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Effect of Voluntary Wheel-running Exercise on Kidney Oxidative Stress in Fluoride-exposed Mice

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ABSTRACT

Purpose: Excessive fluoride induces kidney injury by reducing the antioxidant capacity and causing oxidative stress. Our previous studies have shown that moderately forced treadmill running alleviates oxidative injury. However, the impact of voluntary wheel-running exercise on fluoride-intoxicated mice remains unclear. In this study, we investigated the effects of voluntary wheel-running exercise on oxidative stress-induced renal injury in fluoride-intoxicated mice.

Methods: 80 Institute of Cancer Research (ICR) mice (half male and half female), were randomly divided into one of eight experimental groups: four receiving distilled water and the other four ingesting 100 mg/L sodium fluoride. Each group was subdivided to incorporate different exercise regimens—no wheel-running, daytime, nighttime, and all-day wheel-running exercises. After 6 months, mice were euthanized for subsequent analyses.

Results: HE staining revealed that 6 months of fluoride exposure caused structural damage in the kidneys of mice, elevated blood urea nitrogen (BUN), and uric acid (UA) levels in the serum. Levels of malondialdehyde (MDA), reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), and catalase (CAT) enzyme activity significantly increased. In contrast, glutathione (GSH) content and total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-PX) enzyme activities obviously decreased. Interestingly, these above changes were reversed by free-wheel movement. Correlation analysis demonstrated that negative relationships exist between ROS, MDA, H_2O_2 levels, CAT enzyme activity, and movement distance. Additionally, T-SOD and GSH-PX enzyme activities were positively associated with exercise distance.

Conclusions: Voluntary wheel-running exercise mitigated renal injury induced by sodium fluoride in mice, and the extent of renal recovery was positively correlated with the distance covered during voluntary wheel-running exercise.

Key-words: *Voluntary wheel-running exercise; Fluorosis; Kidney; Oxidative stress; Correlation analysis*

INTRODUCTION

Fluorine is a natural constituent of the biosphere and is the thirteenth most abundant element in the earth's crust. Fluorine atoms have a small radius and the highest effective surface charge of all the elements, so fluorine is the most electronegative and reactive element [1]. Excessive fluoride intake can impair various enzyme activities, disrupt the balance of calcium and phosphorus metabolism, and result in the formation of dental plaque and deformation of bones and joints [2]. Excessive accumulation of fluoride can also result in liver, kidney, testicular, and neurological damage [3-5]. A growing number of studies have shown that fluoride causes damage to the body by primarily causing oxidative stress, cellular damage, apoptosis, and alterations in the intestinal flora [2]. The kidney is the most important excretory organ of fluoride, and the fluoride content in the urine and fluoride intake showed a significant positive correlation [6]. The kidney is the most sensitive soft tissue to fluoride; excessive fluoride accumulation in the kidneys or through the kidneys will cause damage to the kidneys, which is mainly manifested in the form of filtration and reabsorption obstacles, as well as disorders of substance metabolism and other aspects [7]. The pathological examination results revealed that a high dose of fluorine will obviously result in the swelling or even rupture of renal tubular epithelial cells, the disappearance of nuclei, nuclear consolidation, and other observable changes in the kidneys of mice. Glomeruli undergo congestion out and atrophy, and obvious inflammatory cell infiltration and vascular congestion can be seen in the renal interstitial fluid [8-10].

The concept of "Exercise as medicine" encompasses the utilization of physical activity and exercise as a therapeutic intervention for the prevention and management of a spectrum of chronic diseases [11-13]. Exercise has been shown to have a favorable effect on a range of health-related outcomes in chronic kidney disease and can help improve many related conditions such as hypertension, diabetic nephropathy, and hypofunction; aerobic exercise reduces the level of oxidative stress in the kidneys and improves renal function in mice modeling diabetic nephropathy [14, 15]. Voluntary wheel running is a mouse-specific behavioral trait characterized by self-reinforcement and reward effects and is a non-compulsive exercise. Voluntary running wheel exercise provided several advantages over forced treadmill exercise. Firstly, the running pattern closely mimics the natural running behavior of mice. Secondly, it is conducted in non-stressful conditions that align with the rhythmicity of the animal. Lastly, this type of exercise does not require direct intervention from the researcher and can be seamlessly integrated into long-term studies [16]. When provided with a running wheel, rats engage voluntarily, reducing the stress associated with animal exercise and potentially alleviating chronic stress [17].

Excessive accumulation of fluoride in the organism can induce oxidative stress in the kidney and change the normal tissue morphology of the kidney [18]. Voluntary running wheel as a kind of non-forced aerobic exercise, can alleviate or ameliorate renal injury in various unfavorable states and restore its physiological function. Therefore, in the present experiment, we investigated the effects of free-wheeling exercise on renal tissue morphology, renal injury biomarker, and antioxidant capacity in mice with fluorosis by using mice ingesting fluorine during the daytime, nighttime, and all-day voluntary wheel-running exercise, and examined the correlation between the distance of the voluntary wheel-running exercise and the indexes related to oxidative stress. The objective is to demonstrate the beneficial effects of appropriate exercise and to serve as a reference for the concept of "exercise as medicine".

