ANTAGONISTIC EFFECT OF SELENIUM ON OXIDATIVE STRESS, DNA DAMAGE, AND APOPTOSIS INDUCED BY FLUORIDE IN HUMAN HEPATOCYTES

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SUMMARY: To evaluate the toxicity of fluoride and the antagonistic effect of selenium, normal human primary hepatocytes were incubated with sodium fluoride (80 µg/mL) and/or sodium selenite (1.73 µg/mL) for 12 hr *in vitro*. The percentage of apoptotic hepatocytes, the number of cells in S phase, DNA damage in cells, and lipid peroxide (LPO) levels in the cells and culture medium, together with aspartate transaminase (AST) and lactate dehydrogenase (LDH) activities in the cell culture medium, were higher in the fluoride-exposed group than in the control group, but the glutathione (GSH) content in the fluoride-exposed cells was lower than in the control group. However, these toxic effects of fluoride were reduced by selenium, which elevated the GSH content and reduced the LPO levels, the LDH and AST activities, and the percentage of apoptotic and DNA-damaged cells. These findings suggest that fluoride exposure can cause hepatocyte damage, whereas selenium can antagonize fluoride-induced apoptosis, DNA damage, and oxidative stress in hepatocytes.

Keywords: Apoptosis; Cell cycle; DNA damage; Fluoride toxicity; Human hepatocytes; Oxidative stress; Selenium antagonism.

INTRODUCTION

Fluorosis is prevalent in some parts of central and western China where it is caused not only by drinking fluoride-containing groundwater but also by breathing airborne fluoride released from coal burning. Available statistics indicate that throughout China more than 42 million people are seriously afflicted by fluorosis and another 330 million are exposed to it.

Among its adverse biochemical effects, fluoride causes increased lipid peroxidation in the blood of humans[1] and in the blood and tissues of experimental animals.[2] Previous findings indicated that active oxygen and free radical-induced damage play an important role in fluoride-induced toxicity.[1-3] The increase in lipid peroxidation causes damage to cell membranes, and the products of lipid peroxidation can easily penetrate cell membranes by simple diffusion and directly attack DNA, leading to apoptosis.[4-5] Both laboratory and epidemiological investigations indicate that selenium can partially antagonize fluoride-induced lipid peroxidation.[6-7] Recent reports also suggest that fluoride may induce apoptosis in primary animal liver, pancreatic, and renal cells or cell lines.[7-10]

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Considering differences in species response, we have used human hepatocytes to undertake preliminary experiments, the results of which have been published in a Chinese medical journal.[11] This report is in part a translation of that paper with the addition of new material on the effects of selenium and fluoride on DNA damage in human hepatocytes.

MATERIALS AND METHODS

General chemicals: Fetal calf serum, HEPES (N-hydroxyethylpiperazine-N'-2-ethanesulfonate) buffer, and RPMI (Roswell Park Memorial Institute) 1640 medium were obtained from GIBCO (Paisley, Scotland). Percoll was obtained from Pharmacia (Uppsala, Sweden). Propidium iodide**,** RNase A, typsin, collagenase, Tris, Triton X-100, and DMSO (dimethyl sulfoxide) were purchased from Sigma (Deisenhofen, Germany). Sodium fluoride, sodium selenite, sodium phosphate, sodium citrate, Na_2-EDTA, and trypan blue were obtained from Shanghai Chemical Reagent Corporation (Shanghai, China). Penicillin and streptomycin were obtained from North China Pharmaceutical Corporation (Tianjin, China).

Separation and culturing of human hepatocytes: Normal human liver tissue was obtained from surgical operations and delivered to the laboratory on ice. About 5 g of liver tissue was put in a 50-mL beaker, minced to about 1 cm^3 in size with ophthalmologic scissors, and then washed three times with phosphate buffer saline (PBS). To start the digestive action, 20 mL of digestive solution containing 0.5% trypsin and 0.025% collagenase was added to the beaker to re-suspend the tissue, which was then gently stirred at a constant speed for 30–40 min at 37 ºC. RPMI 1640 medium containing 10% v/v heat-inactivated fetal calf serum (FCS) was added to stop the digestive action. Four layers of medical gauze were used as a filter to remove the unlysed tissue, and the crude cell suspension was centrifuged at 50 g for 5 min at 4 ºC. In order to achieve high purity, the cell pellet was re-suspended in PBS and centrifuged again with a 30% Percoll gradient (50 g, 25 min at 4 ºC) to remove non-parenchymal and dead cells. The cell viability was then measured by the trypan blue exclusion assay (over 85%), and the hepatocyte purity was assessed under light microscopy. All laboratory procedures were performed under aseptic conditions.

