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Effects of Selenium and Zinc on Renal Oxidative Stress and Apoptosis Induced by Fluoride in Rats

RI-AN YU[*1], TAO XIA[#], AI-GUO WANG[#], AND XUE-MIN CHEN[#1]

[*]*Department of Occupational and Environmental Health, School of Public Health, Guangdong Pharmaceutical University, Guangzhou 510310, Guangdong, China;* [#]*Department of Occupational and Environmental Health, MOE Key Laboratory of Environment and Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei, China*

Objective To study the effects of selenium and zinc on oxidative stress, apoptosis, and cell cycle changes in rat renal cells induced by fluoride. **Methods** Wistar rats were given distilled water containing sodium fluoride (50 mg/L NaF) and were gavaged with different doses of selenium-zinc preparation for six months. Four groups were used and each group had eight animals (four males and four females). Group one, sham-handled control; group two, 50 mg/L NaF; group three, 50 mg/L NaF with a low dose of selenium-zinc preparation (0.1 mg/kg Na_2SeO_3 and 14.8 mg/kg $ZnSO_4 \cdot 7H_2O$); and group four, 50 mg/L NaF with a high dose of selenium-zinc preparation (0.2 mg/kg Na_2SeO_3 and 29.6 mg/kg $ZnSO_4 \cdot 7H_2O$). The activities of serum glutathione peroxidase (GSH-Px), kidney superoxide dismutase (SOD), and the levels of malondialdehyde (MDA) and glutathione (GSH) in the kidney were measured to assess the oxidative stress. Kidney cell apoptosis and cell cycle were detected by flow cytometry. **Results** NaF at the dose of 50 mg/L increased excretion of fluoride in urine, promoted activity of urine γ-glutamyl transpeptidase (γ-GT), inhibited activity of serum GSH-PX and kidney SOD, reduce kidney GSH content, and increased kidney MDA. NaF at the dose of 50 mg/L also induced rat renal apoptosis, reduced the cell number of G_2/M phase in cell cycle, and decreased DNA relative content significantly. Selenium and zinc inhibited effects of NaF on oxidative stress and apoptosis, promoted the cell number of G_2/M phase in cell cycle, but failed to increase relative DNA content significantly. **Conclusion** Sodium fluoride administered at the dose of 50 mg/L for six months induced oxidative stress and apoptosis, and changes the cell cycle in rat renal cells. Selenium and zinc antagonize oxidative stress, apoptosis, and cell cycle changes induced by excess fluoride.

Key words: Fluoride; Selenium; Zinc; Oxidative stress; Apoptosis; Proliferation

INTRODUCTION

Fluoride is an essential trace element, but excessive fluoride exposure causes fluorosis. Epidemiological study showed that in China, 330 million people are exposed to a high level of fluoride and 42 million suffer from fluorosis, or fluorine poisoning. Fluorosis causes damages to osseous tissue (teeth and bone) and other tissues such as kidney, liver and brain[1]. Although fluorosis can severely damage many systems of human body, its pathogenesis and toxicological mechanism are unclear. Previous studies demonstrated that excess fluoride not only causes lipid peroxidation and DNA damage, but also induces apoptosis and changes cell cycle[1-3]. Yang *et al*.[4] reported that fluoride causes significant increase of lipid peroxides (LPO) and metabolic disorder of trace elements, such as zinc, iron, and copper in the serum and kidney of rats. Selenite possesses significant antagonistic effects on renal damages induced by fluoride and corrects metabolic disorder of iron and copper, but serum level of zinc is still lower than that in control, suggesting that zinc supplementation is important in prevention and treatment of fluorosis. Xue *et al*.[5] reported that sodium selenite and zinc sulphate antagonize the renal impairments induced by fluoride through their antioxidation. The synergistic effect of sodium selenite and zinc sulphate is more powerful

[1]Correspondence should be addressed to Dr. Ri-An YU, Department of Occupational and Environmental Health, School of Public Health, Guangdong Pharmaceutical University, Guangzhou 510310, Guangdong, China, or to Prof. Xue-Min CHEN, Department of Occupational and Environmental Health, MOE Key Laboratory of Environment and Health, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei, China. E-mail: yurian.tj@163.com. Tel: 86-20-34055934. Fax:86-20-34055355

Biographical note of the first author: Ri-An YU, male, born in 1964, Ph. D., professor, majoring in the fields of environmental and molecular toxicology.