MATERIAL AND METHODS

Establishment of animal models: 80 three-week-old Institute of Cancer Research (ICR) mice, half male and half female, were purchased from the Experimental Animal Center of Shanxi Medical University (Taiyuan, Shanxi, China). All mice were maintained in a standard environment with AD libitum access to water and were fed formulated diets purchased from Shanxi Medical University (Taiyuan, Shanxi, China) according to GB 14924.3-2010.

After one week of acclimatization, the mice were randomly divided into eight groups: Control (C) group (drinking distilled water and no exercise), daytime exercise (DE) group (drinking distilled water, running free wheels only during the daytime), nighttime exercise (NE) group (drinking distilled water, running free wheels only at night), all day exercise (AE) group (drinking distilled water, free running exercise throughout the day), fluoride (F) group (drinking 100 mg/L NaF, no exercise), fluoride plus daytime exercise (DEF) group (drinking 100 mg/L NaF, exercise only during the daytime), fluoride + night exercise (NEF) group (drinking 100 mg/L NaF and exercising only at night) and fluoride + all day exercise (AEF) group (drinking 100 mg/L NaF and exercising freely throughout the day). The exercise time of the daytime exercise group was from 8:00 to 20:00, and the exercise time of the nighttime exercise group was from 20:00 to the next day 8:00. During the exercise period

of each group, a running wheel with a counter was provided for voluntary exercise; otherwise, the wheel was removed. The counter on each wheel recorded the number of revolutions, and the daily revolutions of each mouse were recorded to calculate the daily amount of exercise for subsequent experimental use.

Each group was euthanized the same way using cervical dislocation after 6 months. All experimental procedures were permitted by the Animal Care and Use Committee of Shanxi Agricultural University (Jinzhong, Shanxi, China, SXAU-EAW-2021M0429).

Body weight change and kidney viscera coefficient: After the six-month test period, the body weights of the experimental mice were measured, and the weights of the left and right kidneys were quickly weighed and recorded after the mice were executed, and the viscera coefficient of the left and right kidneys were calculated separately.

HE staining: After immersion in a 4% paraformaldehyde solution at 4°C for 24 h, kidney tissues were dehydrated in gradient alcohol and dealcoholized in xylene. The tissue was then immersed in paraffin to make paraffin sections, and 5μm sections were cut with a Leica RM2265 microtome (Leica, Bensheim, Germany). HE staining was conducted on paraffin sections as directed in the kit instructions (Solarbio Science & Technology Co., Beijing, China). An optical microscope (Olympus, Tokyo, Japan) was used to observe changes in kidney morphology.

Renal injury biomarker: The concentrations of BUN and UA in serum were measured according to the instructions of the commercial assay kits (Jiancheng Biotech, Nanjing, China).

Determination of oxidative stress: We detected the oxidative stress index by determining the levels of malondialdehyde (MDA), reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), glutathione (GSH), total superoxide dismutase (T-SOD), glutathione peroxidase (GSH-PX) and the catalase (CAT) according to the instructions of the commercial assay kits (Jiancheng Biotech, Nanjing, China).

Quantitative real-time PCR: Mouse kidney RNA was extracted with Trizol (Takara, Dalian, China), and total RNA was converted into cDNA with Prime ScriptTM RT Master Mix (Takara, Dalian, China). The primer sequences designed by Primer 3.0 plus are listed in Table 1. qRT-PCR used Mx3000PTM QPCR instrument (Stratagene, La Jolla, USA) and Premix Ex Taq™II TB GreenTM (Takara, Dalian, China) to complete the experiment according to the reaction instructions. Use the $2^{-\Delta\Delta Ct}$ method to analyze related genes. The primers are shown in Table 1.

Statistical analysis: The t-test for each indicator was performed using GraphPad Prism8 software (GraphPad Software Inc, San Diego, USA) to compare the differences between the groups. $^{*}P<0.05$, $^{*}P<0.01$ indicates a statistically significant difference when compared with group C; $^{\#}P<0.05$, $^{\#}P<0.01$ indicates a statistically significant difference when compared with group F. Use of linear regression to assess the correlation between exercise and oxidative stress-related index.

RESULTS

The running distance of mice in exercise groups: As illustrated in Figure 1, the total exercise distance of mice across various exercise groups is displayed, with the NE group and the AE group demonstrating significantly greater distances compared to the DE group. Likewise, the NEF group and AEF group showed significantly higher exercise distances than the DEF group($P<0.01$).