RPMI 1640 medium supplemented with 15 mM HEPES, 100 U/mL penicillin, 100 µg/mL streptomycin, 1.4 µM hydrocortisone, and 10% v/v heat-inactivated FCS was prepared in 500-mL batches which were used in amounts needed to bring the final cell concentration to 1 x 10^6 cells/mL. The cell suspension was incubated at 37 ºC in a humidified incubator at 95% RH and 5% CO_2. Approximately 2–3 hr later, the cell suspension was divided into tubes containing 5 mL each. These tubes were divided into four groups (each con-

sisting of three tubes) for the treatments: fluoride-exposed, selenium-treated, combination of fluoride-exposed and selenium-treated, and control (untreated). For the fluoride-exposed group (F group), 0.1 mL of sodium fluoride (NaF) solution (4.0 mg/mL) was added each tube to make the final concentration 80 μg F^-/mL; for the selenium-treated group (Se group), 0.1 mL sodium selenite (Na_2SeO_3) solution (86.5 μg/mL) was added to make the final concentration 1.73 μg Se/mL; for the combined fluoride-exposed and selenium-treated group (F+Se group), 8.0 mg/mL NaF (50 μL) and 173 μg/mL Na_2SeO_3 (50 μL) were added to make the final concentration 80 μg/mL in F^- and 1.73 μg/mL in Se; and for control group, the cells were treated with 0.1 mL PBS only. The concentrations of NaF and Na_2SeO_3 in this experiment are based on the work of Machalinska *et al*[4] and Yu *et al*.[7] The tubes were then put into an incubator for 24 hr (37 ºC, 5% CO2) and monitored by measurements of the toxicity parameters.

Detection of apoptosis: 1 mL of cell suspension aliquot was put into a 10-mL conical tube and centrifuged for 5 min (50 g at 4 ºC). The cell pellet was then fixed with an 80% alcohol, which was pre-cooled at –20 ºC for 24 hr. Prior to toxicity detection, the cell pellet was washed two times with PBS, and then 100 μL of pH 7.8 PC (phosphate-citrate) buffer (0.05 mM NaH_2PO_4 mixed 9:1 (V:V) with 25 mM sodium citrate) was added to re-suspend the cell pellet. After 15 min at room temperature (25 ºC), 0.5 mL of propidium iodide (100 μg/mL) and RNase A (5 μg/mL) were added to the tube, which was kept for 30 min in darkness. For each sample, 5000 cells were counted with a Flow Activated Cell Sorter (FACS) flow cytometry (BD Company, San Jose, CA, USA). In addition, the percentage of apoptosis was analyzed with Cell Quest software and the cell cycle was analyzed with ModFit LT software.

The rest of the cell suspension was centrifuged at 50 g for 5 min at 4 ºC. The cell pellet was dissolved by 0.5 mL of cell lysing solution containing 100 mM Na_2EDTA, 2.5 mM NaCl, 10% sodium sarcosinate, 10 mM Tris, 1% Trition X-100, and 10% DMSO. The supernatant and hepatocyte lysates were stored at –20 °C for further analysis.

Detection of DNA damage: The single cell gel electrophoresis assay (also known as Comet assay) was performed according to Singh *et al*[12] with some modifications. Approximately 1.0 x 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 mM Tris/1% sodium laurylsarcosine/2.5 M NaCl/100 mM Na_2EDTA, pH 10) at 4 ºC for 1–2 hr. The slides were then pretreated for 15 min in electrophoresis buffer (300 mM NaOH/1 mM 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 M Tris at 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, the slides were rinsed in distilled water and covered again for microscopic examination. All steps were conducted under red light to prevent UV-induced DNA damage.

