

RAPID COMMUNICATION

## DNA damage, apoptosis and cell cycle changes induced by fluoride in rat oral mucosal cells and hepatocytes

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### Abstract

**AIM:** To study the effect of fluoride on oxidative stress, DNA damage and apoptosis as well as cell cycle of rat oral mucosal cells and hepatocytes.

**METHODS:** Ten male SD rats weighing 80~120 g were randomly divided into control group and fluoride group, 5 animals each group. The animals in fluoride group had free access to deionized water containing 150 mg/L sodium fluoride (NaF). The animals in control group were given distilled water. Four weeks later, the animals were killed. Reactive oxygen species (ROS) in oral mucosa and liver were measured by Fenton reaction, lipid peroxidation product, malondialdehyde (MDA), was detected by thiobarbituric acid (TBA) reaction, reduced glutathione (GSH) was assayed by dithionitrobenzoic acid (DTNB) reaction. DNA damage in oral mucosal cells and hepatocytes was determined by single cell gel (SCG) electrophoresis or comet assay. Apoptosis and cell cycle in oral mucosal cells and hepatocytes were detected by flow cytometry.

**RESULTS:** The contents of ROS and MDA in oral mucosa and liver tissue of fluoride group were significantly higher than those of control group ( $P < 0.01$ ), but the level of GSH was markedly decreased ( $P < 0.01$ ). The contents of ROS, MDA and GSH were  $(134.73 \pm 12.63)$  U/mg protein,  $(1.48 \pm 0.13)$  mmol/mg protein and  $(76.38 \pm 6.71)$  mmol/mg protein in oral mucosa respectively, and  $(143.45 \pm 11.76)$  U/mg protein,  $(1.44 \pm 0.12)$  mmol/mg protein and  $(78.83 \pm 7.72)$  mmol/mg protein in liver tissue respectively. The DNA damage rate in fluoride group was 50.20% in oral mucosal cells and 44.80% in hepatocytes, higher than those in the control group ( $P < 0.01$ ). The apoptosis rate in oral mucosal cells was  $(13.63 \pm 1.81)$  % in fluoride group, and  $(12.76 \pm 1.67)$  % in hepatocytes, higher than those in control group. Excess fluoride could differently lower the number of oral mucosal cells and hepatocytes at  $G_0/G_1$  and  $S/G_2/M$  phases ( $P < 0.05$ ).

**CONCLUSION:** Excess fluoride can induce oxidative

stress and DNA damage and lead to apoptosis and cell cycle change in rat oral mucosal cells and hepatocytes.

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**Key words:** Fluoride; Oxidative stress; DNA damage; Apoptosis; Cell cycle

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### INTRODUCTION

Fluoride is an essential trace element for human beings and animals. Fluoride can prevent caries and enamel fluorosis. Caries is the demineralization of the enamel by acids produced by plaque bacteria, leading to cavitation. Enamel fluorosis is a subsurface hypomineralization of the dental enamel caused by chronic ingestion of high fluoride concentration while the dentition is forming<sup>[1]</sup>. Other manifestations of fluoride toxic effects include skeletal fluorosis and damage to kidney, liver, parathyroid glands and brain<sup>[2-4]</sup>. Lipid peroxidation is implicated as an important mechanism of fluorosis. A close association between fluoride toxicity and oxidative stress has been reported in human beings<sup>[5]</sup>, experimental animals<sup>[3]</sup> and cultured cells<sup>[2]</sup>. Studies have shown that excess fluoride can cause DNA damage, trigger apoptosis and change cell cycle<sup>[2,6]</sup>. Jeng *et al*<sup>[7]</sup> studied the effects of sodium fluoride on human oral mucosal fibroblasts and found that sodium fluoride is toxic to oral mucosal fibroblasts *in vitro* by inhibiting protein synthesis, disturbing mitochondrial function and depleting cellular ATP. The effects of sodium fluoride on cultured human oral keratinocytes have been investigated with respect to induction of unscheduled DNA synthesis. These researches indicate that fluoride has harmful effects on oral mucosa. In the present study, we investigated the effects of sodium fluoride (NaF) on lipid peroxidation, DNA damage and apoptosis in rat oral mucosal cells and hepatocytes.