Morphological observation by HE staining: As shown in Figure 2, in the normal control group, the epithelial cells of the renal tubules were well arranged, the lumen of the renal capsule was normal, and the renal corpuscles were of uniform texture. Groups DE, NE, and AE were close to the control group without obvious structural alterations. Compared with group C, the cells in group F were more disorganized, with unclear intercellular boundaries, thickening of the tubular wall of the renal tubules, narrowing of the tubular lumen, increasing the area of the cystic lumen of the renal corpuscles (as indicated by the red arrows in the figure), disintegration of the epithelial cells, granular degeneration or swelling of some of the cells, and condensation of the nuclei of the cells. Compared with group F, the cells in the DEF, NEF, and AEF groups were close to normal, the tubular walls of the renal tubules were not thickened, the glomeruli were not significantly swollen, and the cystic lumens of the renal bodies were close to normal.

Voluntary wheel-running exercise ameliorates renal injury indicators: As illustrated in Figure 3, the serum blood BUN levels in the F group were significantly elevated compared to the C group, while the NEF and AEF groups showed a significant reduction in BUN levels compared to the F group ($P<0.05$, $P<0.01$). Similarly, the serum UA levels in the F group were significantly higher than in the C group, and the AEF group showed a significant

decrease in UA levels compared to the F group (P<0.05, P<0.01).

Voluntary wheel-running exercise ameliorates fluoride-exposed oxidative damage in the kidney tissue of mice: As shown in Fig 4, the MDA, ROS, and H_2O_2 relative significantly increased in the F group compared to the C group whereas the oxidative stress product expression levels decreased in the AEF group when compared to the F group ($P < 0.05$, $P < 0.01$). Meanwhile, the NEF group exhibited significantly reduced expression levels of oxidative stress products, including MDA, ROS, and H_2O_2, compared to the F group ($P < 0.05$, $P < 0.01$). These oxidative stress products significantly decreased in the DEF group compared to the F group except MDA ($P < 0.05$, $P < 0.01$).

In Fig 5, the GSH relative decreased in the F group compared to the C group whereas the antioxidant substance expression levels increased in the AEF group when compared to the F group ($P < 0.05$).

The results of the antioxidant enzyme activities were revealed in Fig. 5. The T-SOD GSH-PX and CAT activities significantly decreased in the F group compared to the C group but the enzyme activities increased in the DEF, NEF, and AEF group compared to the F group ($P < 0.05$, $P < 0.01$).

Effects of fluoride exposure and/or voluntary wheel-running exercise on the mRNA expression of oxidative stress: As shown in Fig. 6, with the comparison to the C group, the mRNA expressions of GSH-PX, CAT, SOD1, and SOD2 were significantly reduced in the F group. There was a significant enhancement in the levels of genes listed above in the DEF, NEF, and AEF groups compared with the F group ($P < 0.05$, $P < 0.01$).

Correlation between oxidative stress level and voluntary wheel-running exercise distance: The correlation analysis between exercise distance and oxidative stress biomarkers is shown in Figure 7. In mice in the group drinking distilled water, the contents of MDA, ROS, and H_2O_2 in the body were negatively correlated with the distance of voluntary wheel-running exercise (r=-0.5539, r=-0.4908, r=-0.6636). In mice in the group drinking 100 mg/L NaF, the contents of MDA, ROS, and H_2O_2 were negatively correlated with exercise (r=-0.5220, r=-0.4929, r=-0.5817).

The correlation analysis between exercise distance and renal antioxidant machinery is shown in Figure 8. There was no correlation between the content of GSH and the amount of exercise. The activity levels of three oxidative stress enzymes were not correlated with exercise volume in mice provided with distilled water. In fluorosis mice, renal enzyme activities of T-SOD and GSH-PX showed positive correlations with exercise (r=0.4951, r=0.5899), whereas CAT activity exhibited a negative correlation (r=-0.4978).

Table 1. Primer sequences for QRT-PCR

Gene name	Primer sequence (5'→3')	Product size (bp)	GenBank number
β-actin	F:TGTGGATCAGCAAGCAGGAG R:ACGCAGCTCAGTAACAGTCC	87	NM_007393.5
CAT	F: CCAACAAGATTGCCTTCTCC R: GCTCCTTCCACTGCTTCATC	142	NM_009804.2
SOD1	F: AGATGACTTGGGCAAAGGTG R: AATCCCAATCACTCCACAGG	85	NM_011434.2
SOD2	F: GCGTGACTTTGGGTCTTTTG R: AGCGACCTTGCTCCTTATTG	110	NM_013671.3
GSH-PX	F: CCAGGAGAATGGCAAGAATG R: CATTCCGCAGGAAGGTAAAG	146	NM_001329528.1

