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Original Paper

Combination of Fluoride and SO_2 Induce DNA Damage and Morphological Alterations in Male Rat Kidney

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Key Words
Rat • Sodium fluoride • Sulfur dioxide • Kidney • DNA damage • H&E • Comet assay

Abstract
Background/Aims: We investigated the combined toxic effect of sodium fluoride (NaF) and sulfur dioxide (SO_2) on kidney morphological changes and DNA damage in male Wistar rats. ***Methods:*** In this study we selected totally 96 male Wistar rats (12-week-old) then randomly group-housed them into four cages, treated with deionized water, NaF, SO_2 and co-treatment of NaF and SO_2 respectively. Morphological changes of kidney were detected by hematoxylin and eosin (H&E) staining at 2, 4, 6 and 8 weeks. Correspondingly, tailing ratio and comet length were measured by BAB Bs Comet Assay System, including DNA damage special unit were calculated to evaluate the grades of kidney DNA damage at the same time. ***Results:*** Treated groups showed a body weight decrease when compared to control group. However, no significant difference in the relative weight of kidney was found in all four groups. It is noteworthy that at 2, 4, 6 and 8 weeks after exposure, the morphological alteration of renal tubules were observed in all treated groups, especially in group-IV. Also, at 4 and 6 weeks, notable DNA damage was found in all treated groups, as assessed by significantly increasing trend of comet length tailing ratio. ***Conclusion:*** The study manifests that presence of NaF and SO_2 will not only induce renal tissue lesions but also impact DNA integrity. In addition, this combined exposure exhibits a synergistic effect, characterizing a dose-dependence and time correlation. These findings may provide novel insights regarding perturbations of DNA damage and its functions as a potential new mechanism, by which cautious interpretation of NaF and SO_2 co-exposure evolved in both animals and human beings is necessary.

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Introduction

Both sodium fluoride (NaF) and sulfur dioxide (SO_2) are major environmental toxic factors, [1, 2] which are widely distributed and co-existed in the environment [3]. As reported, the main source of SO_2 is generated from some of the specific areas like coal-burning fluorosis [4], aluminum smelters and volcanic fog [5-7]. Meanwhile, SO_2 is one of the increasing factors threatening the respiratory system of animals and human beings [8]. In terms of NaF, there is no consensus on the role of fluoride as an essential element in the water. The destructive effects of fluoride in population linked with the chronic exposure of low doses fluoride in water which is largely from geological sources, such as dental fluorosis and the exposure of ground water contaminated with the inorganic form of this mineral (30-50 mg/L) [9]. On the other hand, low doses of fluoride could contribute to prevent dental and bony diseases, achieved by modifying water fluoridation programs (1.0 mg/L) and processing ingredients in toothpastes and gels [10]. In recent reports, accumulated toxic factors of NaF and SO_2 in environment could induce morphological and genetic alterations in respiratory, reproductive, immune, and cardiovascular systems [11-15], widely existing in bone and tooth of animals and humanity [16]. Furthermore, cautions must be exercised when evaluating toxic effects. Soft tissues other than respiratory system and bone are ubiquitously affected, even though NaF and SO_2 act as systemic poisons for animals and human beings [17]. Therefore, soft tissue toxicity deserves deeper investigations.

Kidney is the largest excretory organ in the body [18], which plays a major role in filtration of body waste. Approximately 50-80% of Sodium fluoride is excreted through the kidney [19], of which, renal tubes play an important key in the process of waste removal. However, the dysfunction of renal tissue aroused by chronic fluoride exposure may cause polycystic and some other kidney diseases [20]. Such absorbability is consistent with previous studies that environmental or occupational fluoride could stimulate renal damage [21]. Meanwhile, as a result of industry-related high dose exposure, mainly in the form of NaF, fluoride-induced renal toxicity had been clearly explained by experimental models [22]. The activity of fluoride is restricted at low dose of renal toxicity, while, this situation could be changed with participation of mixtures that increase Na and Ca ratio to linked with high fluoride level or their own renal implications [23]. Apart from the toxicity of NaF and their impact on kidney and other tissues from some studies, paucity information has been indicated the influence of fluoride and sulfur dioxide on sensitive organ of kidney, including tissue injure, oxidative stress and DNA damage [20-25]. Hence, this study will focus and investigate the NaF and SO_2 toxicity and their impacts on the morphological alterations and DNA integrity in rat kidney.