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than that of either sodium selenite or zinc sulphate alone. Chen *et al*.[6] showed that joint antagonistic effect of selenium and zinc against DNA damage in pallium neural cells of rats induced by fluoride is more obvious. In the present study, we investigated the effects of selenium and zinc on rat renal oxidative stress, apoptosis, and cell cycle changes induced by fluoride.

MATERIALS AND METHODS

Animal Handling

Wistar rats weighing approximately 80-120 g were used in all the experiments. Male and female rats were housed separately in polycarbonated cages with compressed fiber bedding. Commercial pelleted diet and water were provided *ad libitum.* Contents of selenium, zinc, and fluoride in pelleted diet were 0.1 mg/kg, 30 mg/kg, and 10.6 mg/kg, respectively. Concentration of zinc in water was lower than 50 μg/L, and concentrations of selenium and fluoride were 2.0 μg/L and 7.7 μg/L, respectively. Four groups of eight animals each (four males and four females) were used. The first group was sham-handled control. The second group (fluoride group) was provided with distilled water containing 50 mg/L sodium fluoride. The third group (F+Se-Zn, low dose) was given distilled water containing 50 mg/L sodium fluoride by gavage with a low dose of selenium-zinc preparation (0.1 mg/kg sodium selenite and 14.8 mg/kg zinc sulfate). The fourth group (F+Se-Zn, high dose) was provided with distilled water containing 50 mg/L sodium fluoride by gavage with a high dose of selenium-zinc preparation (0.2 mg/kg sodium selenite and 29.6 mg/kg zinc sulfate). Sodium selenite and zinc sulfate, when given, were prepared in 0.9% normal saline. Animals in control group and fluoride group received an equal volume of 0.9% normal saline. The gavage volume was 1 mL/200 g body weight. Once every day for six days per week. The animals were sacrificed six months later and kidneys were removed immediately for use.

Determinations of Fluoride Concentration and γ-Glutamyl Transpeptidase Activity in Urine

At the end of experiment, the animals were held in plastic metabolic cages for 24 hours, and urine was collected in the container put over ice to preserve the activity of gamma-glutamyl transpeptidase (γ-GT). Concentration of fluoride in urine was determined with fluoride ion-selective electrodes as described by Zhang *et al.*[7]. γ-GT activity of urine was measured by an enzymatic assay as described by Meister *et al.*[8]. Creatinine concentration was measured by colorimetry after Jackson[9]. γ-GT in urine was corrected for creatinine concentration (Cr).

Preparation of Tissue Extract

Kidneys were minced and homogenized in 50 mmol/L cold sodium phosphate buffer (pH 7.0) containing 0.1 mmol/L EDTA to give 10% homogenate (W/V). The homogenates were then centrifuged at 1000 rpm for 10 min at 4℃. The supernatants were separated and used for enzyme assays and protein determination.

Determinations of GSH-Px in Serum and SOD, GSH, and LPO in Kidney

GSH-Px (glutathione peroxidase) was evaluated by a modified method[10]. A mixture consisting of 650 μL (0.05 mol/L, pH 10.2, 0.1 mmol/L EDTA) sodium phosphate buffer, 100 μL 0.01 mol/L glutathione (GSH), 100 μL 1.5 mmol/L NADPH, and 100 μL glutathione reductase (0.24 units) was added to 50 μL of tissue extract and incubated at 37℃ for 10 min. After incubation, 50 μL of 12 mmol/L t-butyl hydroperoxide was added to 450 μL of the tissue mixture to start the reaction. GSH-Px was measured at 340 nm for 3 min. The molar extinction coefficient of 6.22×103 mol/L • cm^{-1} was used to determine the activity. One unit of activity was equal to the millimoles of NADPH oxidized/min per mg protein.