Image analysis was performed with 200x magnification using a fluorescence microscope (Olympus B-60F5) equipped with a 549-nm excitation filter and a 590-nm barrier filter, coupled to a CCD camera (Kodak, USA). Five hundred randomly selected cells (100 cells from each slide) per group were scored. In this test, DNA damage to the cells was evaluated using the ratio of tail DNA content/the whole cellular DNA content. Data were analyzed by Ridit assay.

Measurement of LDH, AST, and ALT in supernatants: To evaluate the hepatocellular damage following various treatments, the supernatant was placed in Eppendorf tubes and the activities of lactic dehydrogenase (LDH), aspartate transaminase (AST), and alanine transamine (ALT) release were determined by an automatic procedure using an OLYMPUS AU 1000 automatic biochemical analyzer (Tokyo, Japan). The values were calculated from a standard curve, and enzyme activities were expressed as U/L.

Estimation of LPO, GSH, and protein: Lipid peroxidation (LPO) in the supernatants and hepatocyte lysates was assessed by estimation of malondialdehyde (MDA) according to the method of Ohkawa *et al*.[13] Glutathione (GSH) in hepatocyte lysates was assayed by the method of Beutler *et al*,[14] and protein content in supernatants and hepatocyte lysates was measured colorimetrically by the method of Lowry *et al*.[15] The contents of LPO, GSH, and protein were expressed as nM MDA/mg.prot, μg/mg.prot, and mg/mL, respectively.

Statistical analysis: All numerical data are expressed as means ± SD. Determination of significance was performed by ANOVA with subsequent Dunnett's test, except for the DNA damage, for which the Ridit assay was used. Results were considered statistically significant at $p < 0.05$. In all experiments assays were performed in triplicate and repeated three times.

RESULTS

Effect of fluoride and selenium on the percentage of apoptosis and cell numbers of cell cycle in human hepatocytes: As shown in Table 1, the percentage of apoptotic cells in F group was significantly higher than in the control group ($p=0.0085$), Se group ($p=0.016$), and F+Se group ($p=0.024$). The percentage

of apoptotic cells in Se group was higher than in control group, but the difference was not statistically significant (p=0.121).

Table 1. Distribution of apoptosis and cell cycle numbers in human hepatocytes (Mean ± SD of triplicates expressed as percentages)

Group	Apoptosis	G_0/G_1	S	G_2/M
Control	10.31±1.023	96.37 ± 4.32	3.25 ± 0.74	0.34 ± 0.13
F group	15.56±2.06*	95.29 ± 5.64	4.82 ± 0.45*	0.22 ± 0.02
Se group	11.22±1.28†	96.52 ± 5.97	3.03 ± 0.24†	0.32 ± 0.04
F+Se group	12.98±1.15†	97.20 ± 6.31	2.46 ± 0.68†	0.25 ± 0.07

*P<0.05, vs control; †P<0.05, vs F group.

Compared with the control group, the cell number in S phase was significantly increased in F group (p=0.038), while the cell number in G_0/G_1 phase and G_2/M phase was not markedly changed (p=0.236 and p=0.361, respectively). In F-Se group, the cell number in G_0/G_1 phase was increased, while in G_2/M phase it was reduced, but the difference was not statistically significant (p=0.089 and p=0.118, respectively). All cell numbers in each phase in Se group were not markedly changed compared with the control group (p=0.05). However, the cell number in S phase in F+Se group was significantly lower than that of F group (p=0.022).

Effect of fluoride and selenium on DNA damage in human hepatocytes: Table 2 indicates that the percentage of DNA-damaged cells in F group was significantly higher than in the control group (p=0.0046), Se group (p=0.035), and F+Se group (p=0.0085). The actual Ridit values in each group were 0.5000, 0.793, 0.615 and 0.594, respectively. Compared with the control group, the percentage of DNA damage cells in Se group and F+Se group were increased, but the differences were not statistically significant (p=0.154 and p=0.568, respectively).

Table 2. DNA damage in hepatic cells

Group	No.of cells counted	Number of cells damaged					% of cells damaged	Ridit value
		0	1	2	3	4		
Control	500	424	33	23	16	4	15.20	0.50
F group	500	205	104	95	73	23	59.00	0.79*
Se group	500	357	56	52	28	7	21.60	0.61
F+Se group	500	403	42	29	21	5	19.40	0.59†

*P<0.05, vs control; †P<0.05, vs F group.