Materials and Methods

Animals and exposure

96 male Wistar rats (12-week-old) and commercial rodent pellets were purchased from the Experimental Animal center of Shanxi Medical University, Taiyuan, China. All rats were randomly divided into 4 groups, each group contained 24 rats (Table 1), such as group-I (received deionized water alone as control), group-II (treated with NaF, 45 mg F^-/L), group-III (placed with SO_2 in ambient air,15ppm 4hrs/day, chamber-house made of wooden panel cabinet-type with glass windows (1.5×0.7×1.0 m) and fitted two exhaust fans top and bottom for ventilation and PGM-35 with RAE Systems (Inc, USA) used for monitor of the concentration of SO_2) and group-IV (exposed with both high NaF and SO_2) housed in plastic cages on heat-treated hardwood bedding, under environmental conditions of 22°C, 40-70% humidity, and a 12:12hr dark: light cycle. All animal procedures were performed in compliance with the regulations and guidelines of international ethics committee of animal welfare. Pure sulfur dioxide gas (SO_2) (99.99%) was obtained from Foshan Kedi Gas Chemical Industry Co, Ltd, Guangzhou, China. Six rats (per group) were selected randomly at different age point like 2, 4, 6 and 8 weeks respectively to weigh and injected with 20% urethane solution

Table 1. Fluoride levels in diet and sulfur dioxide (SO_2) concentration in ambient air. [a]Sulfur dioxide gas in air could not be detected below 0.1 ppm with gas monitor. [b]15 ppm sulfur dioxide emission was maintained continuously for four hours from 8:00 am to 12:00 pm during exposure days; ±5.0 indicates the maximum (20 ppm) and minimum (10ppm) SO_2 concentration. [c]In the NaF and NaF+SO_2 group, the rats were treated with 45 mg F^-/L in the drinking water

Parameter	Control	NaF	SO_2	NaF+ SO_2
Fluoride in diet (mg F^-/L)	23.39±1.04	23.39±1.04	23.39±1.04	23.39±1.04
SO_2 in ambient air(ppm)	< 0.1[a]	< 0.1[a]	15.0±5.0[b]	15.0±5.0[b]
Fluoride in drinking Water (mg F-/L)	<0.6	45[c]	<0.6	45[c]

for lethal anesthesia, and then implemented by cervical vertebra luxation in accordance with animal ethical standards. The kidneys were carefully removed and blotted free of blood for further study. Meanwhile, the surviving rats were weighed and recorded weekly once.

Histological examination

Kidney samples were rinsed thoroughly before representative samples were fixed in Bouin's solution for 12-24 hours and embedded in paraffin. The paraffin sections (5μm) were cut and de-waxed prior to hematoxylin and eosin (H&E) staining. For scoring sections were stained with hematoxylin/eosin. Finally, the tissue sections were evaluated and images taken by standard optical microscope (Olympus, Japan), and then used for observing histological alterations.

Comet Assay

After anesthetized (Urethane 20%) rats were sacrificed and perfused with 0.9% saline, the kidneys were removed and washed with phosphate buffer solution (PBS:8.0g NaCl, 0.2g KCl,2.8g $Na_2HPO_4 \cdot 12H_2O$, 0.2g KH_2PO_4, pH 7.4) for three times. The isolated kidney organs were chopped into pieces and homogenized with PBS at 4°C (pH 7.4) in the glass homogenizer, and filtrated with a 300-mesh sieve to obtain the single cell suspension. This entire process maintained under 4^0C to avoid additional cell damage. Finally, the sample slides were stained with Trypan blue and then evaluated the cell viability. When the viability was up to 90%, the cells were used for comet assay.

