

Fluorine-Induced Apoptosis and Lipid Peroxidation in Human Hair Follicles In Vitro

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Abstract Fluoride is an essential trace element for human body; however, exposure to high amounts of fluoride has been documented to be correlated with an increasing risk of hair loss. To date, little is known about the mechanism(s) of how fluoride affects hair follicles. Here, we demonstrated that middle (1.0 mmol/L) and high (10.0 mmol/L) concentrations of sodium fluoride (NaF) significantly inhibited hair follicle elongation in vitro, but low NaF (0.1 mmol/L) showed little influence. Moreover, treatment with high levels of NaF resulted in a marked increase in terminal dUTP nick end labeling-positive cells in the outer layer of the outer root sheath, the dermal sheath, and the lower bulb matrix surrounding dermal papilla. Furthermore, the enhanced apoptosis was coupled with an increased oxidative stress manifested as higher malondialdehyde content. Additionally, the presence of selenium considerably antagonized the effects of middle NaF on hair follicles, with regard to either the suppression of hair growth or the induction of oxidative stress and apoptosis. In conclusion, exposure to high levels of fluoride compromises hair follicle growth and accelerate cell apoptosis in vitro. The toxicity of fluoride can be reduced by selenium, at least partially via the suppression of intracellular oxidative stress.

Keywords Hair follicle · Apoptosis · Sodium fluoride · Lipid peroxidation

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Introduction

Fluoride (F), widely distributed in the environment, is an essential trace element for normal tooth and bone development. At high concentrations, however, it can cause severe problems, such as dental fluorosis [1], bone fractures [2], renal toxicity [3], and reproductive defects [4]. The toxic effects of F have been linked to the inhibition of protein synthesis and cell cycle progression [5–7] and the induction of lipid peroxidation and cell apoptosis [8–10]. The underlying molecular mechanisms for F function have been studied to some extent. Several reports [11–13] have documented that F complexed with aluminum induces the activation of guanosine triphosphate-binding G-proteins and activates numerous downstream signaling effector proteins such as protein kinase C, the kinase p38, and a serum response factor. Fluoride may also elicit some of its effects through inhibition of phosphatase, thus consequently activating protein kinases [14, 15].

High fluoride intake is also correlated with an increased risk of hair loss [16], which commonly results from aberrant hair follicle apoptosis [17, 18]. However, the effects of F on hair follicles are poorly studied. Hence, in this study we aimed to investigate the influence of different concentrations of sodium fluoride (NaF) on hair follicles *in vitro* as well as the underlying mechanism(s). Since selenium can antagonize fluoride-induced lipid peroxidation [19] and memory damage [20], we also explored whether sodium selenite (Na_2SeO_3) counteracted the effects of NaF on cultured hair follicles.

Methods

Culture and Treatment of Human Hair Follicles

Scalp skin specimens were obtained from patients who underwent plastic surgery in the department of head–neck surgery. Written informed consent was obtained for the study. Human hair follicles were isolated and cultured as described [21, 22]. Briefly, the intact hair follicle bulb was removed from the subcutaneous fat, under a stereo dissecting microscope, using watchmakers' forceps, by gently gripping the outer root sheath of the follicle in the forceps and pulling the hair follicle from the subcutaneous fat. This results in the isolation of intact hair follicle bulbs without sustaining any visible damage, which is essential for successful maintenance of hair follicles *in vitro*. Isolated anagen follicles were cultured in serum-free Williams E medium (Gibco, USA) supplemented with 2 mmol/mL L-glutamine, 10 ng/mL hydrocortisone, 10 $\mu\text{g}/\text{mL}$ transferrin, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin at 37°C in a humidified 5% (v/v) CO_2 incubator.