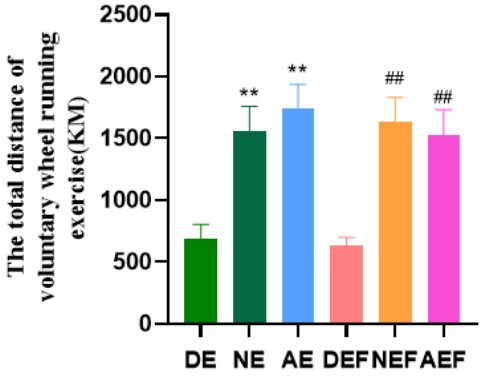

Fig.1 The running distance of mice in exercise groups. DE: daytime exercise group; NE: nighttime exercise group; AE: all day exercise group; DEF: daytime exercise plus fluoride group; NEF: nighttime exercise plus fluoride group; AEF: all day exercise plus fluoride group. Data represents the mean ±SEM (n=6). "**" indicates a statistically significant difference compared to group DE and "##" indicates a statistically significant difference compared to group DEF (P<0.01).

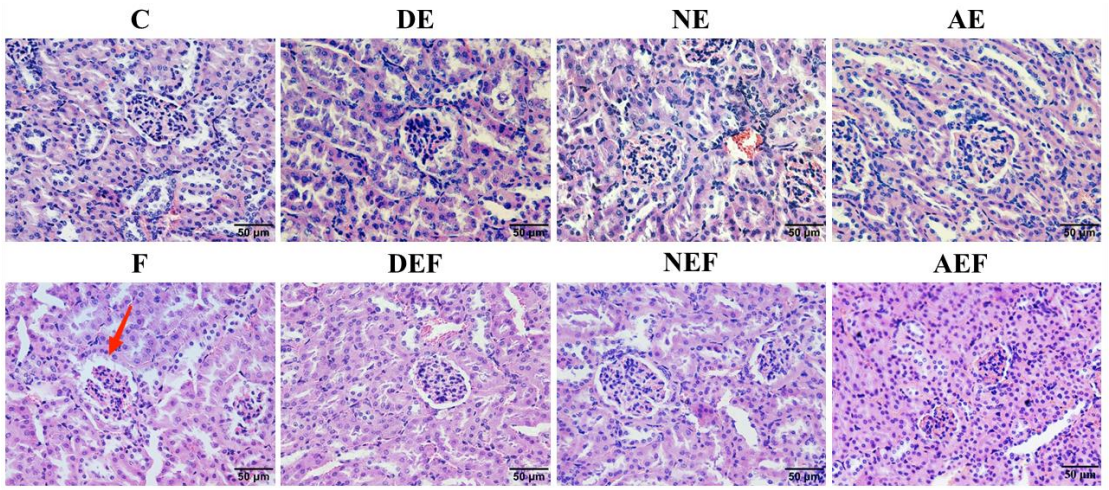

Fig. 2 HE staining of kidney tissues (400 ×). (n = 3 mice in each group). C: control group; DE: daytime exercise group; NE: nighttime exercise group; AE: all day exercise group; F: fluoride group; DEF: daytime exercise plus fluoride group; NEF: nighttime exercise plus fluoride group; AEF: all day exercise plus fluoride group. Scale bar: 50 μm.

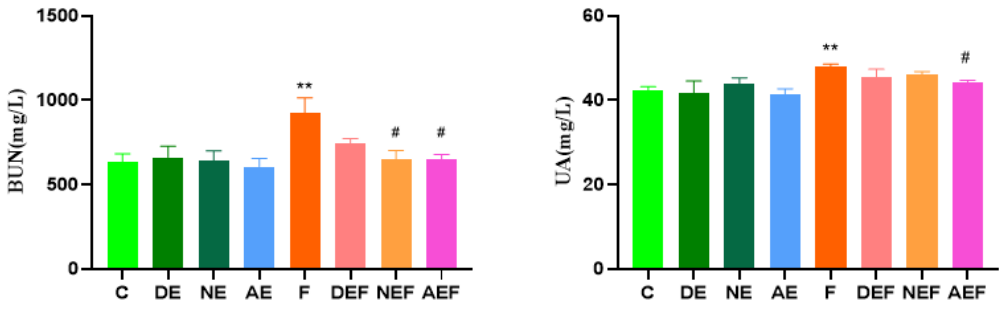

Fig. 3 The serum levels of blood urea nitrogen (BUN) and uric acid (UA) in mice. C: control group; DE: daytime exercise group; NE: nighttime exercise group; AE: all day exercise group; F: fluoride group; DEF: daytime exercise plus fluoride group; NEF: nighttime exercise plus fluoride group; AEF: all day exercise plus fluoride group. Data represents the mean ±SEM (n=6). "*" indicates a statistically significant difference compared to group C and "#" indicates a statistically significant difference compared to group F (P<0.05).