Alkaline comet assay was carried out by published protocol with slight modifications. Brief description with modified protocol as follow: the slides were covered with 0.5% normal agarose, subsequently added the mixture of 10μL cell sample and 75μL 0.5% low melting-point agarose to the slides, which were immediately sealed with cover slips. And then, immersed in a 4°C lysing solution (100mM EDTA, 1% Triton X-100, 2.5M NaCl, 10mM Tris, 1% N-laurylsarcosine, 10% DMSO were freshly added) for 1 hour in the dark. After that, removed the coverslips and the slides were performed with electrophoresis for 20 min at 300 mA and 25 V in alkaline solution (300 mM NaOH, 1 mM EDTA, pH >13). After electrophoresis, the slides were removed and washed 3 times with neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min each time. After this, the washed slides were fixed in ethanol for 5 min and stained with ethedium bromide (20μL/mL). The entire process was performed in the dark to prevent additional DNA damage. Finally, the three image-analysis parameters were used to estimate the results. For cyclophosphamide treatment, considered as a positive control during the experiment.

Tailing ratio, comet length and DNA damage grade were used to evaluate DNA damage. Moreover, BAB Bs Comet Assay System was applied to measure the grades of DNA damage. More than 100 cells per slide were surveyed through a fluorescent microscope (BX51, Olympus) installed with a green light excitation at 590-nm barrier filter. The negatively charged DNA fragments migrated towards the anode to form a comet, which is characterized by fluorescence staining. Initially, 100 cells per sample were chosen at random to assess the tailing ratio by counting the tailing DNA, and then 25 cells were selected and photographed to measure the distance of DNA migration in each sample. As for the grade of DNA damage, it was graded from the length of DNA fragments migration, which was computed by subtracting the diameter of the nucleus from the total length of the comet. Grading was done as follows: grade I: tailing length/diameter of the nucleus<1, grade II: 1< tailing length/diameter of the nucleus<2 and grade III: tailing length/diameter of the nucleus ≥2. Grades I and II indicate generic rupture of the DNA chain. Grade III indicates severe damage with a small head and a large, bright tail which looks like a broom.

Statistical analysis

Experimental data were shown as mean± SD and analyzed using Student's t-test. *P*-values of< 0.05 were considered as significant.

Results

Changes Trends of body weight and kidney coefficient by the influence of NaF and SO_2

The experimental workflow assessing body weight and kidney coefficient were conducted in combination exposure of NaF and SO_2. Compared to control group indicated in Table 2, body weight of group-III rats was significantly decreased after 2 weeks exposure and further body weight reduction was observed in group-IV after 2, 4, 5 and 8 weeks. However, no significant changes were found in kidney coefficient from all groups (Fig. 1).

Morphological changes in the kidney by the impact of NaF and SO_2

After being administered with NaF/SO_2, swelling of tubular epithelia cells and glomeruli appeared in all treated groups, which was invisible in the control group. Besides, this accompanied by the appearance of other lesions in treated groups. Renal interstitial congestion was found when attacked by NaF at 2 weeks (Fig. 2B, B1); granular degeneration was detected in cytoplasm of renal tubular epithelium at 4 weeks (Fig. 2F, F1); neutrephil infiltration and more severe congestion in glomeruli and renal interstitial were checked out at 6 weeks (Fig. 2J, J1); lymphocytic infiltration and congestion were observed in glomeruli and renal interstitial at 8 weeks (Fig. 2N, N1). After another process of SO_2, hyaline cast, formed by the solidification of Tamm-Horsfall protein, and renal interstitial congestion were found at 2 weeks (Fig. 2C, C1); more serious hyaline cast occurred at 4 weeks (Fig. 2G, G1); lymphocytic infiltration and congestion were observed in glomeruli and renal interstitial at 6 weeks (Fig. 2K, K1); renal tubular epithelial cell exfoliation and more severe congestion in glomeruli and renal interstitial were presented at 8 weeks (Fig. 2O, O1). In addition, the combination of NaF and SO_2 induced renal interstitial congestion and renal tubular epithelial cell hyalinization at 2 weeks, compared to the

Table 2. Change in body weight (g) of male rats (mean±SD). *P<0.05 (compared with the control group in all groups)