Superoxide dismutase (SOD) activity was determined at room temperature according to the epinephrine method[11] based on the capacity of SOD to inhibit autoxidation of adrenaline to adrenochrome. Ten μL of tissue extract were added to 970 μL (0.05 mol/L, pH 10.2, 0.1 mmol/L EDTA) of sodium carbonate buffer. And 20 μL of 30 mmol/L epinephrine (dissolved in 0.05% acetic acid) were added to the mixture to start the reaction. SOD was measured at 480 nm for 4 min. One unit of SOD was defined as the amount of protein causing 50% inhibition of the autoxidation of adrenaline.

The reduced glutathione (GSH) was determined in tissues by the method of Kum-Talt and Tan using dithionitrobenzoic acid (DTNB) to measure the absorbance at 412 nm[12]. Lipid peroxidation products (LPO) were determined by measuring the levels of malondialdehyde (MDA)[13]. To 0.2 mL of homogenate 0.2 mL 8.1% (w/v) sodium dodecyl sulphate and 1.5 mL 20% acetic acid were added, with pH was adjusted to 3.2 with 20% (w/v) sodium acetate solution. After the addition of 1.5 mL of thiobarbituric acid (0.8%; w/v), the mixture was diluted to 4 mL with water and then heated for 60

min in a boiling water bath. It was cooled to room temperature and 1 mL of water was added followed by a mixture of n-butanol and pyridine (15:1). The mixture was shaken vigorously and centrifuged at 1500×g for 15 min. The absorbance of organic layer was measured at 532 nm and the results were expressed as μmol MDA/mg.protein.

Protein content was determined by the method of Lowry, and bovine serum albumin was used as a reference[14].

Detection of Apoptosis

Apoptosis was analyzed by the determination of sub-G1 cells. At the end of various designated treatments (such as cell separation), cells were washed, fixed and permeated with 70% ice-cold ethanol at 4℃ for 2 hours, and then incubated with freshly prepared propidium iodide (PI) staining buffer (0.1% Triton X-100, 200 μg/mL RNase A, and 20 μg/mL PI in PBS) for 15 min at 37℃, followed by flow cytometry analysis of 20 000 cells from each animal. The histogram was abstracted and percentage of cells in the sub-G1 phase was then calculated to reflect the percentage of apoptotic cells. In addition, cell cycle was analyzed with ModFit LT software. DNA relative content (DNA_{RC}) and proliferation index (PI) were evaluated by the methods described in our previous publication[15].

Statistical Analysis

Data calculated separately for each group were expressed as $\bar{x} \pm s$. The significance of the difference between two groups was evaluated by Student's *t*-test. $P<0.05$ was considered statistically significant.

RESULTS

Concentration of Fluoride And Activity of γ-GT in Rat Urine

Urine fluoride levels are shown in Table 1. The results indicated that after the whole experimental period, urine fluoride concentrations in groups of fluoride (F), F+Se-Zn (low dose) and F+Se-Zn (high dose) were significantly higher than that in control group ($P<0.05$). Although urine fluoride concentrations in groups of F+Se-Zn (low dose) and F+Se-Zn (high dose) were higher than that in fluoride group, statistical analysis yielded $P>0.05$.

TABLE 1

Concentration of Fluoride and Activity of γ-GT in Rat Urine ($\bar{x} \pm s$, n=8)

Groups	Urine Fluoride (mg/L)	Urine γ-GT (U/mmol.Cr)
Control	5.14±0.48	38.44±5.04
Fluoride Group (F)	13.05±3.01[a]	132.42±21.05[a]
F+Se-Zn (Low Dose)	22.58±3.79[a,b]	84.98±9.64[a,b]
F+Se-Zn (High Dose)	19.73±2.49[a,b]	79.68±12.80[a,b]

Note. [a]$P<0.05$ compared with the control group, [b]$P>0.05$ compared with the fluoride group.

