and hygienic conditions. All procedures were ethically approved by the Ethics Committee of Henan University of Science and Technology according to the Chinese Animal Management Guidelines.

The mice were given distilled water and fed SPF grade mice breeding feed ad libitum (Huanyu Bio, Beijing, China). After a week of acclimatization, the experimental mice were randomly allotted into four groups ($n = 12$ each): the control group drank distilled water without F; the F group was given drinking water containing 25, 50, and 100 mg/L F. After 90 days of treatment with F, the mice were anesthetized with a 20% urethane (ethyl carbamate) solution, and their kidneys were gently isolated and rapidly fixed in 10% formaldehyde for further observations.

Histopathological Observation and Injury Score

The paraffinized tissue sections were cleared in xylene, dehydrated in graded ethanolic solutions (100, 95, 90, 80, 70, and 50%), washed with distilled water (2 min), and stained with H&E, PAS, PASM, ALP, and PB, according to the manufacturer's protocol. As mentioned, the PAS stain was used to measure renal cast formation in fluorosis mice. Images were computed using a computer-supported imaging system connected to a light microscope (OlympusAX70). The following lesions were evaluated for renal injury scores. The percentage of these injuries was counted on a scale from 0 to 10: 0, absence of lesions (typical); 1–4, 10–40% (mild); 5–6, 50–60% (moderate); 7–8, 70–80% (severe); and 9–10, 90–100% (very severe) [25].

TdT-Mediated dUTP Nick End Labeling (TUNEL) Staining

Apoptosis in the kidney of fluorosis mice was evaluated using the TUNEL method. According to the manufacturer's instructions, paraffin sections were serially cut and stained with a commercially available TUNEL reaction kit (Promega DeadEnd™ Colorimetric TUNEL System and Roche Cell Death Detection Kit) and DAPI. The sections were observed using an Olympus IX51 fluorescence microscope (Olympus America, Center Valley, PA), and green fluorescence staining was considered positive for nuclear DNA fragmentation.

Immunohistochemistry (IHC)

The paraffinized renal tissue slides (5 μm) were cleared in xylene, dehydrated in graded ethanolic solutions (see the "TdT-Mediated dUTP Nick End Labeling (TUNEL) Staining" section), and washed with distilled water. The cleaned sections were blocked with 5% bovine serum albumin (BSA) to prevent nonspecific antibody binding. The blocked sections were incubated with specific Fis1 (1:1000, 10956–1-AP, Proteintech, Wuhan China), Mff (1:1000, 17090–1-AP, Proteintech, Wuhan, China), MiD49 (1:1000, 16413–1-AP, Proteintech, Wuhan, China), and MiD5(1:1000, bs-12634R, Bioss, Beijing, China) at 4 ℃ in a humidified chamber overnight. Afterward, the incubated sections were washed with a phosphate-buffered solution thrice. The sections were incubated with a secondary antibody for 50 min at room temperature and stained with 3,3-diaminobenzidine. The loaded sections were counterstained with hematoxylin and washed with tap water. Ultimately, immunohistochemical

micrographs were viewed using an Olympus IX51 fluorescence microscope (Olympus America, Center Valley, PA).

Statistical Analysis

All data are illustrated as mean ± standard deviation (SD). Statistical evaluation was done by one-way variance analysis (ANOVA) using GraphPad Prism 8.0.2 (GraphPad Software, La Jolla, CA, USA), followed by Tukey's multiple comparison tests. $*P < 0.05$, $**P < 0.01$ indicated statistically significant differences. Pearson's correlation analysis was obtained using Origin2022 software (Origin Lab, Northampton, MA, USA).

Results

Renal Histological Alterations

In the control group, the glomerular space (the lumen within the glomerular capsule) was typical without apparent damage, and the renal tubules were structurally intact and closely packed with epithelial cells (Fig. 1). The kidney tissue of fluorosis mice presented nucleolytic activity, rupture of Bowman's capsule and renal tubule epithelial cells, and atrophy of glomeruli. These results suggest that F can dose-dependently increase nephrotoxicity.

F increased Glycogen Distribution

The PAS staining showed a uniform distribution of glycoproteins in the mouse kidneys of the control group (Fig. 2A). However, after 90 days of F treatment, dose-dependent increments were observed in the number of glycoproteins in the glomerular, tubular basement membranes, and kidney injury ($P < 0.01$; Fig. 2B). The renal tubular injury score results showed that excessive F significantly increased renal tubular damage ($P < 0.05$ or $P < 0.01$; Fig. 2B).