Treatment weeks	N	Control	SO_2	NaF	NaF+SO_2
1	24	172.05±17.37	171.89±13.57	169.41±12.87	169.19±14.51
2	24	285.30±14.70	270.70±3.63*	274.86±7.08	268.16±7.00*
3	18	277.55±10.49	253.53±9.48	271.88±6.68	256.60±8.97
4	18	285.74±21.53	246.64±11.81	277.00±7.96	225.40±9.13*
5	12	304.62±12.28	268.23±9.32	297.56±10.59	260.25±14.13*
6	12	315.88±20.45	280.07±5.73	291.18±13.90	280.12±10.55
7	6	312.96±15.90	285.87±11.12	324.36±18.15	287.24±11.30
8	6	311.38±17.12	279.73±16.36	337.03±10.27	262.62±39.33*

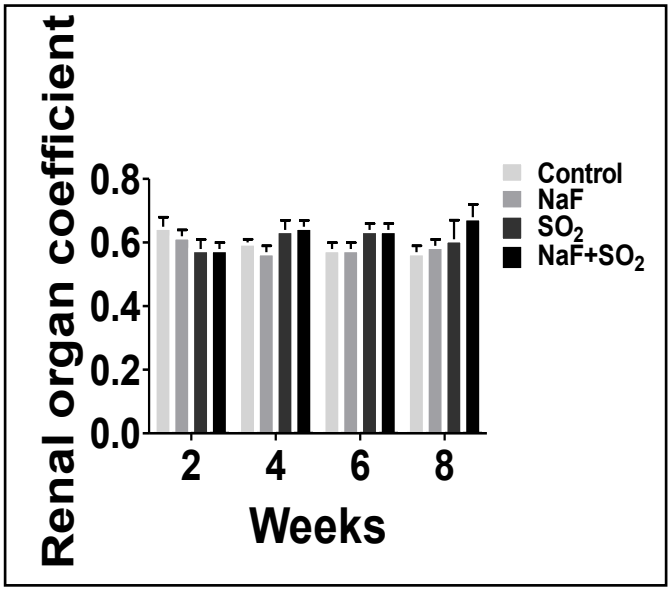

Fig. 1. Effects of NaF and/or SO_2 on renal organ coefficient (n=6; mean ± SD).