Urine γ-GT activities in groups of fluoride (F), F+Se-Zn (low dose) and F+Se-Zn (high dose) were significantly higher than that in control group ($P<0.05$). Although urine γ-GT activities in groups of F+Se-Zn (low dose) and F+Se-Zn (high dose) were lower than that in fluoride group, statistical analysis yielded $P>0.05$.

Activity of GSH-Px and SOD, contents of GSH and LPO

Table 2 shows the effects of fluoride and its joint action with selenium-zinc on activity of GSH-Px in serum and SOD in kidney, and contents of GSH and LPO in kidney. Serum GSH-Px activity in fluoride group was lower than that in control group ($P<0.05$), but serum GSH-Px activities in groups of F+Se-Zn (low dose) and F+Se-Zn (high dose) were significantly higher than that in fluoride group ($P<0.05$).

TABLE 2

Effects of Fluoride and Its Synergistic Action With Selenium-zinc on Activity of GSH-Px and SOD Activities, and GSH and LPO Contents ($\bar{x} \pm s$, n=8)

Groups	Serum GSH-Px (U/mL)	Kidney SOD (nU/mg.Pr)	Kidney GSH (mg/mg.Pr)	Kidney MDA (μmol/ mg.Pr)
Control	108.13±32.55	22.70±2.83	33.70±5.92	307.29±32.71
Fluoride Group (F)	56.40±14.84[a]	16.29±3.75	20.67±6.21[a]	409.83±56.79[a]
F+Se-Zn (Low Dose)	84.58±21.55[a,b]	18.14±5.18	28.76±4.88	322.89±50.57[b]
F+Se-Zn (High Dose)	90.97±28.73[b]	24.64±4.11[b]	32.32±4.89[b]	299.25±58.57[b]

Note. [a]$P<0.05$ compared with the control group; [b]$P<0.05$ compared with the fluoride group.

Kidney SOD activity and GSH content in fluoride group were lower than those in control group. Compared with fluoride group, selenium and zinc increased SOD activities and promoted GSH contents; statistical analysis yielded $P>0.05$ in F+Se-Zn group (low dose) and $P<0.05$ in F+Se-Zn group (high dose).

Kidney LPO content in fluoride group was higher than that in control group ($P<0.05$), but kidney LPO contents in groups of F+Se-Zn (low dose) and F+Se-Zn (high dose) were significantly lower than that in fluoride group ($P<0.05$).

Percentage of Apoptosis and DNA Relative Contents in Rat Renal Cells

Table 3 shows the rates of apoptosis and relative contents of DNA induced by fluoride and its joint action with selenium-zinc in rat renal cells. The results indicated that the rate of apoptosis in fluoride group was higher than that in control group, but DNA_{RC} was lower than that in control group ($P<0.01$). The rates of apoptosis in groups of F+Se-Zn (low dose) and F+Se-Zn (high dose) were significantly lower than that in fluoride group ($P<0.01$), but DNA_{RC} was higher than that in fluoride group ($P>0.05$).

TABLE 3

Effects of Fluoride and Its Joint Action With Selenium-zinc on Apoptosis Rates and DNA Relative Contents in Rat Renal Cells ($\bar{x} \pm s$, n=8)

Groups	Rates of Apoptosis	DNA_{RC}
Control	5.78±1.95	190.52±10.05
Fluoride Group (F)	13.84±0.75[a]	168.74±5.73[a]
F+Se-Zn (Low Dose)	8.38±0.48[c]	172.54±4.38[b]
F+Se-Zn (High Dose)	9.54±0.83[c]	173.04±9.28[b]

Note. [a]$P<0.01$ compared with the control group; [b]$P>0.05$, [c]$P<0.01$ compared with the fluoride group.