Effect of fluoride and selenium on the biological membrane system in human hepatocytes: As shown in Table 3, the activities of LDH and AST in F group were significantly increased (p=0.016 and p=0.0021, respectively) and the activity of LDH in F+Se group was markedly increased (p=0.039) compared with the control group. Compared with the control group, the activities of LDH and AST in Se group were increased, but the differences were not statistically significant (p=0.358 and p=0.782, respectively). There was no significant difference in the activity of ALT among the four groups (p>0.05).

Table 3. Activity (U/L) of LDH, ALT, and AST in supernatants (Mean ± SD of triplicate determinations)

Group	LDH	ALT	AST
Control	126.4 ± 2.6	8.0 ± 1.2	54.5 ± 3.2
F group	140.4 ± 7.6*	7.6 ± 1.8	91.1 ± 36.4*
Se group	131.4 ± 1.7†	7.6 ± 1.1	64.4 ± 2.3†
F+Se group	137.4 ± 1.6	7.2 ± 1.7	70.6 ± 3.1

*P<0.05, vs control; †P<0.05, vs F group.

Effect of fluoride and selenium on lipid peroxidation in human hepatocytes: As shown in Table 4, the concentration of GSH in F group was significantly lower than in the control group (p=0.0056) and F+Se group (p=0.013). The levels of LPO in the cells and supernatants in F group were significantly higher than in the control group (p=0.0068 and p=0.0004, respectively) and Se group (p=0.0042 and p=0.026, respectively).

Table 4. GSH and LPO concentrations in liver cells and supernatants (Mean ± SD of triplicate determinations)

Group	GSH conc.	LPO conc. (nM MDA/mg.prot)	
	(μg/mg protein)	hepatocyte	supernatant
Control	7.595 ± 1.042	1.473 ± 0.401	1.694 ± 0.443
F group	4.225 ± 0.781*	2.884 ± 0.589*	3.547 ± 0.561*
Se group	8.859 ± 1.195†	1.408 ± 0.415†	2.109 ± 0.684†
F+Se group	6.218 ± 1.492†	2.015 ± 0.618	2.468 ± 0.396

*P<0.05, vs control; †P<0.05, vs F group.

There were differences in the level of LPO between F group and F+Se group in the cells and supernatants, but these differences were not statistically significant (p=0.561 and p=0.086, respectively).

Interestingly, we observed that fluoride had caused not only lipid peroxidation but also cellular apoptosis. There was a significantly positive correlation between lipid peroxidation and apoptosis induced by fluoride (r=0.856, p=0.0037) (data not shown).

DISCUSSION

An association between fluoride toxicity and increased oxidative stress has been reported in the blood of humans[1,16,17] and in the blood and liver tissue of experimental animals.[2,17,18] The results of the present study showed that *in vitro* treatment of human primary hepatocytes by fluoride resulted in increased activities of LDH and ALT measured in the cell culture medium of treated cells. These data suggest that fluoride may cause damage to biological membrane system in human hepatocytes as previously shown in animals.[19] On the other hand, such damage can be alleviated by simultaneous treatment with selenium, suggesting a possible measure of protection.

Fluorosis is a well-defined clinical entity characterized by toxic effects of high-fluoride intake on teeth, bones, and soft tissues. Many theories about the mechanism of fluorosis have been proposed in recent years, one of which is the free-radical theory supported by animal experiments. In this theory, the active unpaired electron on free radicals can interact with many biomacromolecules, such as unsaturated fatty acids, DNA, proteins, etc. These interactions can lead to lipid peroxidation of the biological membrane and DNA denaturation.[20] Although some data on the toxicity of fluoride from human blood also provide supportive evidence for this free-radical theory,[1,15,16] only meager data are available from human tissues other than blood.[11] Indeed, our study provides further evidence that fluoride can reduce the content of GSH and increase the level of LPO in both the hepatocytes and the culture medium after treatment by fluoride. Therefore, our results are consistent with findings from animal experiments.[2,21,22] It is known that free radicals induces lipid peroxidation of biological membranes and that malondialdehyde (MDA), a product of lipid peroxidation, causes cross-linking and formation of polymers in these membranes. In agreement, our results strongly suggest that fluoride can cause severe damage to the hepatocytes which will decrease membrane fluidity and penetrability. The appearance of LDH and AST in the culture medium indicates that the biological membrane of the cells was not intact after apparent attack by free radicals resulting from excessive exposure to fluoride. Therefore, our findings from liver tissues confirm and extend the results of other reports using human blood and animal tissues.[2,16,17,23] How-

ever, whether decreased content of antioxidants such as GSH causes enhanced lipid peroxidation or results from exhaustion due to lipid peroxidation warrants further investigation.