Renal Basement Membrane Alterations

As shown in Fig. 3, renal basement membrane injury was detected by PASM staining. In the control group, there

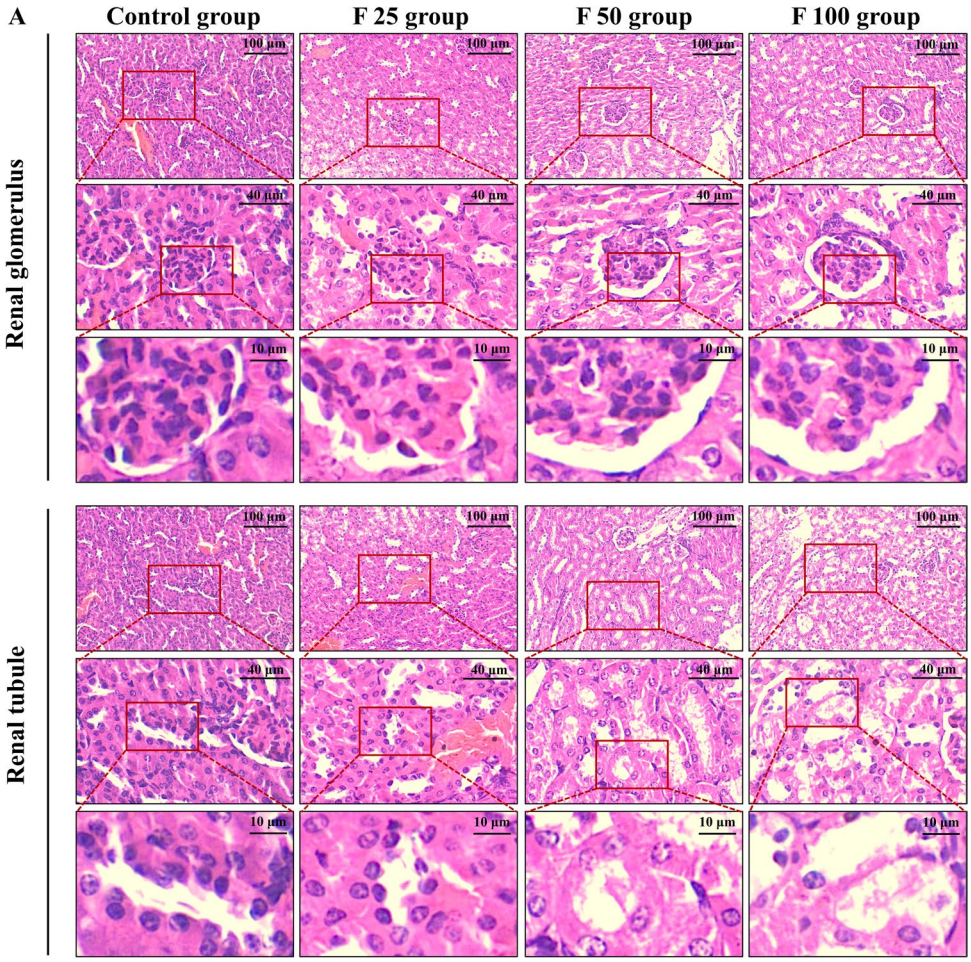

Fig. 1 Effects of F on renal morphology and structure using H&E staining. Renal tubular cells were irregularly arranged with swollen cells and pale nuclei. The results showed that excessive F damaged the kidney, resulting in glomeruli atrophy and renal capsule space increased