The hair follicles were randomly divided into seven groups ($n=12$ for each group) to be left untreated (negative control) or be treated with different concentrations of NaF in the presence or absence of 0.01 mmol/L Na_2SeO_3 : i.e., LF (0.1 mmol/L NaF), MF (1.0 mmol/L NaF), HF (10.0 mmol/L NaF), LFS (0.1 mmol/L NaF+0.01 mmol/L Na_2SeO_3), MFS (1.0 mmol/L NaF+0.01 mmol/L Na_2SeO_3), and HFS (10.0 mmol/L NaF+0.01 mmol/L Na_2SeO_3).

Morphological Observation and Length Measurement

The morphological changes of hair follicles were observed by an inverted microscope (LEICA, Germany). The length of each hair follicle was measured by ocular micrometer every day.

Terminal dUTP Nick End Labeling Assay

The frozen tissue sections were rehydrated and treated by 0.4% pepsin (v/v 0.01 N hydrochloric acid) at room temperature for 30 min. After washing with phosphate-buffered saline, sections were incubated with equilibration buffer for 10–15 min and reaction buffer containing TdT enzyme (Trevigen, Gaithersburg, MD) for 60 min at 37°C. Terminal dUTP nick end labeling (TUNEL)-positive staining was examined by two independent investigators who were blind to this study. Three random fields for each slide were analyzed at $\times 1,000$ magnification using a fluorescent microscope (Olympus, Japan). The apoptotic index was expressed as a percentage of TUNEL-positive cells.

Lipid Peroxidation Assay

Lipid peroxidation (LPO) was assessed using a lipid peroxidation assay kit (Calbiochem, San Diego, CA) to measure the formation of malondialdehyde (MDA), the main decomposition product of peroxides derived from polyunsaturated fatty acids, as described previously [23]. The products generated by the reaction of thiobarbituric acid with MDA were quantified at 532 nm with Bio-Tek Instruments Biotrak-2 microplate reader (Amersham, England). The content of MDA was calculated using the following formula: $\text{MDA}(\text{nmol/mL}) = [\Delta A_{532}(\text{sample}) - \Delta A_{532}(\text{control})] / [\Delta A_{532}(\text{standard}) - \Delta A_{532}(\text{control})] \times 10 \text{ nmol/mL} \times (\text{dilution multiple})$.

Transmission Electron Microscope

The hair follicles were fixed with 4% paraformaldehyde at 4°C overnight and 1% osmium tetroxide at 4°C for 1 h, dehydrated in graded acetones, and flat-embedded in Epon plastic 812 in a cross-sectional orientation. Ultrathin sections (approximately 85 nm in thickness) were stained with 0.25% lead citrate and 5% uranyl acetate in 50% acetone and photographed using a JEOL 100CX transmission electron microscope (Los Angeles, CA, USA).

Statistic Analysis

All data were presented as mean \pm standard deviation. Differences among multiple groups were determined using the one-way analysis of variance (ANOVA) test. Content of MDA was analyzed using the Kruskal–Wallis *H* test. $P < 0.05$ was considered to be statistically significant.

Results

Hair Follicle Growth in Vitro

Figure 1a showed the basic structure of an anagen hair follicle. The dermal papilla (DP), which is composed of specialized fibroblasts located at the bottom of the follicle, is thought to control the number of bulb matrix cells and thus the size of hair [24]. Hair follicle length was calculated every day for 8 days. Exposure to low NaF (0.01 mmol/L) had little influence on hair follicle growth ($P > 0.05$ versus the control; Fig. 1b). After 2 days of culture, the mean length of hair follicles was significantly lower in both middle (1.0 mmol/L) and high