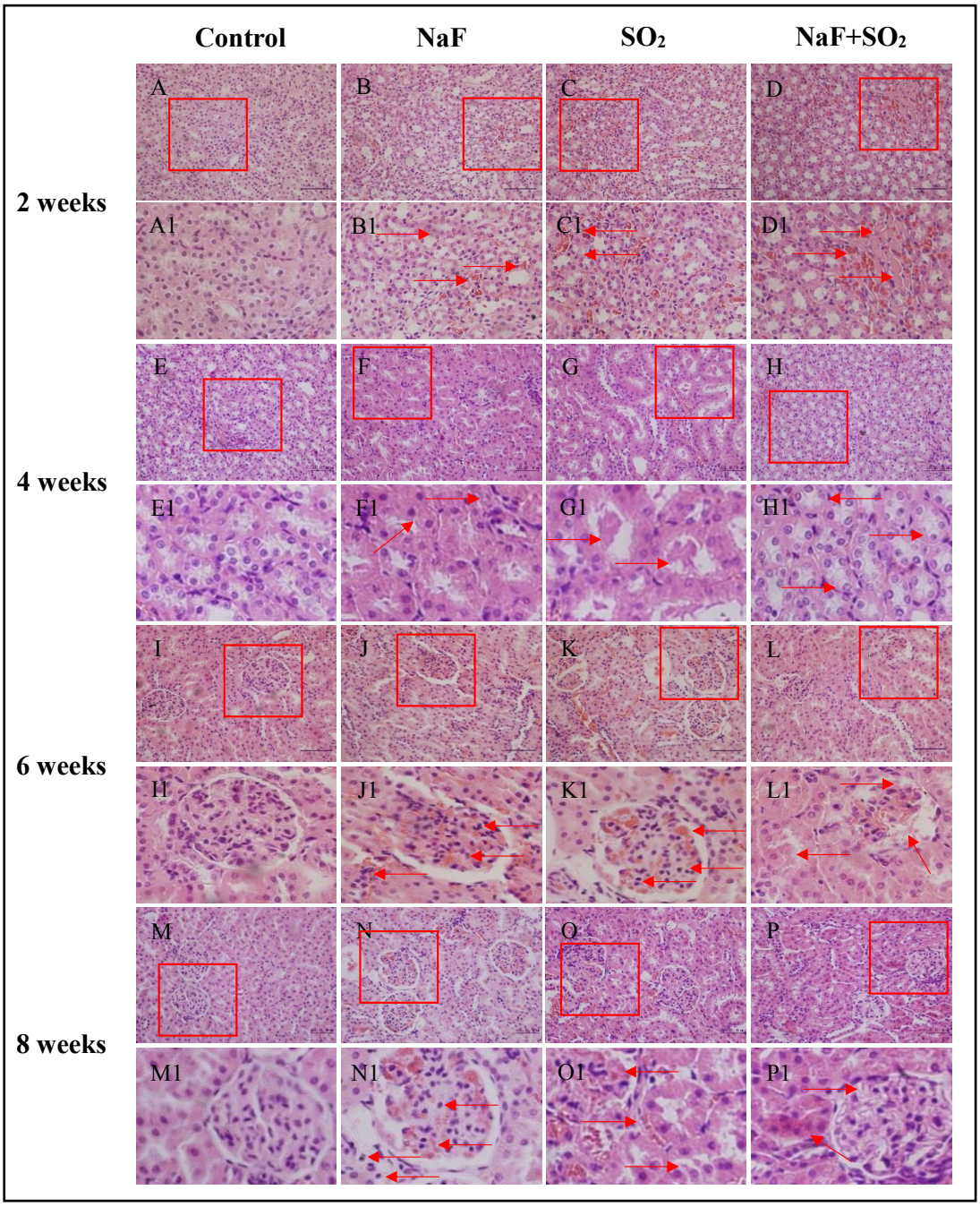

Fig. 2. Histopathology of the kidney in male rats induced by NaF and SO_2 (H.E. ×40). A, E, I and M are the control group sections of renal tissue at 2, 4, 6 and 8 weeks respectively; B, F, J and N are the sections of group-II at 2, 4, 6 and 8 weeks respectively; C, G, K and O are the sections of group-III at 2, 4, 6 and 8 weeks respectively; D, H, L and P are the sections of group-IV at 2, 4, 6 and 8weeks respectively. A1, B1, C1, D1, E1, F1, G1, H1, I1, J1, K1, L1, M1, N1, O1 and P1 are their corresponding magnifications. And the pathological lesions are pointed out with red arrows.

individual effects of NaF and SO_2 (Fig. 2D, D1); severe vacuolar degeneration was observed in renal tubular epithelial cell at 4 weeks (Fig. 2H, H1); severe congestion occurred in disorganized glomeruli at 6 weeks (Fig. 2L, L1); karyopyknos and cytoplasmic red-dyed particles were detected in necrosis of renal tubular epithelia, except the swollen glomeruli at 8 weeks (Fig. 2P, P1).