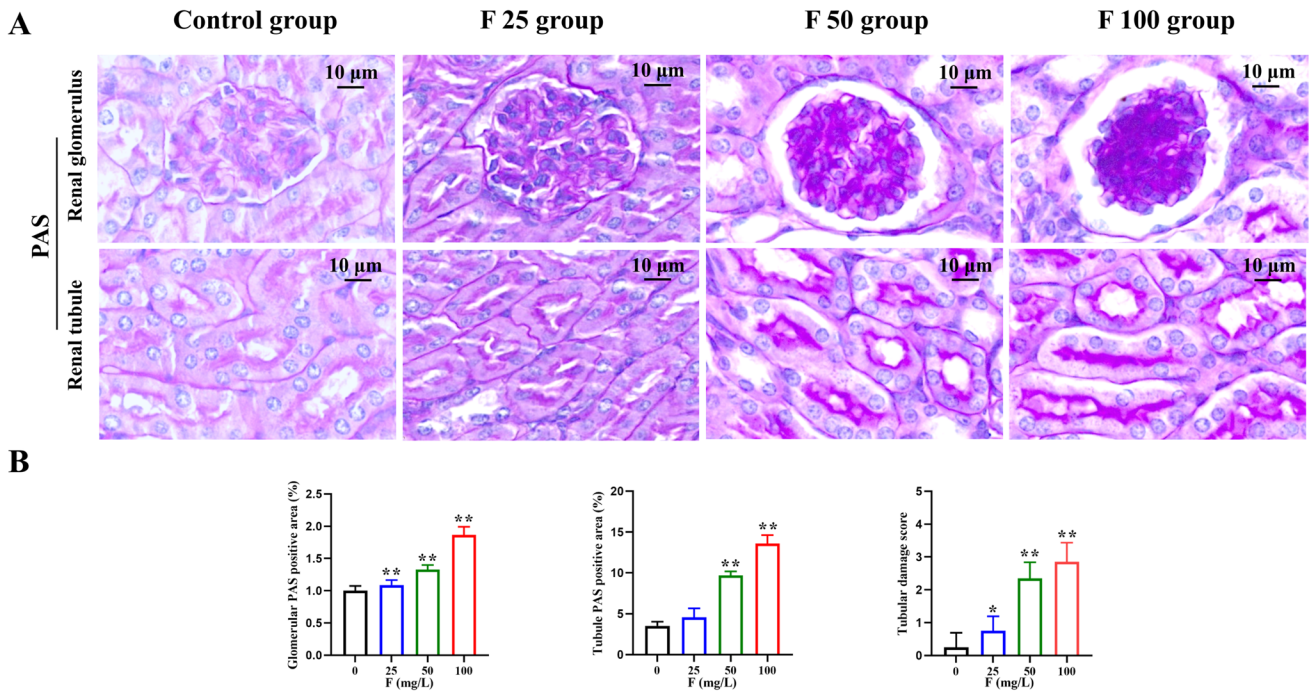

Fig. 2 The effect of F on the distribution of glycogen in kidneys. **A** PAS staining of the kidney. **B** The statistical data of glomerular PAS-positive area, renal tubular-positive area, and renal tubular injury score. Glycoproteins were accumulated in glomeruli and tubules of mice treated with excess F. All data were expressed as mean ± SD ($n = 6$). (*, **) above columns indicated a significant difference ($P < 0.05$ and $P < 0.01$, respectively) compared to the control group

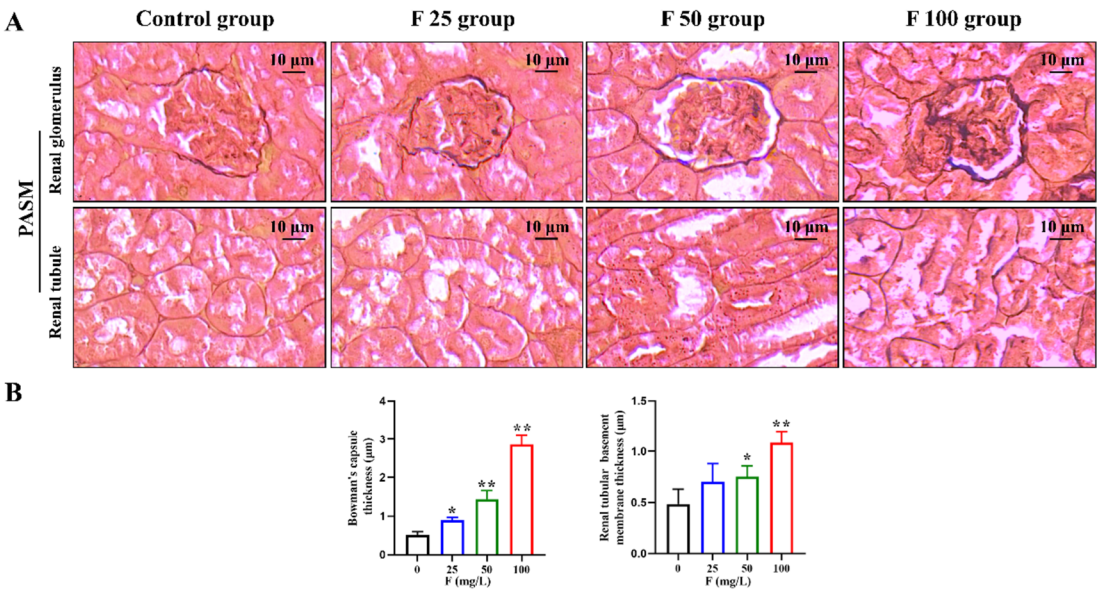

Fig. 3 The effect of F on the basal membrane of the kidney. **A** PASM staining of the kidney. **B** The basement membrane thickness of renal sacs and the renal tubular epithelium. All data were expressed as mean ± SD ($n = 6$). *$P < 0.05$; **$P < 0.01$

were no noticeable pathological changes in glomeruli and renal tubules; the basement membrane of the glomeruli and the brush border of renal tubules were intact (Fig. 3A). With the increase in F dose, the mesangial matrix of glomerulus increased and basement membrane thickened. Concomitantly, the basement membrane of the renal tubules is thickened, and the brush boundary is shortened or even broken ($P < 0.05$ or $P < 0.01$; Fig. 3A and B). These findings indicate that F exposure can damage the brush border of renal tubules in a dose-dependent manner.