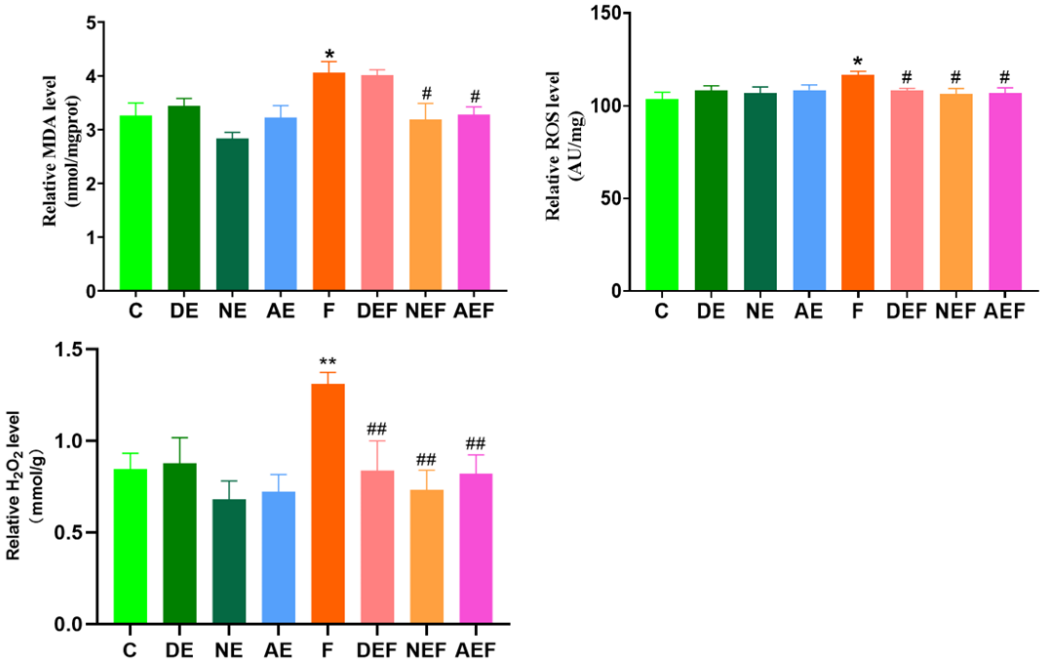

Fig. 4 The renal levels of malondialdehyde (MDA), reactive oxygen species (ROS), and hydrogen peroxide (H2O2) in mice. C: control group; DE: daytime exercise group; NE: nighttime exercise group; AE: all day exercise group; F: fluoride group; DEF: daytime exercise plus fluoride group; NEF: nighttime exercise plus fluoride group; AEF: all day exercise plus fluoride group. Data represents the mean ±SEM (n=6). "*" indicates a statistically significant difference compared to group C and "#" indicates a statistically significant difference compared to group F (P<0.05).

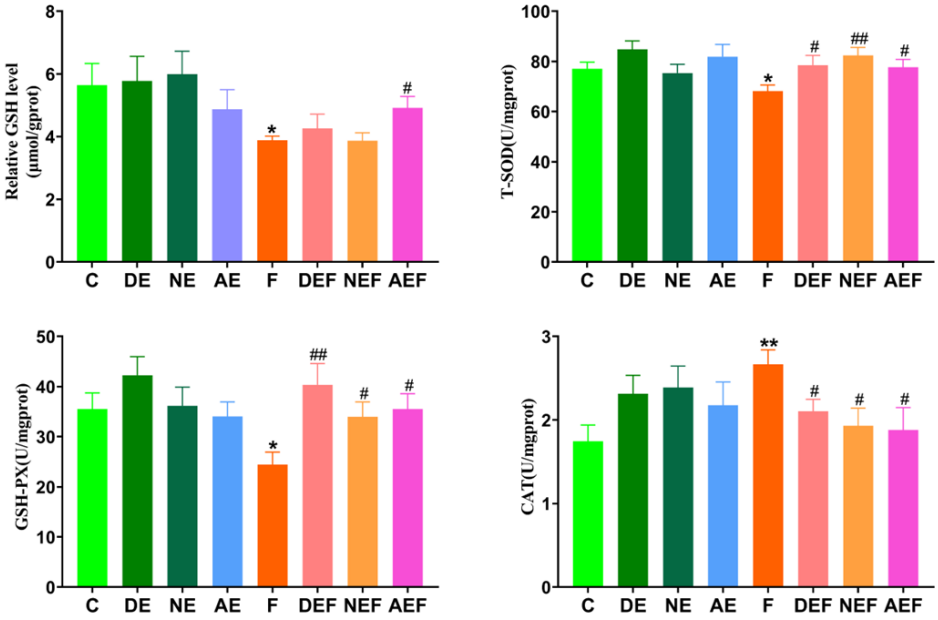

Fig. 5 Renal levels of reduced glutathione (GSH), total superoxide dismutase (T-SOD) activity, glutathione peroxidase (GSH-PX) activity, and catalase (CAT) activity in mice. C: control group; DE: daytime exercise group; NE: nighttime exercise group; AE: all day exercise group; F: fluoride group; DEF: daytime exercise plus fluoride group; NEF: nighttime exercise plus fluoride group; AEF: all day exercise plus fluoride group. Data represents the mean ±SEM (n=6). "*" indicates a statistically significant difference compared to group C and "#" indicates a statistically significant difference compared to group F (P<0.05).