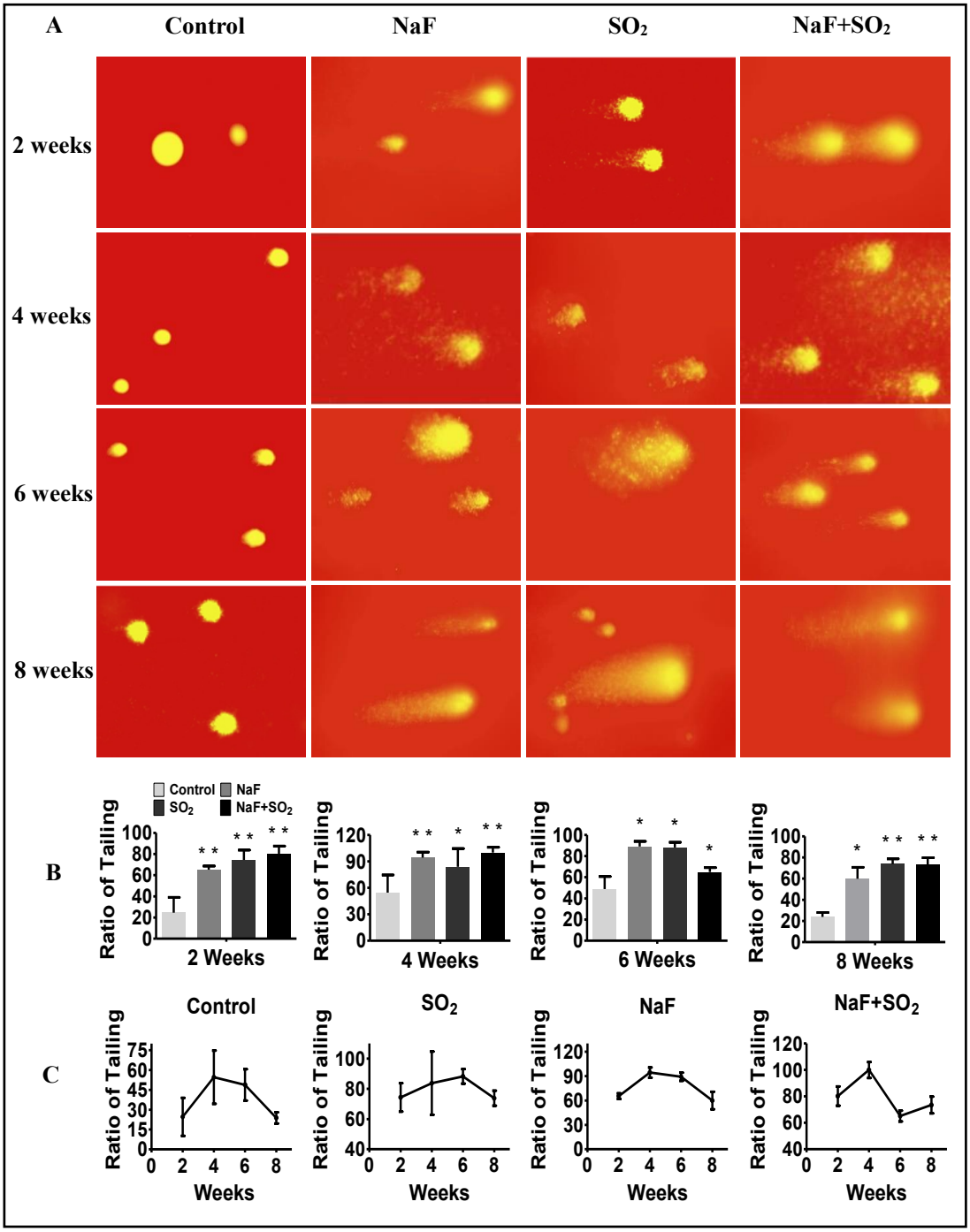

Fig. 3. Comet assay and ratio of tailing induced by NaF and SO_2 in male rats (n=100, mean± SD). A showed the representative photos of comet assay to renal cells in control, NaF, SO_2 and NaF+SO_2 groups at four different times respectively. B presented the ratios of tailing in NaF, SO_2 and NaF+SO_2 groups compared with control group at 2, 4, 6 and 8 weeks respectively. C showed the changes of the comet ratio in each group at 2, 4, 6 and 8 weeks severally. P<0.05 or P<0.01indicates significant differences and is shown with * or ** (compared with control).