Renal Alkaline Phosphatase (ALP)

ALP was accumulated in the renal glomerulus of the mice exposed to the medium (50 mg/L) and high (100 mg/L) doses of F, while dose-dependently decreased in the renal tubules of all F treatment mice ($P < 0.01$; Fig. 4B).

F increased Renal Iron Metabolism

The results of the PB demonstrated a large number of iron ions distributing around renal tubules in the medium (50 mg/L) and high (100 mg/L) exposed groups ($P < 0.01$; Fig. 5A, B). However, no footprint of iron ions was found in the renal glomerulus.

Renal Apoptotic Rate in Fluorosis Mice

The damage of apoptosis-related DNA in renal cells was observed by the TUNEL assay, as shown in Fig. 6. A few signs of DNA damage or apoptosis were recorded in the control group. However, the apoptotic rate was dose-dependently increased in the kidneys of the mice exposed to F ($P < 0.01$; Fig. 6B). Therefore, these observations suggested that excessive F could aggravate apoptosis in the renal system of fluorosis mice.

Renal Expression of Mitochondrial Fission-Related Proteins

As shown in Fig. 7, the expression of mitochondrial fission protein in renal tissue was assessed upon exposure to F. IHC results showed that the renal expressions of Fis1, Mff, MiD49, and MiD51 were dose-dependently upregulated in fluorosis mice ($P < 0.01$; Fig. 7B).

Correlation Analysis

Correlation analysis was approved to evaluate the correlation between renal mitochondrial fission-related proteins and indicators of kidney injury and apoptosis. As shown in Fig. 8, there was an apparent positive correlation between renal mitochondrial fission-related proteins

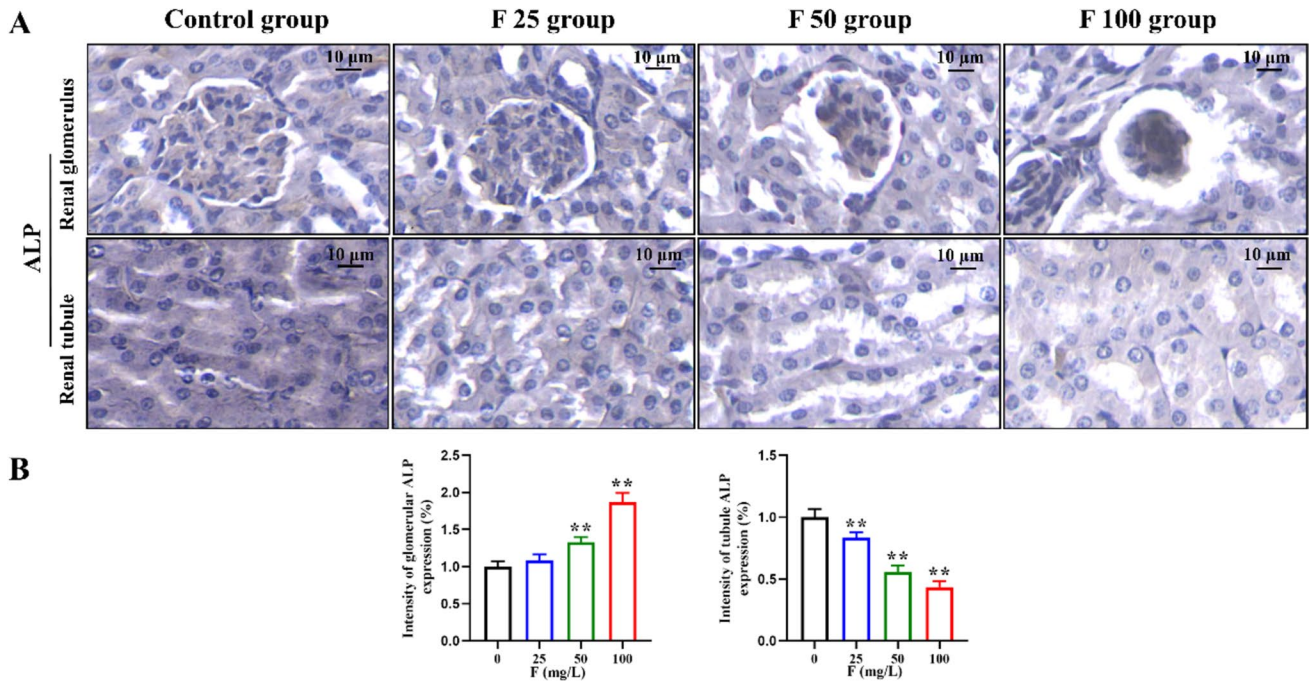

Fig. 4 Effect of F on ALP in the kidney. **A** Representative images of ALP-stained kidney tissue sections. **B** The intensity of glomerular and tubule ALP expression. All data were expressed as mean ± SD ($n = 6$); $^{**}P < 0.01$