Cell Cycle and Proliferation Index in Rat Renal Cells

Table 4 shows the effects of fluoride and its joint action with selenium-zinc on rat renal cell cycle and proliferation index (PI). The numbers of cells in G_0/G_1, S and G_2/M phase induced by fluoride were lower than those in control group, but statistical analysis yielded $P<0.05$ only in G_2/M phase. Compared with fluoride group, F+Se-Zn (low dose) increased the cell number in G_2/M phase ($P<0.05$). PI was lower in fluoride group than in control group ($P>0.05$). Compared with fluoride group, F+Se-Zn (low dose) and F+Se-Zn (high dose) increased PI, but the difference was not significant ($P>0.05$).

DISCUSSION

Many studies have shown that fluoride induces excessive production of oxygen free radicals and decreases the biological activities of some substances, such as catalase, superoxide dismutase, xanthine oxidase, and glutathione peroxidase, which play an important role in antioxidation and elimination of free radicals. Liu *et al.*[16] suggested the mechanism of fluoride in injuring soft tissue as follows: fluoride causes excessive production of nitric oxide (NO), lipid peroxides (LPO), and oxygen free radicals, leading to the reduced capability of scavenging free radicals and antioxidating, which seriously damage the structure, especially the biological membrane structure, functions of cells, and biomacromolecules, such as proteins and nuclei acids, and even the entire soft tissues. The stimulation of γ -GT activity seems to contribute to the renal morphological damage at the level of proximal tubular cells[17]. In our study, fluoride at the dose of 50 mg/L administered for six months resulted in increased excretion of fluoride and increased activities of γ -GT in urine, indicating that kidneys are damaged by excess fluoride. Wang *et al.*[1] reported that fluoride causes lipid peroxidation, DNA damage, and apoptosis using L-02 cells as an experimental model and found that there exists a significant positive correlation between fluoride concentration and these changes. An increase in the level of lipid peroxides along with a concomitant decrease in the activities of superoxide dismutase

TABLE 4

Effects of Fluoride and Its Joint Action With Selenium-zinc on Rat Renal Cell Cycle ($\bar{x} \pm s$, n=8)

Groups	G_0/G_1 (%)	S (%)	G_2/M (%)	PI
Control	63.86±3.90	9.36±2.65	8.68±1.21	22.01±3.72
Fluoride Group (F)	59.62±2.29	8.10±1.42	6.30±1.27[a]	19.46±0.98
F+Se-Zn (Low Dose)	57.58±3.55	8.22±1.06	8.18±0.23[b]	22.22±2.11
F+Se-Zn (High Dose)	59.66±4.95	8.60±2.39	6.98±1.49	20.72±5.05

Note. [a]$P<0.05$ compared with the control group; [b]$P<0.05$ compared with the fluoride group.

(SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and reduced glutathione content were observed in fluoride-treated groups of rats[18]. Karaoz *et al.* [19] have also observed the effects of chronic fluorosis on lipid peroxidation and histology of kidney tissues in first- and second-generation rats. In this study, excess fluoride increased excretion of fluoride in urine, promoted activity of urine γ-GT, inhibited activity of serum GSH-P_X and kidney SOD, reduced kidney GSH content, and increased kidney LPO, suggesting that excess fluoride causes kidney impairment and oxidative stress.

Apoptosis is a programmed cell death accomplished by specialized cellular machinery and is essential for cell development and tissue homeostasis in eukaryotic organisms. Previous studies have shown that fluoride induces apoptosis in human embryo hepatocytes[1], epithelial lung cells[20], human and rat pancreatic islets and RINm5F cells[21], and HL-60 cells[22]. In the present study, we investigated the effects of fluoride on rat renal apoptosis and found that relative DNA content and cell number in G_2/M phase in fluoride-treated group were significantly lower than those in the control group, although there were no obvious changes in cell number in G_0/G_1 and S phase. Wang *et al.*[1] reported that the number of cells in S phase in all fluoride-treated groups is significantly higher than that in the control group, but there are no changes in cell number in G_0/G_1 phase and G_2/M phase. Jing *et al.*[23] reported that cell number in G_2/M phase is reduced by 100 mg/L sodium fluoride in rat hepatocytes, and Liu *et al.*[24] showed that fluoride at 20.0 μg/mL not only increases the number of cells in S phase, but also decreases the number of cells in G_2/M phase. This may be due to the differences in cell sensitivity to fluoride in different phases in cell cycle[25]. Cells synthesize DNA mainly in S phase, and DNA synthesis reduced by fluoride results in decreased DNA relative contents. This process interrupts the normal signal transduction in cell cycle, inhibits cell progression from S phase to G_2/M phase and causes cell cycle arrest in S phase, and also inhibits cell proliferation. These findings including ours indicate that excess fluoride induces apoptosis and changes cell cycle.