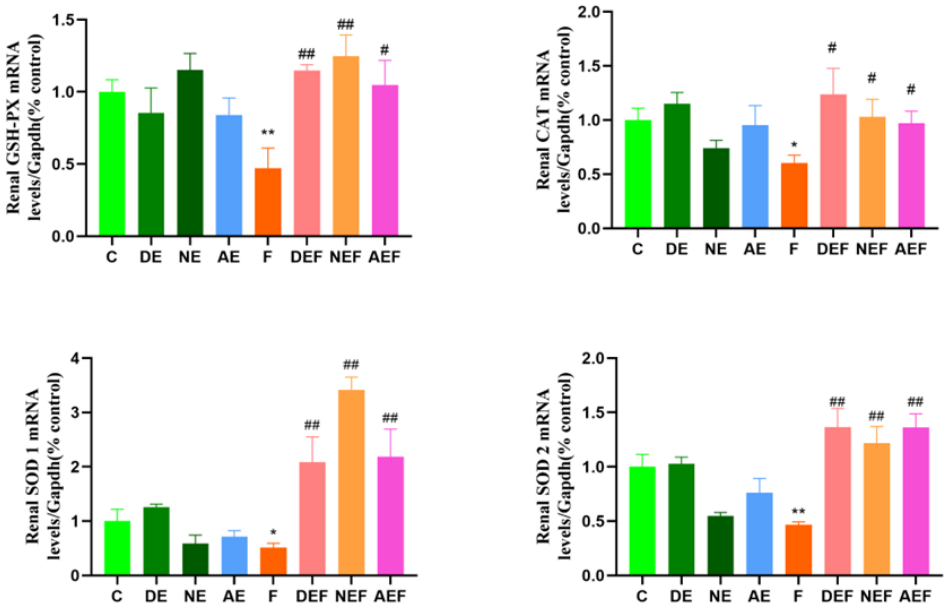

Fig. 6 Expression of oxidative stress-related enzyme mRNA in the kidney. C: control group; DE: daytime exercise group; NE: nighttime exercise group; AE: all day exercise group; F: fluoride group; DEF: daytime exercise plus fluoride group; NEF: nighttime exercise plus fluoride group; AEF: all day exercise plus fluoride group. Data represents the mean ±SEM (n=6). "*" indicates a statistically significant difference compared to group C and "#" indicates a statistically significant difference compared to group F (P<0.05).