### MATERIALS AND METHODS

#### Animals

Male SD rats weighing 80-120 g were used in the

experiments and housed in polycarbonated cages with compressed fiber bedding. Commercial pellet diet and water were provided *ad libitum*. The animals were divided into control group and fluoride group, 5 animals each group. The control group was given distilled water and the fluoride group was provided with distilled water containing 150 mg/L sodium fluoride. The animals were sacrificed four weeks later and their oral mucosa and liver were removed immediately for use.

#### **Determination of fluoride concentration in urine and blood**

At the end of experiments, the animals were held in plastic metabolic cages for 8 h, and urine was collected in the container. Concentration of fluoride in urine and blood was determined with fluoride ion-selective electrodes as described by Zhang *et al.*<sup>[8]</sup>.

#### **Preparation of tissue extract**

Oral mucosa and liver were minced and homogenized in 50 mmol/L cold sodium phosphate buffer (pH 7.0) containing 0.1 mmol/L EDTA to produce 10% homogenates (W/V). The homogenates were then centrifuged at 1000 r/min for 10 min at 4 °C. The supernatants were separated and used for enzyme assays and protein determination.

#### **Determination of GSH, LPO and ROS**

The reduced glutathione (GSH) was determined in tissues by the method of Kum-Talt and Tan<sup>[9]</sup> using dithionitrobenzoic acid (DTNB) reagent and the absorbance at 412 nm was measured. Lipid peroxidation products (LPOs) were determined by measuring the levels of malondialdehyde (MDA)<sup>[10]</sup>. To 0.2 mL of homogenates 0.2 mL 8.1% (w/v) sodium dodecyl sulphate and 1.5 mL 20% acetic acid were added, and 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, heated for 60 min in boiling water bath and cooled to room temperature. Then 1 mL of water and a mixture of n-butanol and pyridine (15:1) were added. The mixture was shaken vigorously and centrifuged at 1500 r/min for 15 min. The absorbance of organic layer was measured at 532 nm and the results were expressed as mmol MDA/mg protein. Reactive oxygen species (ROS) was measured with test kit (Nanjing Jiancheng Biological Technology Company) by Fenton reaction. Gress reagent was used to initiate color reaction and the absorbance was read at 510 nm on a spectrophotometer. Protein content was determined by the method of Lowry using bovine serum albumin as a reference<sup>[11]</sup>.

#### **Detection of DNA damage**

Single cell gel electrophoresis assay (also known as comet assay) was performed as previously described<sup>[12]</sup> with some modifications. Approximately  $1.0 \times 10^5$  cells were embedded in low melting agarose (0.65%) that was layered onto fully frosted microscope slides coated with a layer of 0.75% normal agarose (diluted in Ca<sup>2+</sup> and Mg<sup>2+</sup> free PBS buffer). A final layer of 0.65% low-melting agarose was placed on top. Slides were immersed in a jar containing cold lysate solution (1% Triton X-100, 10% DMSO and 89% of 10 mmol/L Tris, 1% sodium laurylsarcosine, 2.5 mol/L NaCl,

100 mmol/L Na<sub>2</sub>EDTA, pH 10) at 4 °C for 1-2 h. Then, slides were pretreated for 15 min in electrophoresis buffer (300 mmol/L NaOH/1 mmol/L Na<sub>2</sub>EDTA, pH 12) and exposed to 25 V/300 mA for 20 min. Pre-incubation and electrophoresis were performed in ice bath. Slides were neutralized for 3 - 5 min in 0.4 M Tris, pH 7.5 and DNA was stained by adding 50 µL of ethidium bromide (20 µg/mL) onto each slide. After staining for 5 min, slides were rinsed in distilled water and covered again for microscopic examination. All steps were conducted under red light to prevent additional DNA damage.

Image analysis was performed with 200 × magnification using a fluorescence microscope (Olympus B-60F5) equipped with an excitation filter of 549 nm and a 590 nm barrier filter, coupled to a CCD camera (Kodak, USA). One hundred randomly selected cells per slide were scored. In this test, DNA damage of the cells was evaluated using the ratio of tail DNA content/the whole cellular DNA content.

#### **Detection of apoptosis**

DNA fragmentation during apoptosis could lead to extensive loss of DNA content and a distinct sub-G<sub>1</sub> peak when analyzed by flow cytometry. Apoptosis was analyzed by the determination of sub-G<sub>1</sub> cells. At the end of designated treatments (such as cell separation), cells were washed, fixed and permeated with 70% ice-cold ethanol at 4 °C for 2 h. Cells were 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) at 37 °C for 15 min, followed by flow cytometry of 20 000 cells from each animal. The histogram was abstracted and percentage of cells in the sub-G<sub>1</sub> phase was then calculated to reflect the percentage of apoptotic cells. In addition, cell cycle was analyzed with ModFit LT software.