Selenium and zinc are essential trace elements for humans and animals. Zinc or selenium can partially diminish the oxidative stress induced by cadmium in kidneys, and simultaneous administration of zinc and selenium with cadmium may efficiently protect kidneys from cadmium-induced oxidative damage[10]. Selenium and zinc can antagonize the renal impairments induced by fluoride through their antioxidation and joint antagonistic effect against DNA damage in pallium neural cells of rats induced by fluoride. The joint effect of selenium and zinc is more powerful than the effect of selenium or zinc alone[5-6]. In this research, the ameliorative effects of selenium and zinc on rat renal oxidative stress, apoptosis, and cell cycle changes induced by excess fluoride were observed.

Reactive oxygen species (ROS) have been implied as potential modulators of apoptosis. Anuradha *et al.*[22] suggested that sodium fluoride (NaF) possibly induces apoptosis by oxidative stress-induced lipid peroxidation, thereby releasing cytochrome C into the cytosol and further triggering the caspase cascade leading to apoptotic cell death in HL-60 cells. Thrane *et al.*[26] found that activation of mitogen-activated protein (MAP) kinase p38 and possibly c-Jun N-terminal kinase (JNK) is involved in NaF-induced apoptosis of epithelial lung cells, whereas extracellular signal regulated kinase (ERK) activation seems to counteract apoptosis in epithelial lung cells. Refsnes *et al.*[20] suggested that NaF induces an apoptotic effect and an increase in PI-positive A549 cells *via* similar mechanisms, involving protein kinase C (PKC), protein kinase A (PKA), tyrosine kinase, and Ca^{2+}-linked enzymes. Selenium has protective effect against the apoptosis induced by superoxide anion[27] and has a protective role in caspase-3-dependent apoptosis induced by H_2O_2 in cultured primary pig thyrocytes[28]. Zinc is a potent inhibitor of apoptosis, whereas zinc depletion induces apoptosis in many cell lines. Zinc inhibits apoptosis by maintaining caspase-3 inactive[29]. Selenium attenuates oxidative stress responses through modulation of p38 MAPK and nuclear factor kappaB (NF-κB) signaling pathways[30]. Selenite suppresses hydrogen peroxide-induced cell apoptosis through inhibition of ASK1/JNK and activation of PI3-K/Akt pathways, and differentially modulates the mammalian mitogen-activated protein kinase pathways and represses the JNK/SAPK signaling pathway by inhibiting JNK/SAPK through a thiol redox mechanism[31-32]. Zinc finger proteins play an important role in a variety of cellular functions, including cell growth, proliferation, apoptosis, and intracellular signal transduction, thus repressing mitogen-activated protein kinase signaling pathways[33]. Selenium compounds can inhibit phospholipid/Ca^{2+}-dependent PKC[34], and PKC may be involved in the relationship between zinc and apoptosis[35]. In addition, there is a positive relationship between DNA damage and apoptosis as well as between lipid peroxidation and apoptosis induced by fluoride[1]. Selenium and zinc have antagonistic effect on DNA damage induced by fluoride in pallium neural cells of rats[6]. In a word,

fluoride induces apoptosis through oxidative stress, caspase and PKC activation, MAPK signal pathway, and DNA damage. Selenium and zinc can influence oxidative stress, caspase and PKC activation, MAPK signal pathway, and DNA damage.

In conclusion, excess fluoride induces oxidative stress, apoptosis and cell cycle changes in rat renal cells, and selenium and zinc may have ameliorative effects on these disturbances caused by fluoride, but exact mechanisms need to be further investigated.

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