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Effects of Fluoride on Lipid Peroxidation, DNA Damage and Apoptosis in Human Embryo Hepatocytes[1]

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Objective To investigate the effects of fluoride on lipid peroxidation, DNA damage and apoptosis in human embryo hepatocyte L-02 cells. **Methods** Lipid peroxide (LPO) level, reduced glutathione (GSH) content, DNA damage, apoptosis, and cell cycle analysis were measured after *in vitro* cultured L-02 cells were exposed to sodium fluoride at different doses (40 µg/mL, 80 µg/mL, and 160 µg/mL) for 24 hours. **Results** Fluoride caused an increase of LPO levels and a decrease of GSH content in L-02 cells. There appeared to be an obvious dose-effect relationship between the fluoride concentration and the observed changes. Fluoride also caused DNA damage and apoptosis and increased the cell number in S phase of cell cycle in the cells tested. There was a statistically significant difference in DNA damage and apoptosis when comparing the high dose of fluoride treated cells with the low dose of fluoride treated cells. **Conclusion** Fluoride can cause lipid peroxidation, DNA damage, and apoptosis in the L-02 cell experimental model and there is a significant positive correlation between fluoride concentration and these pathological changes.

Key words: Fluoride; Human embryo hepatocytes; Lipid peroxidation; DNA damage; Apoptosis

INTRODUCTION

Fluorosis is a serious public health problem in many parts of the world as a result of a high fluoride content in groundwater and airborne fluoride released from the burning of fluoride-load coal. Epidemiological evidence shows that about 330 million people in China are exposed to a high level of fluoride and about 42 million people are suffering from fluoride intoxication. Fluoride intoxication causes damages to osseous tissue and other tissues such as liver, kidney and brain. Previous studies have demonstrated that the damage caused by fluoride intoxication is mainly mediated through lipid peroxidation[1,2]. Excessive fluoride causes not only DNA damage in rat cells[3,4], but also triggers apoptosis[5,6]. Due to biological differences among species, it is not known whether excessive fluoride leads to lipid peroxidation, DNA damage and apoptosis in human embryo hepatocytes. In the present study, we investigated the effects of different doses of sodium fluoride (NaF) on lipid

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peroxidation, DNA damage and apoptosis in human L-02 embryo hepatocytes and the relationship among these pathological changes *in vitro*.

MATERIALS AND METHODS

General Chemicals

Fetal bovine serum, HEPES buffer, agarose, and DMEM cell culture powder were obtained from GIBCO BRL (Paisley, Scotland). Propidium iodide, ethidium bromide, RNase A, trypsin, triton X-100, and DMSO were purchased from Sigma (Deisenhofen, Germany). Sodium fluoride, sodium phosphate, sodium citrate, and Na_2-EDTA were purchased from Shanghai Chemical Reagent Corporation (Shanghai, China).

L-02 Cell Culture and NaF Treatment

L-02 cells were cultured in DMEM supplemented with 10% v/v heat-inactivated fetal bovine serum, 15 mmol/L hepes, and antibiotic supplement (penicillin 100 U/mL and streptomycin 0.1 mg/mL) at 37℃ in an incubator with humidified atmosphere containing 5% CO_2. Exponentially growing cells were divided into four groups: treated with PBS as a control group and with sodium fluoride at 40, 80,160 μg/mL (named low, medium, and high dose of fluoride groups, respectively). After 24 h of incubation, the cells were rinsed twice in 1× PBS, trypsinized and resuspended in DMEM medium containing 10% v/v heat-inactivated fetal bovine serum. The cells were then kept on ice for further assays. The cell viability was assessed by trypan blue exclusion and >90% of the cells were alive for all groups.

Detection of Apoptosis

Approximately 1.0×10^6 cells from each group were centrifuged for 5 min at 4℃. The cell pellet was then fixed for 24 h with 80% alcohol, which was pre-chilled at -20℃. Prior to toxicity detection, the cell pellet was washed twice with 1×PBS, and then 100 μL of PC buffer (9 volume of 0.05 mmol/L $NaPO_4$ was mixed with one volume of 25 mmol/L sodium citrate, pH 7.8) was added to re-suspend the cell pellet. After 15 min at room temperature, 0.5 mL propidium Iodide (100 μg/mL) and RNase A (5 μg/mL) were added and cell suspension was incubated for 30 min in the dark. For each group, 5000 cells were counted with fluorescence activated cell sorter (FACS) flow cytometry (BD Company, San Jose, CA, USA). In addition, apoptosis was analyzed with Cell Quest software and cell cycle was analyzed with ModFit LT software.