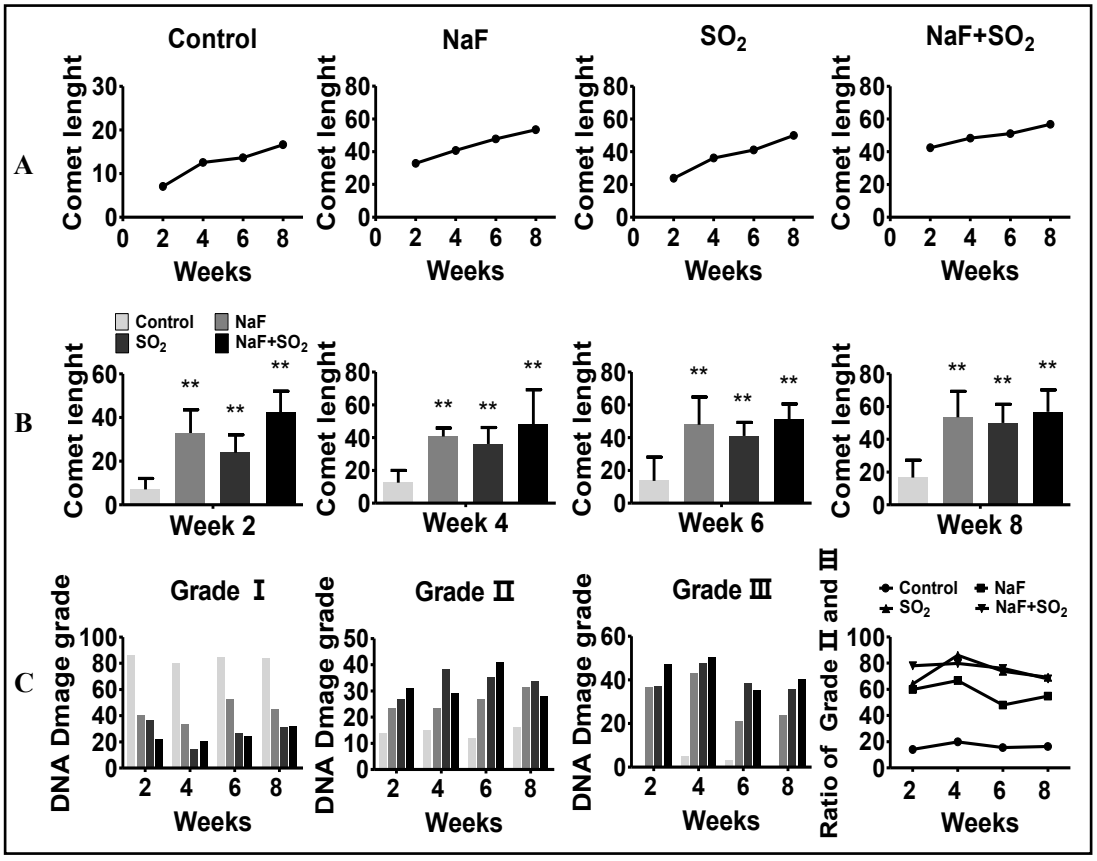

Fig. 4. The length of comet and DNA damage grade in renal cells induced by NaF and SO_2 (n=25, mean± SD). A presented the changes of comet length in each group during the experimental period. B demonstrated the changes of comet length in NaF, SO_2 and NaF+SO_2 groups compared with control group at 2, 4, 6 and 8 weeks severally. C revealed the three DNA damage grades in all the groups at 2, 4, 6 and 8 weeks respectively. Besides, the last Fig. of C showed the total percentage of Grade II and Grade III in treated groups at 2, 4, 6 and 8 weeks respectively. Grade I and II indicated that, generic rupture of the DNA chain with a small comet head. Grade III shows a large and bright tail that looks like a broom, indicates severe DNA damage. The criterion of grade is Grade II: tailing length/diameter of the nucleus<1; Grade II: 1< tailing length/diameter of the nucleus < 2; Grade III: tailing length/diameter of the nucleus ≥ 2. P<0.05 or P<0.01indicates significant differences and is shown with * or ** (compared with control).

Identification of DNA damage in renal tissue in NaF and SO_2 induced rat

Damaged DNA fragments appeared in all treated groups respectively shown in Fig. 3A when compared to the control group and relatively more abundant DNA damaged fragments were found in group-IV. These results were consistent with pathological outcomes. According to the analysis of tailing ratio, there was a significant increase trend in all treated groups during 8 weeks of exposure (Fig. 3B). Simultaneously, the maximum tailing ratio had been obtained at 4 weeks exposure in group-I, group-II and group-IV as well as 6 weeks exposure in group-III (Fig. 3C).

In further analysis of comet length, a similar pattern was generated as a significant increase was observed in all experimental groups at 2, 4, 6 and 8 weeks (Fig. 4A, B). Besides, grade II (1< tailing length/diameter of the nucleus < 2) and grade III (tailing length/diameter of the nucleus ≥ 2) increased dramatically when compared with control group and group-IV got the most significant raise. In addition, more than 50% of Grade II and Grade III damages were observed in all treated groups and for up to 4 weeks reaching the maximum (Fig. 4C), except for group-II postpone for 2 weeks.