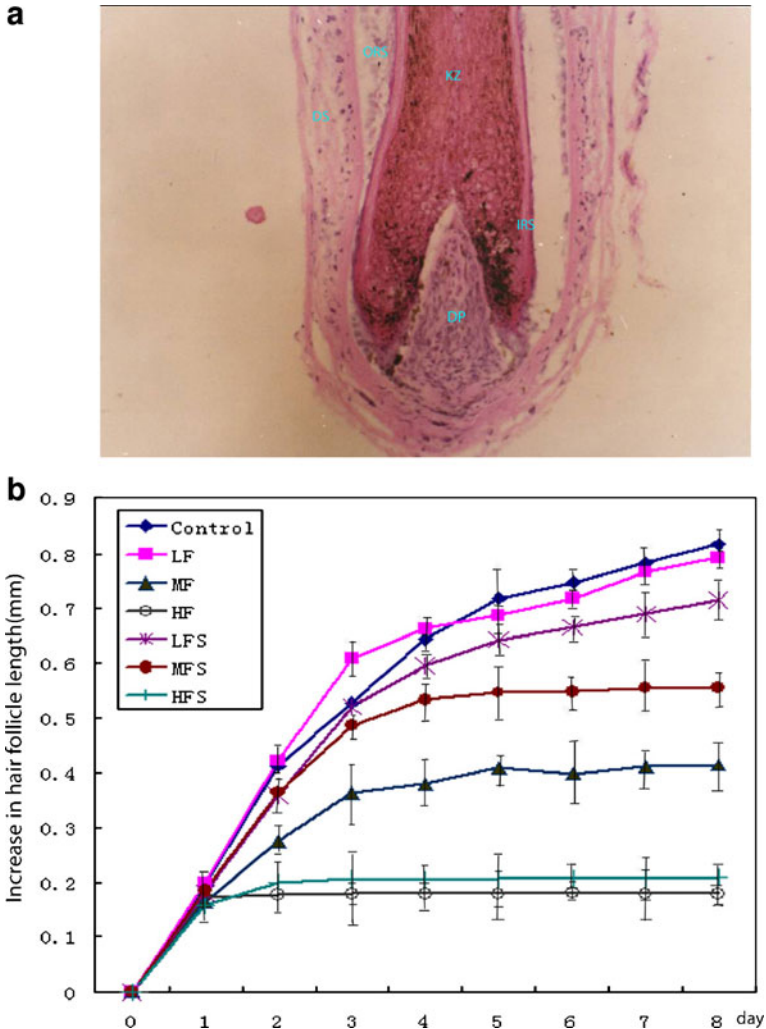


Fig. 1 The morphology and growth of hair follicles *in vitro*. **a** The basic structure of a representative anagen hair follicle. *DP* dermal papilla, *DS* dermal sheath, *IRS* inner root sheath, *KZ* keratogenous zone, *ORS* outer root sheath. **b** The growth curve of hair follicles treated with different concentrations of NaF with or without Na_2SeO_3 . *Control* no treatment; *LF*, *MF*, and *HF* treatment with 0.1, 1.0, and 10.0 mmol/L NaF, respectively; *LFS*, *MFS* and *HFS* treatment with 0.1, 1.0, and 10.0 mmol/L NaF in the presence of 0.01 mmol/L Na_2SeO_3 , respectively

(10.0 mmol/L) NaF treatment groups than that in the control group ($P < 0.05$). Additionally, the presence of Na_2SeO_3 substantially counteracted the inhibitory effects exerted by middle NaF but had little influence on the function of high NaF.

TUNEL Staining

In a representative anagen hair follicle, TUNEL-positive cells were detected in the cuticle, Henle's layer, and Huxley's layer of the inner root sheath (IRS) as well as the keratogenous zone of the upper bulb matrix (Fig. 2a). In a catagen hair follicle, intense TUNEL staining