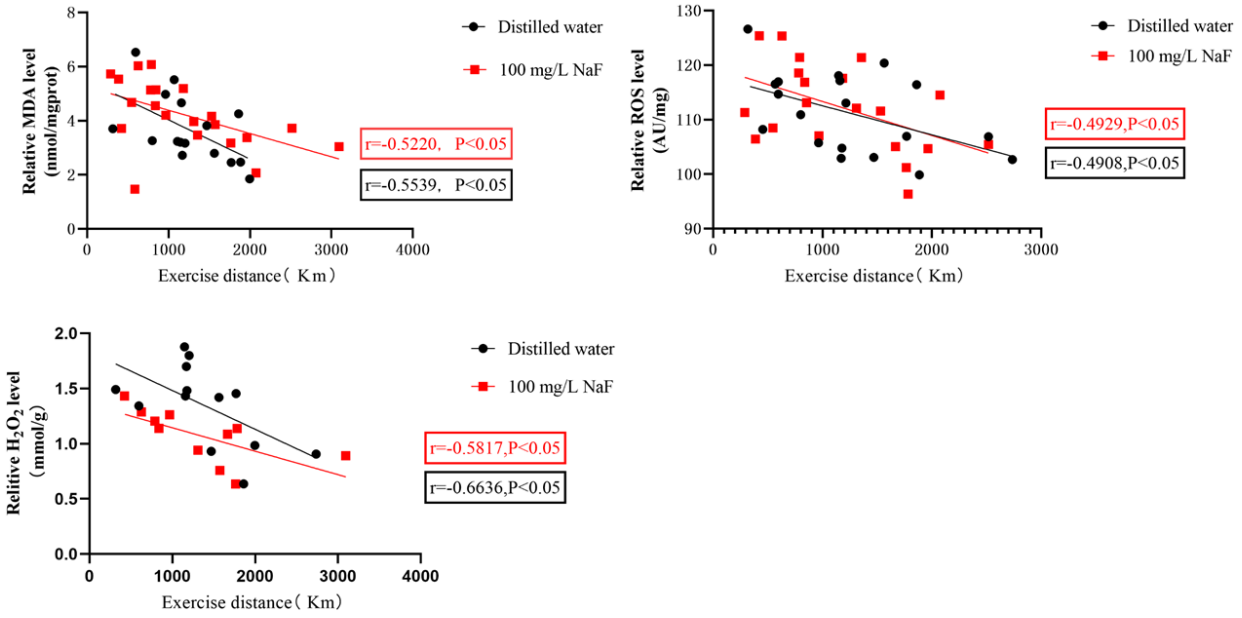

Fig. 7 Correlation analysis between exercise distance duration and renal levels of malondialdehyde (MDA), reactive oxygen species (ROS), and hydrogen peroxide (H_2O_2). The black dots and lines represent groups with distilled water, and the red dots and lines represent groups with 100 mg/L NaF in drinking water.

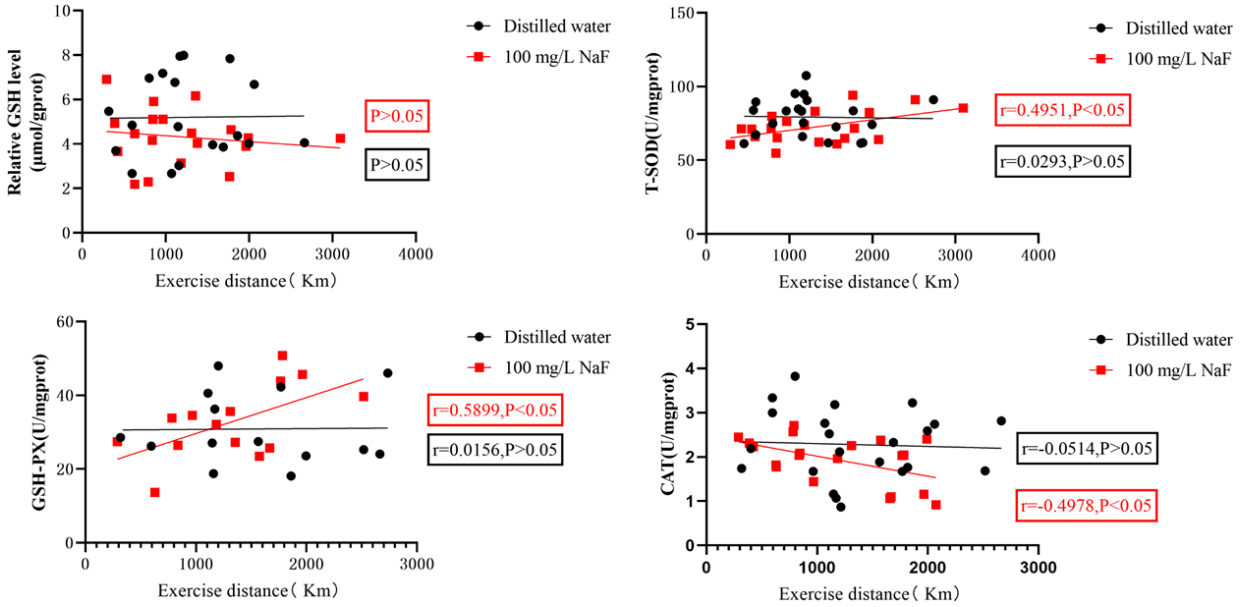

Fig. 8 Correlation analysis between exercise distance duration and renal levels of reduced glutathione (GSH), total superoxide dismutase (T-SOD) activity, glutathione peroxidase (GSH-PX) activity, and catalase (CAT) activity. The black dots and lines represent groups with distilled water, and the red dots and lines represent groups with 100 mg/L NaF in drinking water.

DISCUSSION

Studies have shown that excessive fluoride intake results in detrimental histopathological changes in the kidney, including renal tubular cell degeneration and necrosis, inflammatory cell infiltration, glomerular hypertrophy, and the formation of glassy casts within renal tubules [19, 20]. In this study, mice exposed to fluorine exhibited disrupted morphological structure in the kidney, narrowing of the tubular lumen in renal tubules, increased cystic lumen area in renal corpuscles, and condensation of cell nuclei. In contrast, no pathological changes were observed after adding NaF followed by exercise, suggesting a potential protective effect of exercise against fluoride-induced renal injury.

BUN and UA are the end products of protein and purine metabolism, respectively. They are typically excreted from the body after filtration by the renal glomeruli. In instances of kidney damage, renal insufficiency, or diminished renal function, the excretion of these substances is impaired, resulting in elevated serum concentrations of BUN and UA. It has been demonstrated through research that excessive intake of fluoride can lead to increased levels of BUN and UA in the serum [9, 20]. In this study, excessive fluoride led to an increase in serum BUN and UA levels, while the NEF group reduced the content of BUN, and the AEF group reduced the levels of both BUN and UA. This suggests that high fluoride levels can cause changes in kidney biochemical indicators, leading to renal insufficiency, while exercise can improve indicators. Additionally, the results indicate that voluntary wheel-running exercise (especially the higher degree of freedom of all-day voluntary wheel exercise) has a significant alleviating effect on fluoride-induced kidney damage in rats.