Discussion

Fluoride and SO_2 have been identified as hazardous substances for years, which are widely existing in the environment, especially in developing countries [3]. These components could be obtained from drinking water and breathing air, so that they can easily enter human body, play a crucial role and cause severe diseases [22, 26]. Depending on the traits of systemic toxins, these two systemic toxins can damage the renal tissue [1, 8]. In a recent study, the morphological alterations such as degeneration, necrosis of the tubular cells, renal tubular hyaline casts and swollen glomeruli abnormalities were detected in 12, 24, 48 mg/kg body weight NaF exposure mice after 42 days [27]. Interestingly, a previous study reported that, NaF could reduce the integrity of tight connections in M-1 cell monolayers of mice [28]. The impact of SO_2 changed the ultra-structural lesions in renal proximal tubular lining cells [22]. Moreover, glomeruli and distal tubular lining cells were also damaged in a dose-dependent manner [29]. In present study, we investigated that pathological and morphological alterations in renal tissue affected by the influence of NaF and SO_2, which was coincided with previous studies. But we found the exposure of combined chemicals of NaF and SO_2 lead more severe damages in kidney than previous expectations mentioned in other studies.

In this study, comet assay was used to quantify damage and level of DNA molecules in soft tissues of kidney. Likewise, several studies estimated damaged DNA and their levels with mice kidneys by the comet assay at different concentrations of NaF (4, 12, 20mg/L; 50, 100, 200 mg/L) [30, 31]. Some of these studies revealed that varying degrees of DNA damage in kidney apoptosis were found in renal cells, which induced by 50, 100, 200 mg/L NaF [31]. And several studies proved that DNA damaged in SO_2 induced mice kidney for 7 days (125, 250, 500 mg/kg body weight) [24]. In addition, the damaged DNA was also found in male and female mice kidney after 7 days' SO_2 inhalation (14.00 ± 1.25, 28.00 ± 1.98, 56.00 ± 3.11, 112.00 ± 3.69 mg/m^3, 6 hr/day) [25]. In our study, we obtained similar results consistently with previous studies. First of all, NaF and SO_2 leaded to DNA damage in kidney with increasing comet length and tailing rate. Besides, DNA damage was more severe in treated groups at 4 and 6 weeks, especially in group-IV during the experiment. However, DNA damage had a certain degree of relief at the end of the trial, which may be benefited from its own DNA repair mechanism [32].

Our findings proved the necessity of exploring the mechanism of NaF-SO_2 interaction. On the basis of current understanding [33-35], the mechanism of combined effect on kidney may be explained as following: (1) Kidney is the largest excretory organ and the renal tubule plays an important role in the process of absorption, secretion and excretion [19]. Fluoride, sulfur dioxide and its derivatives can affect the kidney, especially the renal tubules [21, 22]. When SO_2 enters the body through breathing, food or water, the renal tubules can quickly switch to an acidic environment where F^- will combine with H^- and part of the F^- will exist in the form of HF. This transformation of F^- can be easily absorbed by the body [36]. Moreover, similar studies have been proved in multiple vitro intestinal cell models [37]. In this regard, the interaction of fluoride and SO_2 may be synergistic. (2) The main ways of DNA damage are deamination, depurination, depyrimidine and so on. Fluoride may directly bind to DNA chain, owing its powerful affinity for uracil and amide bond through –NH···F – interactions based on its active biochemical oxidant [38, 39]. After SO_2 enters the body, via breathing, food or water, it could be converted to HSO_3^-, SO_3^{2-} and H^- quickly in the interstitial fluid. This formation could induce cytosine deamination into uracil, which further lead to DNA damage [25, 40]. Meanwhile, Fluoride is an exogenously oxidative inducer, which can trigger the production of free radicals [38]. Similarly, a mass of radicals like HSO_3^- will appear in the process of cellular oxidization of SO_2. Indirectly, free radicals could attack the hydrogen bonds of DNA in forming various DNA adducts. Despite this, excessive free radicals can up-regulate oxidative stress, which can result in tissue injuries like hyaline lesions and fibrosis [41, 42].

Conclusion

In this research, we identified outcomes of combination exposure of NaF and SO_2 on male Wistar rat in different periods. It is a synergistic effect since more severe morphological alterations and DNA damages had been observed on kidney in comparison to their individual effect. Also, it shows in a dose and time dependence manner, especially at 4 weeks. Our findings may arouse attention to control the combined exposure on animals and human beings meanwhile have thrown up many questions in need of further investigation.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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