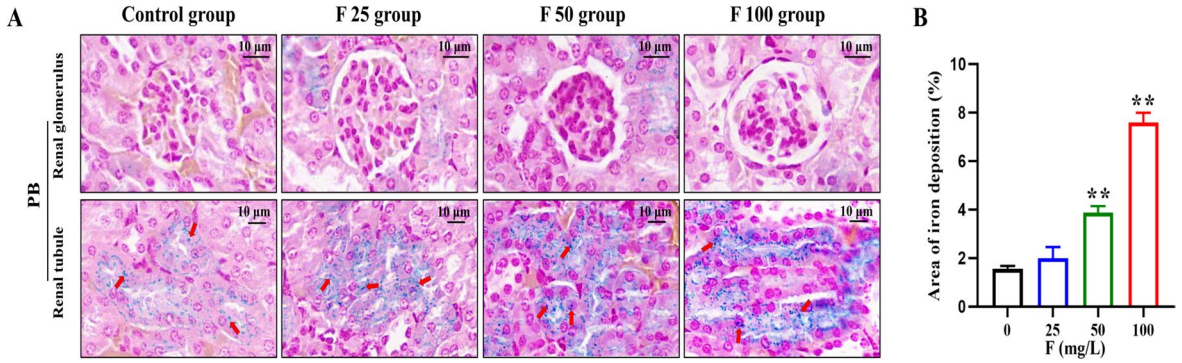

Fig. 5 Effects of F on renal iron metabolism. **A** Representative images of PB-stained kidney tissue sections. **B** The area of iron deposition in renal tubules. Iron deposition (red arrow) in renal tubules increased with the increase of F. All data were expressed as mean ± SD ($n = 6$). $^{**}P < 0.01$

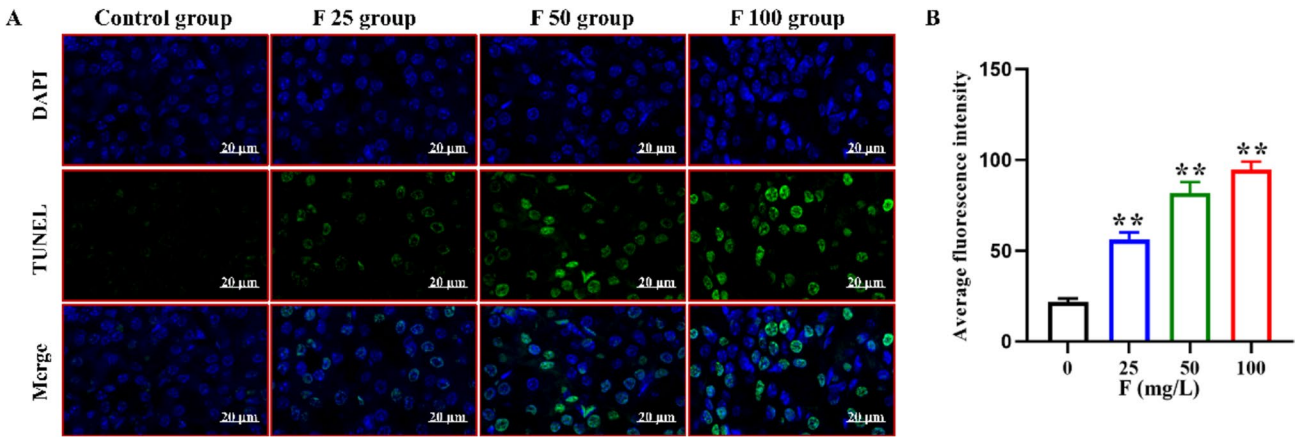

Fig. 6 Effect of F on renal DNA damage. **A** Positive expression of apoptosis-related DNA in renal cells. **B** The average fluorescence intensity of TUNEL. All data were expressed as mean ± SD ($n = 6$). $^{**}P < 0.01$

with apoptosis intensity (AI), iron deposition (ID), capsular space width (CSW), glomerular PAS-positive area (GPPA), tubule PAS-positive area (TPPA), glomerular ALP expression (GAE), and tubular basement membrane thickness (TBMT). However, apoptosis-related indices of the thickness of the parietal layer of Bowman's capsules (TBLBC), tubule ALP expression (TAE), and renal mitochondrial fission-related proteins showed a negative correlation (Fig. 8).