#### **Statistical analysis**

The data were tested with statistical programs. Student's *t* test or Chi-square test was used. *P* < 0.05 was considered statistically significant.

## **RESULTS**

#### **Amount of fluoride in urine and concentration of fluoride in blood**

Animals in fluoride group were provided with distilled water containing 150 mg/L NaF for four weeks. The amount of fluoride in urine and concentration of fluoride in blood were higher than those in control group (*P* < 0.01), demonstrating that animals in fluoride group were in excess fluoride status (Table 1).

#### **Content of ROS, MDA and GSH in oral mucosa and liver**

ROS and MDA contents in oral mucosa and liver were higher in fluoride group than in control group, but GSH content in oral mucosa and liver was lower in fluoride group than in control group, indicating that oxidative stress was induced in fluoride group (Table 2).

#### **DNA damage in oral mucosal cells and hepatocytes**

Table 3 shows the effects of fluoride at the dose of 150

**Table 1 Amount of fluoride in urine and concentration of fluoride in blood (mean ± SD)**

Groups	Fluoride in urine (μg)	Concentration of fluoride in blood (mg/L)
Control	14.22 ± 1.33	0.334 ± 0.023
Fluoride	53.02 ± 5.45 <sup>b</sup>	1.101 ± 0.123 <sup>b</sup>

<sup>b</sup>P < 0.01 vs control group.**Table 2 Content of ROS, MDA and GSH in oral mucosa and liver (mean ± SD)**

Groups	ROS (U/mg protein)	MDA (mmol/mg protein)	GSH (mmol/mg protein)
Control (oral mucosa)	81.21 ± 7.87	0.66 ± 0.05	127.50 ± 13.11
Fluoride (oral mucosa)	134.73 ± 12.63 <sup>b</sup>	1.48 ± 0.13 <sup>b</sup>	76.38 ± 6.71 <sup>b</sup>
Control (liver)	75.57 ± 8.05	0.71 ± 0.06	130.08 ± 12.65
Fluoride (liver)	143.45 ± 11.76 <sup>b</sup>	1.44 ± 0.12 <sup>b</sup>	78.83 ± 7.72 <sup>b</sup>

<sup>b</sup>P < 0.01 vs control group.**Table 3 Effects of fluoride on DNA damage in rat oral mucosal cells and hepatocytes**

Groups	Cells	Grade of DNA damage					Rates of comet assay
		0	1	2	3	4	
Control (oral mucosal cells)	500	448	39	6	5	2	10.40
Fluoride (oral mucosal cells)	500	249	119	49	54	29	50.20 <sup>b</sup>
Control (hepatocytes)	500	453	23	12	9	3	9.40
Fluoride (hepatocytes)	500	276	127	52	31	14	44.80 <sup>b</sup>

<sup>b</sup>P < 0.01 vs control group.**Table 4 Effects of fluoride on apoptosis in rat oral mucosal cells and hepatocytes (mean ± SD)**

Groups	Oral mucosal cells (%)	Hepatocytes (%)
Control	5.61 ± 1.98	5.72 ± 1.82
Fluoride	13.63 ± 1.81 <sup>b</sup>	12.76 ± 1.67 <sup>b</sup>

<sup>b</sup>P < 0.01 vs control group.**Table 5 Effects of fluoride on proliferation in rat oral mucosal cells and hepatocytes (mean ± SD)**

Groups	G <sub>0</sub> /G <sub>1</sub> (%)	S(%)	G <sub>2</sub> /M(%)	PI
Control (oral mucosal cells)	62.75 ± 3.89	9.28 ± 2.56	8.74 ± 1.23	21.89 ± 3.61
Fluoride (oral mucosal cells)	58.53 ± 2.18	8.09 ± 1.31	6.28 ± 1.25 <sup>a</sup>	19.51 ± 0.89
Control (hepatocytes)	64.79 ± 3.91	10.28 ± 2.64	10.67 ± 1.42	22.18 ± 3.59
Fluoride (hepatocytes)	60.58 ± 2.27	9.07 ± 1.42	7.98 ± 1.36 <sup>a</sup>	19.91 ± 1.02

<sup>a</sup>P < 0.05 vs control group.

mg/L for 4 wk on the grades of DNA damage based on the comet assay. Statistical analysis yielded significant

differences between oral mucosal cells and hepatocytes based on chi-square test ( $P < 0.01$ ). Although the grades of DNA damage in oral mucosal cells were higher than those in hepatocytes, there was no significant difference ( $P > 0.05$ ).