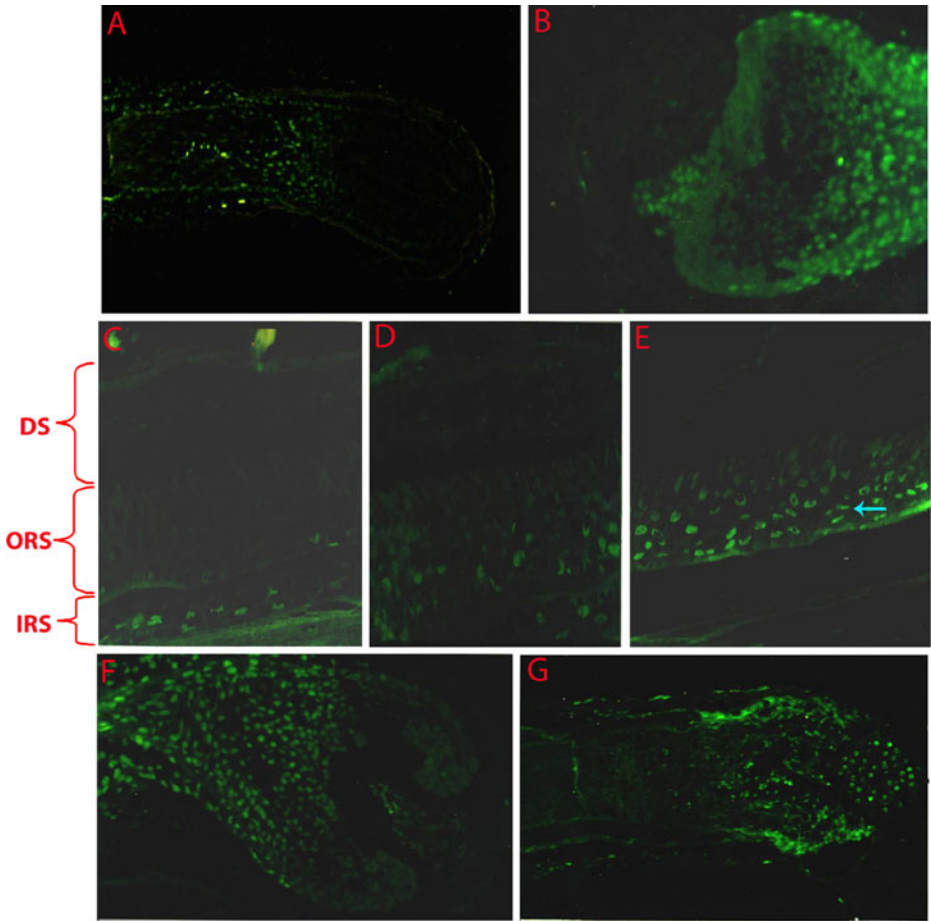


Fig. 2 The apoptotic cells in hair follicle by TUNEL methods under fluorescent microscopy ($\times 40$). **a** The TUNEL-positive cells in a representative anagen hair follicle. **b** The apoptotic cells in a representative telogen hair follicle. **c–g** TUNEL staining of hair follicles treated with indicated concentrations of NaF for 4 days. **c** The TUNEL-positive cells in hair follicles treated with 0.1 mmol/L of NaF. **d, f** The TUNEL-positive cells in the outer root sheath (ORS) and dermal papilla (DP) of hair follicles treated with 1.0 mmol/L of NaF. **e, g** The TUNEL-positive cells distributed in all the layers of hair follicles treated with 10.0 mmol/L of NaF (apoptotic body indicated by arrow)

was detected in the lower bulb matrix cells around the DP and the outer layer cells of the outer root sheath (ORS) (Fig. 2b), suggesting that apoptosis in catagen hair is different from that in anagen hair.

Similar to the untreated hair follicles, those treated with low NaF showed apoptotic cells mainly located in the IRS (Fig. 2c). Notably, middle and high NaF treatment caused a significant increase in apoptosis in the outer layer of the ORS, the dermal sheath (DS), and the lower bulb matrix surrounding the DP (Fig. 2d–g). The apoptotic index of each group was determined and shown in Table 1. Compared to the control group, the treatment with middle or high NaF substantially increased cell apoptosis in the ORS, hair bulb, DS, and DP of hair follicles ($P < 0.05$). However, low NaF had little effect on apoptosis. Interestingly, the presence of Na_2SeO_3 considerably attenuated the inductive effects of