Discussion

Fluorine (F) is a small polar molecular element that readily diffuses into kidney cells, leading to sustained damage to the kidneys [26]. Recent studies have delineated that F affects the excretion of waste and reabsorption of essential substances in the renal tubules, ultimately causing kidney-related anomalies [27–29]. In the current study, F exposure caused renal tubular epithelial cell degeneration and vacuolation. Simultaneously, excessive F shortened or even broke the brush boundary of renal tubules and increased the thickness of the basement membrane. Moreover, an increased distribution of glycogen in the renal tubules is documented. Erstwhile studies have also reported that glycogen accumulation could inevitably intensify the possible impairments in the structure and function of the kidneys, leading to various complications [30]. Additionally, our observations confirmed that excessive F could induce abnormal morphological and structural changes in renal tubules, suggesting another proof of the strong renal toxicity for F.

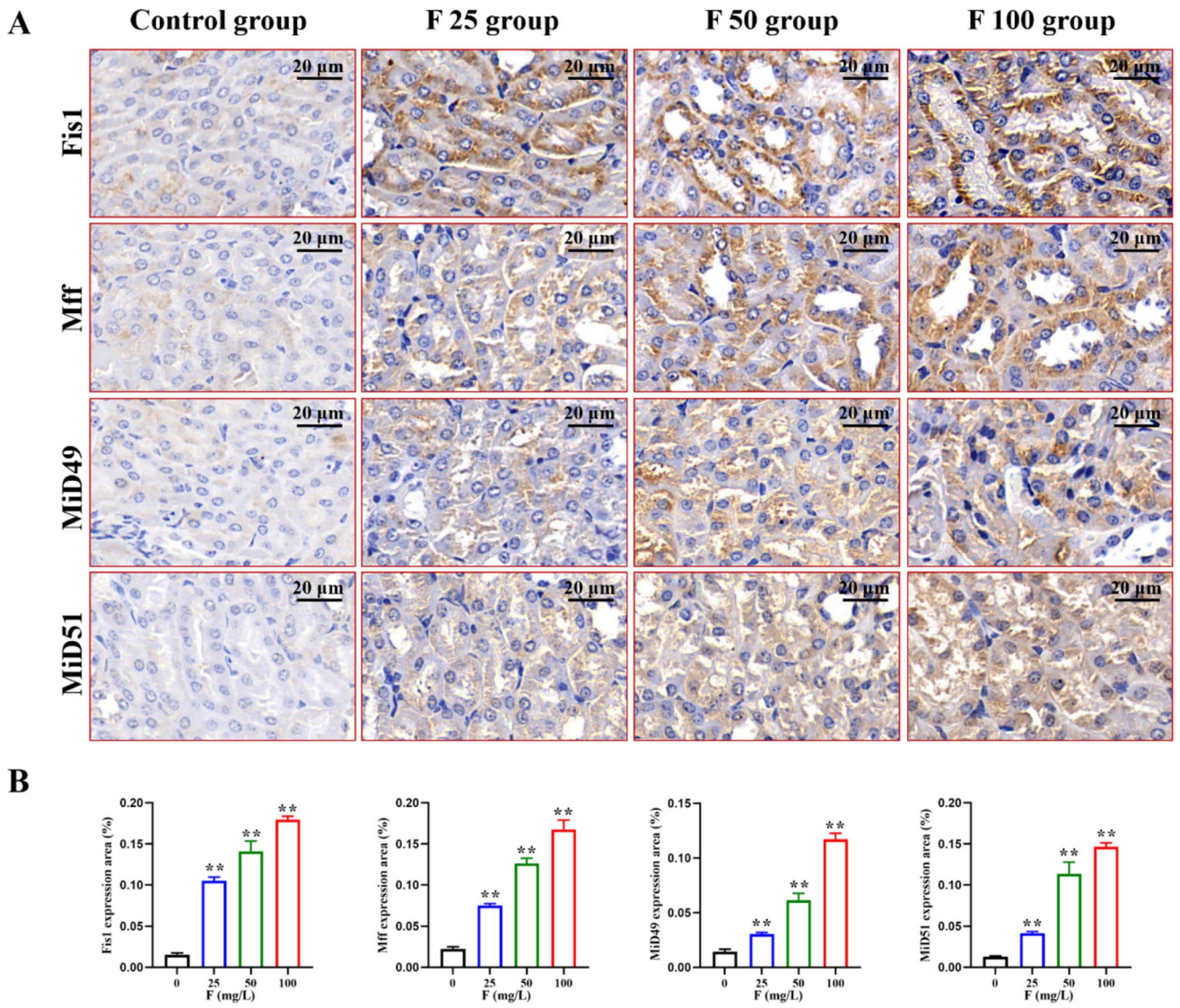

Fig. 7 Effects of F on renal expression of some mitochondrial fission-related proteins. **A** The protein expressions of Fis1, Mff, MiD49, and MiD51 in renal tissues were detected by IHC. **B** Renal Fis1, Mff, MiD49, and MiD51 expression levels. All data were expressed as mean ± SD ($n = 6$). (**) above columns indicated a significant difference ($P < 0.01$) as compared to the control group