Detection of DNA Damage

Single cell gel electrophoresis assay (also known as the Comet assay) was performed according to Singh *et al.*[7] with some modifications. Approximately 1.0×10^5 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- and Mg-free PBS buffer). A final layer of 0.65% low-melting agarose was added on top. Slides were immersed in a jar containing cold lysis 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_2EDTA, pH 10) at 4℃ for 1-2 h. Then, slides were pretreated for 15 min in electrophoresis buffer (300 mmol/L NaOH/1 mmol/L Na_2EDTA, pH 12) and exposed to 25 V/300 mA for 20 min. Preincubation and electrophoresis were performed in an ice bath. Slides were neutralized for 3 to 5 min in

0.4 mol/L Tris, pH 7.5, and DNA was stained by adding 50 μL of ethidium bromide (20 μg/mL) onto each slide. After stained for 5 min, slides were rinsed in distilled water and covered again for microscopic examination. All steps were performed under red light to prevent additional DNA damage.

Image analysis was performed with 200× magnification by 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). A total of 150 randomly selected cells (50 cells from each slide) per group were scored microscopically. In this test, DNA damage of the cells was evaluated by using the ratio of tail DNA content/the whole cellular DNA content and data were analyzed by the Ridit assay.

Measurement of LPO Level, GSH and Protein Content

The rest of cell suspension was centrifuged at 50 xg for 5 min at 4℃. The cell pellet was dissolved in 0.5 mL cell lysis solution. Lipid peroxide (LPO) level was assessed by estimation of malondialdehyde (MDA) according to the method of Ohkawa *et al.*[8]. Glutathione (GSH) content was measured by the method of Beutler *et al.*[9], and protein content was measured colorimetrically by the methods of Lowry *et al.*[10]. The contents of LPO, GSH, and protein were expressed as nmol MDA/mg.prot, μg/mg.prot, and mg/mL, respectively.

Statistical Analysis

Analysis was performed by using ANOVA with subsequent Dunnett's test except for the DNA damage, which used the Ridit assay. A difference at $P<0.05$ was considered statistically significant. All experiments were performed in triplicate and repeated 3 times.

RESULTS

Effects of Fluoride on GSH Content and LPO Level in L-02 Cells

Table 1 demonstrates that LPO level in cells treated by different doses of fluoride was significantly higher than that in the control group ($P<0.05$). In contrast, GSH content in cells treated by different doses of fluoride was markedly lower than that in the control group ($P<0.05$). There existed an obvious dose-effect relationship between the concentration of fluoride and the LPO and GSH contents.

TABLE 1

Contents of GSH and LPO in Cell Lysates ($\bar{x}\pm s$, n=5)

Groups	GSH content (μg/mg.prot)	LPO Level (nmol MDA/mg. prot)
Control (1×PBS)	0.095±0.016	0.3726±0.1141
Low Dose of Fluoride (40 μg/mL)	0.063±0.008*	1.4635±0.3128**
Medium Dose of Fluoride (80 μg/mL)	0.051±0.006*	1.9462±0.1330***
High Dose of Fluoride (160 μg/mL)	0.041±0.007**	2.4768±0.1454***a

Note. Compared to the control group, *$P<0.05$, **$P<0.01$, ***$P<0.001$. a Compared to low dose of fluoride group, $P<0.05$. Data were given as $\bar{x}\pm s$ of the triplicates. GSH content and LPO level were expressed as μg/mg.prot and nmol MDA/mg.prot, respectively.

Effect of Fluoride on DNA Damage in L-02 Cells

Table 2 suggests that the percentage of DNA damage in L-02 cells exposed to different doses of fluoride was higher than that in the control group. The Ridit value in each dose group was 0.5815, 0.8145, 0.8923, and 0.5000, respectively. There was a significant difference in the percentage of cells with DNA damage compared to the control group ($P<0.05$). In addition, there existed a statistically significant difference in DNA damage by comparing medium and high dose groups with low dose group ($P<0.05$). There was an obvious dose-effect relationship between the concentration of fluoride and the level of DNA damage in the cells.

TABLE 2

Percentage of DNA Damage in Cells

Groups	Total Cell Number Counted	Number of Damaged Cells					Percentage of Damaged Cells (%)	Ridit Value
		0	1	2	3	4		
Control (1×PBS)	150	105	34	11	0	0	30.00	0.5000
Low Dose of Fluoride (40 μg/mL)	150	84	39	26	1	0	44.00	0.5815*
Medium Dose of Fluoride (80 μg/mL)	150	30	33	62	19	6	80.00	0.8145**a
High Dose of Fluoride (160 μg/mL)	150	18	15	48	38	31	88.00	0.8923**a

Note. Compared to the control group, *$P<0.05$, **$P<0.01$. Compared to low dose of fluoride group, a$P<0.05$. Data were expressed as percentage of cells with damaged DNA (%).