### Apoptosis in oral mucosal cells and hepatocytes

Table 4 shows the effects of fluoride at the dose of 150 mg/L for 4 wk on apoptosis in oral mucosal cells and hepatocytes based on flow cytometry. There was a statistically significant difference between fluoride and control groups in apoptotic rate of oral mucosal cells and hepatocytes. Although the apoptotic rate was higher in oral mucosal cells than in hepatocytes, there was no significant difference ( $P > 0.05$ ).

### Changes of cell cycle in oral mucosal cells and hepatocytes

Table 5 shows the effects of fluoride on rat cell cycle and proliferation index (PI) of oral mucosal cells and hepatocytes. The number of oral mucosal cells and hepatocytes at G<sub>0</sub>/G<sub>1</sub>, S and G<sub>2</sub>/M phase induced by fluoride was lower than that in control group. PI of oral mucosal cells and hepatocytes was lower in fluoride group than in control group ( $P > 0.05$ ), suggesting that fluoride at the dose of 150 mg/L in drinking water for 4 wk interfered with normal cell cycle in oral mucosa and liver.

## DISCUSSION

Fluorosis is one of the diseases caused by biogeochemical factors. Fluorosis in human beings is mainly caused by drinking water, burning coal and drinking tea. China is one of the countries where fluorosis is most endemic<sup>[2]</sup>. Fluoride intoxication causes damages to osseous tissue (teeth and bone) and soft tissues (liver, kidney, brain, mucosa, etc.). There are many reports on the mechanisms of skeletal and dental fluorosis<sup>[13, 14]</sup>, but how fluoride interferes with soft tissue has not been clarified. In our study, male SD rats were provided with distilled water containing 150 mg/L sodium fluoride for four weeks. The concentration of fluoride in blood and the amount of fluoride in urine in fluoride group were significantly higher than those in control group, suggesting that the experimental animals are in excess fluoride status. The content of ROS and MDA was increased, but content of GSH was decreased in oral mucosa and liver, demonstrating that lipid peroxidation can be induced by fluoride intoxication in oral mucosa and liver.

Studies have shown that fluoride can induce excessive production of oxygen free radicals and decrease the biological activities of some substances, such as catalase, superoxide dismutase, xanthine oxidase and glutathione peroxidase, which play an important role in antioxidation and eliminating free radicals. Karaoz *et al*<sup>[15]</sup> showed that chronic fluorosis can lead to lipid peroxidation and kidney tissue change in first- and second-generation rats<sup>[15]</sup>. Shanthakumari *et al*<sup>[3]</sup> showed that the level of lipid peroxides is increased but the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) are decreased in rats after administered fluoride. Shivarajashan-

kara *et al*<sup>[16]</sup> reported that long-term high-fluoride intake at the early developing stages of life enhances oxidative stress in blood, disturbing the antioxidant defense of rats, suggesting that increased oxidative stress may be one of the mediating factors in the pathogenesis of toxic manifestations of fluoride. Reddy *et al*<sup>[17]</sup> evaluated the antioxidant defense system (both enzymatic and non-enzymatic) and lipid peroxidation both in human beings from an endemic fluorosis area (5 ppm fluoride in drinking water) and in rabbits receiving water containing 150 ppm of fluoride for six months, and found that there is no significant difference in lipid peroxidation, glutathione and vitamin C in blood of fluorosis patients and fluoride-intoxicated rabbits as compared to the controls. They also found that there are not any changes in the activities of catalase, superoxide dismutase, glutathione peroxidase, or glutathione S-transferase in the blood due to fluoride intoxication (of rabbits) or fluorosis in human beings<sup>[17]</sup>. Our results are concordant with other studies<sup>[2, 3, 5, 15, 16]</sup>. Liver is one of the target organs attacked by excessive amount of fluoride. Evidence of toxic changes in liver has been revealed by long term investigations of industrially-induced fluorosis, including abnormal metabolic functions, reduced activity of detoxication reactions and altered structure of subcellular organelles<sup>[18]</sup>. Our results also displayed that excess fluoride could induce oxidative stress not only in liver, but also in rat oral mucosa.