ALP is present in human and plays a key role in intracellular destructive processes and cellular damage [31]. Renal tubular epithelial cells contain a considerable amount of ALP, and its secretion is common after xenobiotics-induced injuries to renal cells [32, 33]. As ALP plays a vital role in the secretion and reabsorption of renal tubules; hence, its release is considered a reliable marker for kidney injury [34]. In the same vein, this study confirmed the decline of ALP in renal tubular epithelial cells and its accumulation in the glomerulus. This explains the concentration-dependent increase in ALP in the luminal fluid due to F [35]. The impact of F on renal tubular epithelial cells leads to their impairment, facilitating the release of ALP into the bloodstream and subsequent accumulation within the glomeruli. This elucidates the underlying mechanism behind the elevation of ALP levels in serum upon F exposure [36]. In addition, F can significantly change the level of ion metabolism [37]. The kidneys are essential in iron metabolism and homeostasis [38]. Iron can be filtered through the kidneys' glomeruli and reabsorbed by proximal tubules, the loop of Henle, and distal tubular cells [39]. Our study found a pronounced iron deposition in renal tubules in the F supplemented group. Under pathological conditions, oxidative stress and subsequent intracellular mechanisms may cause excessive iron accumulation in the renal tubules, leading to iron-induced kidney injury. An in-depth in vitro experiment showed an increment in heme degradation and reported that the fluorinated red blood cells produced methemoglobin and oxyhemoglobin, releasing