Effect of Fluoride on Apoptosis and Distribution of Cell Cycle in L-02 Cells

Table 3 indicates that the percentage of apoptotic L-02 cells treated by medium and high doses of fluoride was significantly higher than that in the control group ($P<0.05$) and were elevated with increasing fluoride concentration. The percentage of apoptotic cells treated with low dose of the fluoride was higher than that in the control group, but the difference was not statistically significant ($P>0.05$).

The cell number in S phase of L-02 cells treated by the different doses of fluoride was much higher ($P<0.05$), but the cell number in G_0/G_1 and G_2/M phases among different groups was not statistically significantly different ($P>0.05$).

DISCUSSION

Fluoride is an essential trace element widely distributed in nature. Intake of excessive fluoride can cause lipid peroxidation, which leads to cellular oxidative stress. The generation of reactive oxygen species further could cause damage to the human body due to decreased availability of antioxidants (such as GSH) and free radical cleaning enzymes (such as SOD)[2,11,12]. In this study, we observed an increasing level of LPO and a decreasing level of GSH content in the lysates of L-02 cells treated by different doses of fluoride. This result indicates that fluoride causes lipid peroxidation.

TABLE 3

Percentage of Apoptosis and Cell Cycle Analysis

Groups	Percentage of Apoptotic Cells	G_0/G_1	S	G_2/M
Control (1×PBS)	4.341 ± 0.782	85.393 ± 3.724	14.500 ± 1.038	0.107 ± 0.038
Low Dose of Fluoride (40 μg/mL)	6.710 ± 2.031	78.827 ± 5.369	20.923 ± 2.787*	0.250 ± 0.181
Medium Dose of Fluoride (80 μg/mL)	9.292 ± 1.713*	80.461 ± 4.368	19.433 ± 1.227*	0.106 ± 0.049
High Dose of Fluoride (160 μg/mL)	11.731 ± 1.202**,a	79.674 ± 5.544	20.123 ± 1.355*	0.203 ± 0.093

Note. Compared to the control group, $^{*}P<0.05$, $^{**}P<0.01$. Compared to low dose of fluoride group, $^{a}P<0.05$. Data were given as $\overline{x} \pm s$ of the triplicates and expressed as %.

Up to now, studies have shown that lipid peroxidation itself does not lead to cell death. However, a variety of free radicals were formed during the lipid peroxidation process. These free radicals could enter the nuclei and cause base modifications and DNA breakage in addition to causing more lipid peroxidation[13,14]. More importantly, fluoride could directly form covalent bonds with mammalian DNA and cause DNA damage due to its active chemical property[15]. We demonstrated that sodium fluoride (40 mg/mL) caused damage to L-02 cell DNA. The percentage of cells with damaged DNA was 44.0%. As cells were exposed to higher doses of fluoride, the percentage of L-02 cells with DNA damage increased. This result is consistent with other studies[6,16].

Apoptosis is also known as a programmed cell death. Previous studies have shown that fluoride could induce many types of cellular apoptosis[4,5,6,17]. In the present study, we investigated the effects of fluoride on apoptosis by using flow cytometry and showed that there was no difference in celllular apoptosis between cells treated with low dose (40 μg/mL) of fluoride and cells in the control group. However, the percentage of apoptotic cells treated with medium and high doses of fluoride was much higher than that in the control group. This indicates that fluoride could cause apoptosis in L-02 cells. Meanwhile, the result also demonstrates that the cell number in S phase in all fluoride treated groups was significantly higher than that in the control group, although there were no changes in the cell number in G_0/G_1 and G_2/M phases. This might be due to the differences in cell sensitivity to fluoride in different phases in the cell cycle[18]. This finding indicates that fluoride could disturb signal transduction in cell cycle, cause the cells to arrest in S phase, and inhibit cell proliferation.