It was reported that DNA damage results from excess fluoride in human embryo hepatocytes<sup>[2, 6]</sup> and pallium neurons<sup>[19]</sup>. Our results showed that DNA damage was induced in rat oral mucosal cells and hepatocytes by sodium fluoride at the dose of 150 mg/L in drinking water for four weeks. Apoptosis is a programmed physiological process of cell death characterized by a distinct set of morphological and biochemical changes, including cytoplasmic membrane blebbing, apoptotic body formation, nuclear condensation and chromosomal DNA fragmentation. Apoptosis can be triggered in a wide variety of cell lines by diverse stimuli, ranging from extracellular signals to intracellular events<sup>[20]</sup>. Previous studies have shown that fluoride induces apoptosis in human embryo hepatocytes<sup>[2, 6]</sup>, human epithelial lung cells<sup>[21]</sup>, human and rat pancreatic islets and RINm5F cells<sup>[22]</sup> as well as in HL-60 cells<sup>[23]</sup>. In the present study, we investigated the effects of fluoride on apoptosis of rat oral mucosal cells and hepatocytes. As regards to the mechanisms of apoptosis induced by fluoride, Wang *et al*<sup>[2]</sup> concluded that fluoride could cause lipid peroxidation, DNA damage and apoptosis, and there is a positive relationship among these changes. Lipid peroxidation and apoptosis may co-exist in the beginning when tissues are exposed to excess fluoride and generates a lot of free radicals that may be sufficient to cause apoptosis. Anuradha *et al*<sup>[23]</sup> showed that sodium fluoride (NaF) induces apoptosis by oxidative stress-induced lipid peroxidation, and thereby releasing cytochrome C into the cytosol and further triggering caspase cascade leading to apoptotic cell death in HL-60 cells. Refsnes *et al*<sup>[21]</sup> reported that NaF induces apoptotic effects and increases PI-positive A549 cells via similar mechanisms, involving protein kinase C (PKC), protein kinase A (PKA), tyrosine kinase and Ca<sup>2+</sup>-linked enzymes. Thrane *et al*<sup>[24]</sup>

found that activation of mitogen-activated protein (MAP) kinases p38 and possibly c-Jun N-terminal kinase (JNK) are involved in NaF-induced apoptosis of epithelial lung cells, whereas extracellular signal regulated kinase (ERK) activation seems to counteract apoptosis in epithelial lung cells. These studies indicate that fluoride induces apoptosis through mechanisms of oxidative stress, caspase and PKC activation, MAPK signal pathway and DNA damage.

Our study also demonstrated that the number of oral mucosal cells and hepatocytes in G<sub>2</sub>/M phase was significantly lower in fluoride group than in control group, although there were no obvious changes in cell number in G<sub>0</sub>/G<sub>1</sub> and S phase. In our study, proliferation index of oral mucosal cells and hepatocytes was also decreased in fluoride group. Wang *et al*<sup>[2]</sup> showed that the number of human embryo hepatocytes in S phase is significantly higher in fluoride treated groups than in control group, but there were no changes in cell number in G<sub>0</sub>/G<sub>1</sub> and G<sub>2</sub>/M phase. This may be due to the differences in cell sensitivity to fluoride in different phases of cell cycle<sup>[25]</sup>.

In the present study, the results in oral mucosal cells were not different from those in hepatocytes significantly. Oxidative stress, DNA damage, apoptosis and modifications of membrane lipids can be induced in hepatocytes by excess fluoride<sup>[2, 3, 6, 18]</sup>. It is difficult to get liver tissue but easy to get oral mucosal cells from patients with fluorosis. Squier *et al*<sup>[26]</sup> reported that ethanol exerts its effect on lipid metabolism and epithelial permeability barrier of oral mucosa in rats. Hansson *et al*<sup>[27]</sup> have analyzed proliferation, apoptosis and keratin expression in cultured normal and immortalized human oral mucosal keratinocytes. Dhillon *et al*<sup>[28]</sup> showed that oral mucosa cells have a smaller increase in gamma-ray-induced DNA strand breaks than lymphocytes, suggesting that oral mucosal cells can be used as experimental materials to study oxidative stress, DNA damage and apoptosis as well as other effects of fluoride *in vivo*.

In conclusion, excess fluoride induces oxidative stress, DNA damage, apoptosis and cell cycle changes in rat oral mucosal cells and hepatocytes. Further investigation is needed to clarify the exact mechanisms.

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