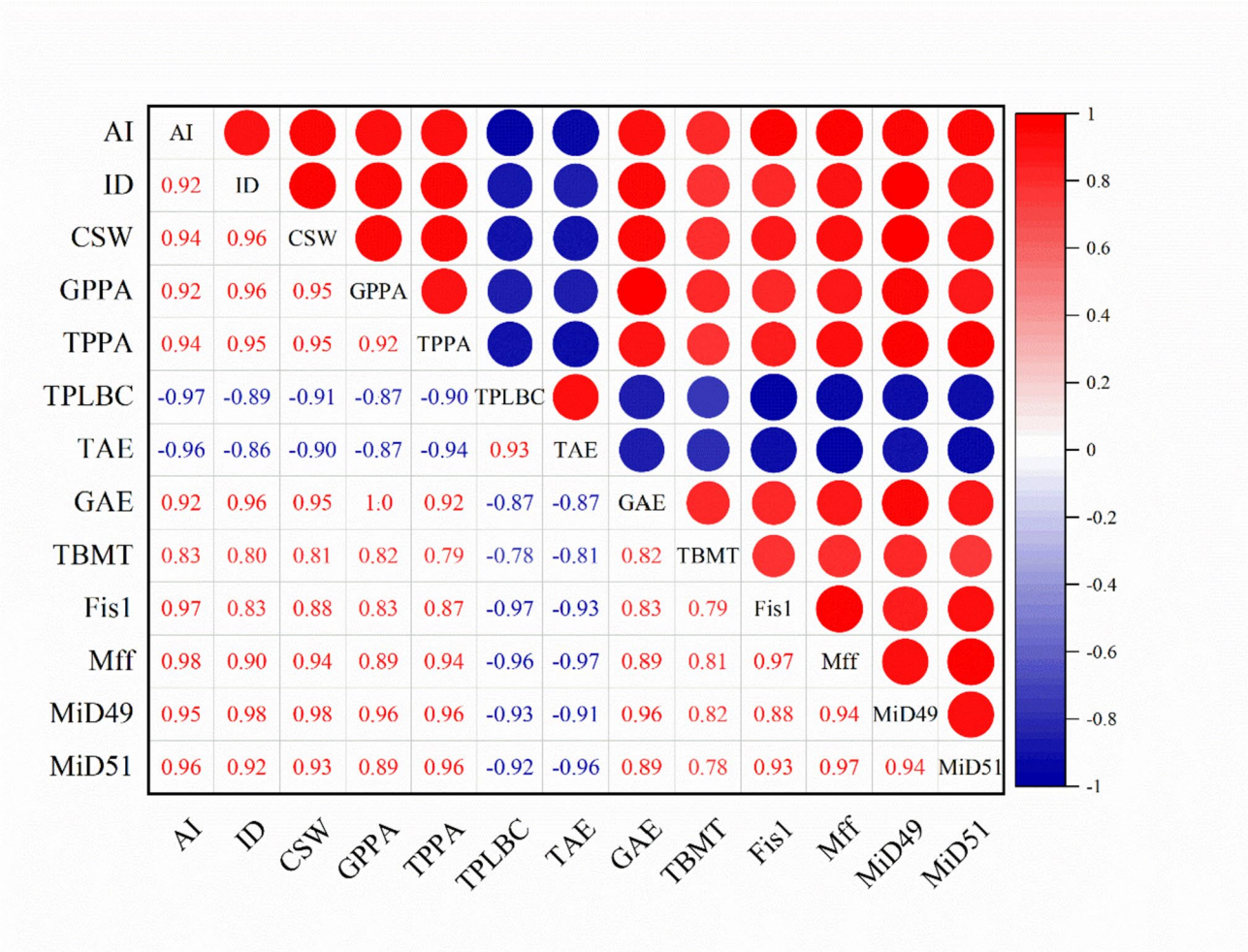

Fig. 8 Correlation analysis of mitochondrial fission-related proteins with indicators of renal injury and apoptosis after fluoride treatment. AI, Apoptosis intensity; ID, iron deposition; CSW, capsular space width; GPPA, glomerular PAS positive area; TPPA, tubule PAS positive area; TBLBC, thickness of the parietal layer of Bowman's capsules; TAE, tubule ALP expression; GAE, glomerular ALP expression; TBMT, tubular basement membrane thickness

iron ions from the porphyrin ring [40]. The excess iron ions in the kidney are possibly caused by increased accumulation of free iron ions in the proximal and distal tubules. This accumulation can be a result of high hemoglobin filtration and a low resorption rate of renal tubule epithelial cells.

We have repeatedly reported the crucial role of mitochondria in maintaining the normal function of the renal system [41, 42]. Renal cells are rich in mitochondria, the main sites of energy conversion and oxidative phosphorylation [43]. Mitochondrial fission and mitochondrial fusion are dynamic processes [44]. To maintain the functionality of the kidney tissue, renal cells regulate the fission and fusion of mitochondria to neutralize the damage caused by F. Dysfunction of mitochondrial fission is an important link in the development of chronic kidney disease, and persistent mitochondrial dysfunction leads to persistent tubular atrophy and atresia [45]. Mitochondrial fission can mitigate damaged mitochondria during cellular stress by promoting mitophagy and apoptosis [46, 47]. Fis1, Mff, MiD49, and MiD51, located on the surface of mammalian mitochondria, are vital proteins that control and regulate mitochondrial fission [48]. Evidence substantiates that mitochondrial fission and elongation are induced, respectively, by upregulation and downregulation of Mff and Fis1 in cells [49]. High expression of Fis1 promotes mitochondrial fission, apoptosis, and pyroptosis of cells, thus inducing nephrotoxicity [50, 51]. MiD49 and MiD51 are considered to be essential components of mitochondrial fission mechanisms. Meanwhile, the Drp1 recruitment activity of MiD49 and MiD51 appeared stronger than that of Mff or Fis1 [52]. We have also reported that mitochondrial structural damage in hepatocytes results in the mitochondrial release of Cyt-c into the cytoplasm, stimulating a cascade reaction between Caspase 9 and Caspase 3 and activating the mitochondria-mediated apoptotic

pathway [53]. Mitochondrial and kidney damage caused by increased mitochondrial fission are complementary and form a vicious cycle. In this regard, in the current study, excessive F also triggered apoptosis through the induction of mitochondrial fission via upregulation of the renal Fis1, Mff, MiD49, and MiD51 levels. Hence, it can be assumed that mitochondrial fission might be a physiological response to compensate for mitochondrial dysfunctionality upon exposure to F and may be involved in regulating fluorine-induced nephrotoxicity damage.

In conclusion, excessive fluoride intake causes morphological damage of renal, interfering with mitochondrial fission and causing apoptosis of renal tubular epithelial cells, which showed substantial renal toxicity. This provides an important scientific basis for further studying of fluoride-induced renal dysfunction.

Author Contribution Qiyong Zuo: Formal analysis, writing the original draft, preparation of the data presentation. Lin Lin: Visualization, validation, data curation. Yuling Zhang: Validation, project administration. Mohammad Mehdi Ommati: Ideas, investigation, critical review. Hongwei Wang: Conceptualization, methodology, review and editing, provision of resources. Jing Zhao: Funding acquisition, validation.

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Data Availability All data generated or analyzed during this study are included in this published article.

Code Availability Not applicable.

Declarations

Ethics Approval The experimental design was approved by the Institutional Animal Experiment Committee of Henan University of Science and Technology, China.

Consent to Participate Written informed consent for publication was obtained from all participants.

Consent for Publication Written informed consent for publication was obtained from all participants.

Competing Interests The authors declare no competing interests.

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