Oxidative stress has been identified as a significant contributor to kidney damage in mice exposed to fluorosis [21, 22]. The results of this experiment showed that relative to normal mice in group C, the kidneys of mice in group F showed a significant increase in the content of stress products, a significant decrease in the antioxidant content of GSH, and a significant decrease in the activity of the antioxidant enzymes T-SOD and GSH-PX. Previous findings have shown that the activity of antioxidant enzymes GSH-PX, and SOD is reduced in the kidneys of fluoride-intoxicated mice and is accompanied by a decrease in GSH content [23, 24]. After excess fluoride was ingested, voluntary wheel-running exercise at different time periods affected the different enzyme activities of mice to different degrees. Compared with the NEF and AEF groups, the DEF group recovered the activity of GSH-PX extremely significantly, which indicated that the daytime voluntary wheel-running exercise was more favorable to the recovery of the activity of GSH-PX in the mice with fluorosis, while for

T-SOD, it was the nighttime voluntary wheel-running exercise group that was more favorable to the recovery of the enzyme activity compared with the other two exercise groups. All-day exercise groups with higher degrees of freedom were effective steadily in alleviating the adverse oxidative stress consequences of fluoride in mice.

Moderate physical activity exerts beneficial effects on health, whereas excessive or high-intensity aerobic training can potentially impair physiological well-being [25]. Research has demonstrated that following a general exercise load, there are no significant pathologic changes observed other than mild glomerular swelling. Overloading exercise can result in significant pathological changes in the kidneys, including heightened expression of apoptotic proteins and increased apoptosis. Some researchers have proposed that whether to cause damage to the kidneys or to further improve the structural function of the kidneys is largely determined by the exercise load after studying rats swimming with different loads for a long period [26]. Therefore, we used the voluntary wheel running method to let the mice exercise voluntarily, avoiding the pressure caused by human factors on the mice and thus causing them to undergo forced and physiologically unfavorable exercise. Taking into account the circadian rhythm of mice, which affects their activity levels at different times of the day, we established three distinct time periods for exercise and recorded the amount of exercise for each mouse. The results showed that the daytime group of mice had significantly lower levels of exercise compared to the nighttime and 24-hour groups. Subsequently, based on their drinking water, which was either distilled water or 100 mg/L NaF solution, we analyzed the correlation between the amount of exercise and oxidative stress-related indicators to further explore the impact of voluntary wheel exercise on oxidative stress in the kidneys of mice. The results showed that the contents of the three oxidative stress products decreased with the increase of the distance of voluntary wheel-running exercise, regardless of whether fluorine was added to water or not [27]. Following excessive fluoride ingestion, the activities of renal enzymes T-SOD and GSH-PX were found to increase in correlation with the extended distance covered by voluntary wheel-running exercise. Voluntary wheel-running exercise represents an appropriate physical activity for mice, with increased exercise distances potentially offering greater amelioration of kidney damage and oxidative stress induced by fluoride exposure. Several other reports have confirmed a positive correlation between exercise and quality of life with moderate exercise [28, 29].

It is interesting to note that there are different reports on CAT in different literatures: studies have shown a significant decrease in CAT activity in the organism after fluorosis. Nonetheless, P. Mahaboob Basha, et al. reported an increase in CAT activity in the brain, heart, liver, and kidneys of rats exposed to a NaF concentration of approximately 600 mg/L for one month [30]. This is consistent with the results of the present experiment. Thus, CAT activity may correlate with the duration and concentration of fluoride exposure. Initially, upon exposure to high fluoride levels, there is an increase in oxidative stress products like H_2O_2. The body counters this by upregulating CAT activity to detoxify H_2O_2 and mitigate cellular damage. Prolonged oxidative stress, however, can deplete the body's antioxidant defenses, potentially resulting in a downregulation of CAT activity [31]. Therefore, it is necessary to investigate the temporal dynamics of CAT activity reduction and the potential collapse of the antioxidant defense system in response to fluoride-induced stress.

CONCLUSIONS

In conclusion, voluntary wheel-running exercise can alleviate oxidative stress injury in the kidney of 6-month-old mice exposed to 100 mg/L NaF, and the longer the distance of voluntary wheel-running exercise, the better the alleviating effect. Consequently, moderate exercise is a feasible and inexpensive approach for combating oxidative stress in the kidneys induced by fluoride toxicity.

FUNDING

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CONFLICT OF INTERESTS

The authors declare no competing interests.

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