In addition, the present study addressed the relationship between fluoride-induced lipid peroxidation and apoptosis as well as the relationship between DNA damage and celllular apoptosis. The results showed that using the same dose of fluoride, there was a positive relationship between LPO level and percentage of apoptotic cells (r=0.882, P<0.01), a negative relationship between GSH content and percentage of apoptotic cells (r=-0.629, P<0.05), and a positive relationship between DNA damage and percentage of apoptotic cells (r=0.9709, P=0.029), although apoptosis was a less sensitive marker than DNA damage induced by fluoride. Therefore, considering previous studies[1,4,11], we think that fluoride can cause lipid peroxidation, DNA damage and apoptosis, and that there is a positive relationship among these changes. We believe that lipid peroxidation and apoptosis may co-exist at the

beginning when human tissues are exposed to excessive fluoride, and lipid peroxidation generates a lot of free radicals that may be sufficient to cause apoptosis.

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REFERENCES

1. Rzeuski, R., Chlubek, D., and Machoy, Z. (1998). Interactions between fluoride and biological free radical reactions. *Fluoride* **31**, 43-45.
2. Shivarajashankara, Y. M., Shivashankara, A. R., Gopalakrishna, Bhat, P., and Hanumanth, Rao, S. (2001). Effect of fluoride intoxication on lipid peroxidation and antioxidant systems in rats. *Fluoride* **34**, 108-113.
3. Zeiger, E., Shelby, M. D., and Witt, K. L. (1993). Genetic toxicity of fluoride. *Environ. Mol. Mutagen*. **21**, 309-318.
4. Chen, J., Chen, X., Yang, K., Xia, T., and Xie, H. (2002). Studies on DNA damage and apoptosis in rat brain induced by fluoride. *Chin. J. Prev. Med*. **36**, 222-224. (In Chinese)
5. Jing, L., Shao, Z. J., Ren, L.Q., and Li, G. S. (1999). Hepatocyte apoptosis in fluoride rats. *Chin. J. Endemiol.* **18**, 84-86. (In Chinese)
6. Hirano, S. and Ando, M. (1997). Fluoride mediates apoptosis in osteosarcoma UMR 106 and its cytotoxicity depends on the pH. *Arch. Toxicol.* **72**, 52-58.
7. Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* **175**, 184-191.
8. Ohkawa, H., Ohishi, N., and Yagi, K. (1979). Assay for lipid peroxides in animal tissues thiobaibituric acid reaction. *Anal. Biochem*. **95**, 351-358.
9. Beultler, E., Duron, O., and Kelly, B. M. (1963). Improved method for the determination of blood glutathione. *J. Lab. Clin. Med*. **61**, 882-888.
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem*. **193**, 265-275.
11. Guan, Z. Z., Xiao, K. Q., Zeng, X. Y., Long, Y. G., Cheng, Y. H., Jiang, S. F., and Wang, Y. N. (2000). Changed cellular membrane lipid composition and lipid peroxidation of kidney in rats with chronic fluorosis. *Arch. Toxicol.* **74**, 602-608.
12. Wang, Y. N., Xiao, K. Q., Liu, J. L., Dallner, G., and Guan, Z. Z. (2000). Effect of long term fluoride exposure on lipid composition in rat liver. *Toxicology* **146**(2-3), 161-169.
13. Schulte-Frohlinde, D. (1986). Mechanism of radiation-induced strand break formation in DNA and polynucleotides. *Adv. Space Res*. **6**, 89-96.
14. Anuradha, C. D., Kanno, S., and Hirano, S. (2001). Oxidative damage to mitochondria is a preliminary step to caspase-3 activation in fluoride-induced apoptosis in HL-60 cells. *Free Radic. Bio. Med*. **31**, 367-373.
15. Gao, X. B., Liu, C. S., Sun, J. X., Zhang, L., Xu, Z., and Li, J. (1998). The effects of sodium fluoride on the UV absorption spectrum of different types of DNA. *Carciog. Teratog. and Mutagen*. **10**, 28-30. (In Chinese)
16. Xia, T., Wang, A., Yu, R., Xiao, D. F., Liu, Q., and Yang, K. D. (2003). Effects of selenium and Vitamin C on hepatocyte DNA damage caused by fluoride in rats. *Chin. J. Endemiol.* **22**, 124-125. (In Chinese)
17. Thrane, E. V., Refsnes, M., Thoresen, G. H., Lag, M., and Schwarze, P. E. (2001). Fluoride-induced apoptosis in epithelial lung cells involves activation of MAP kinases p38 and possibly JNK. *Toxicol. Sci.* **61**, 83-91.
18. Hayashi, N. and Tsutsui, T. (1993). Cell cycle dependence of cytotoxicity and clastogenicity induced by treatment of synchronized human diploid fibroblasts with sodium fluoride. *Mutat. Res*. **290**, 293-320.

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