Many cellular defense systems such as antioxidants and free-radical scavenging enzyme systems exist in most animal organisms including humans. If the balance between antioxidative defense systems and free radicals is broken, oxidation stress will be produced and the cells will be damaged. Following the finding by Rotruck *et al*[24] that selenium was an important component of glutathione peroxidase (GSH-Px), other researchers have demonstrated that selenium has both direct and indirect antioxidative effects on the elimination of free radicals, *i.e.*, an indirect effect through selenium-containing enzymes and a direct effect by itself.[25] The results of our study also showed that through improving the level of internal antioxidant GSH, selenium had an antagonistic action on damage to the cells induced by fluoride. This results is confirmed by Myśliwiec *et al* who measured serum lipid and enzyme activities and the protective action of selenium in fluoride-intoxicated rats.[26]

It has been further reported that the free radicals or the exhaustion of antioxidants can induce apoptosis.[7,27,28] The present study demonstrated that exposure to a high level of fluoride *in vitro* can cause lipid peroxidation and apoptosis in human hepatocytes. Selenium itself has an ability to induce apoptosis, but it also has an antagonistic effect on apoptosis induced by fluoride. Hayashi *et al*[29] reported that the cells in different phases have a different sensitivity to fluoride. The results of our study also showed that fluoride treatment increased the cell number in S phase significantly but had no effect on the cell number in G_0/G phase and G_2/M phase. This finding indicates that fluoride can disturb the signal transduction in cell cycle and can cause the cell arrest in S phase. Our data also show that selenium had an antagonistic effect on the cell cycle arrest induced by fluoride.

Up to now, research has indicated that lipid peroxidation itself does not lead to cell death. A variety of free radicals are formed during the lipid peroxidation process. These free radicals enter the nuclei and cause base modifications and DNA breakage in addition to causing more lipid peroxidation.[20,30] More importantly, fluoride directly forms strong hydrogen bonds with mammalian DNA and causes DNA damage due to this reactive chemical property.[31] In the present study, we demonstrated that sodium fluoride (80 μg/mL) caused 59% damage to DNA in human hepatocytes, a result consistent with other studies.[32] Our work also showed that selenium (1.73μg/mL) produce some damage to human hepatocytes, but the difference between Se group and the control was not statistically significant.

In addition, our data showed that fluoride can cause not only lipid peroxidation but also apoptosis in human hepatocytes, There was a significantly posi-

tive correlation between lipid peroxidation and apoptosis induced by fluoride in human hepatocytes (r=0.856, P=0.0037). Because fluoride itself can attack oxygen and disturb oxygen metabolism, leading to an increased level of oxygen free radicals, we believe that damage to the cells induced by fluoride starts with lipid peroxidation, generating free radicals that may be sufficient to cause apoptosis. Therefore, our data support the free-radical theory. In this process, many lipid free radicals with an intermediate level of activity, such as $L^{\cdot}$, $LO^{\cdot}$, $LOO^{\cdot}$, etc., can easily pass into the nucleoli and then attack DNA directly. As a result, some regulatory genes (such as p53, bcl-2) may be damaged, causing the cell cycle to be disturbed and the balance between growth and death broken, leading to apoptosis. Indeed, our data suggest a role of fluoride in this cellular process, but sequential events in the fluoride-induced biological effects need to be further investigated.

ACKNOWLEDGMENTS

The authors would like to express their sincere thanks to Dr. Qingyi Wei for his critical review of the manuscript. The work was supported by grants from the National Nature Science Foundation of China (No. 30271155), and the China National Key Basic Research and Development Program (No. 2002CB512908).

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