Table 1 The Mean AI of Hair Follicle on the Fifth Day of Different Groups

	Hair bulb below Auber line	ORS	DS and DP
Control	5.1±3.3	24.5±5.1	7.5±4.3
LF	4.4±2.8	23.4±6.0	6.8±5.1
MF	18.9±9.1*	48.7±9.4*	19.4±6.2*
HF	29.3±9.6*	79.5±19.6*	26.4±10.9*
LFS	4.6±2.1	23.8±5.6	8.2±2.9
MFS	11.9±5.6*	33.4±8.8*	15.5±9.5*
HFS	32.5±13.3*	87.0±9.6*	27.9±12.3*

* $P < 0.05$, compared with control group

middle NaF on cell apoptosis ($P < 0.05$). However, the same concentration of Na_2SeO_3 had little influence on the effects exerted by high NaF.

Transmission Electron Microscope

Melanocytes and fibers were found in isolated hair follicle by electron microscope (Fig. 3a). Transmission electron microscope (TEM) analysis of a hair follicle on day 5 of culture revealed the presence of chromatin margination (Fig. 3b) and apoptotic body (Fig. 3c). Similar to the control hair follicle, low NaF-treated hair follicle showed few apoptotic cells (Fig. 3d). However, high NaF treatment resulted in a marked increase in apoptosis, manifested as the presence of vacuoles, disordered cell junctions, and damaged desmosomes (Fig. 3e and f).

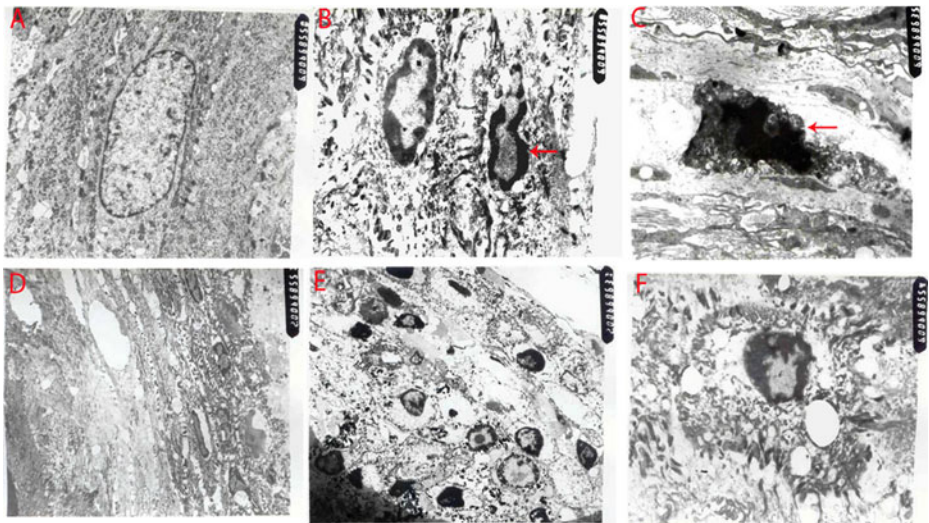


Fig. 3 TEM analysis of hair follicles. **a** A representative anagen hair follicle ($\times 6,000$). **b** Chromatin margination (*arrow*) and **c** apoptotic nucleus (*arrow*) in a hair follicle cultured for 5 days ($\times 6,000$). **d** Few apoptotic cells in hair follicles treated with 0.1 mmol/L of NaF for 5 days ($\times 2,000$). **e, f** Increased apoptosis in hair follicles treated with 10.0 mmol/L of NaF for 5 days ($\times 6,000$)

LPO Assay

MDA is a well-established indicator of lipid peroxidation. Middle and high NaF treatment resulted in a considerable elevation in the MDA levels compared to the control group ($P < 0.05$; Fig. 4), indicating an increased oxidative stress. Low NaF appeared not to affect the content of MDA. Importantly, the presence of Na_2SeO_3 significantly inhibited the oxidative stress induced by middle NaF, as evidenced by lower MDA levels in the MFS group compared to the MF group ($P < 0.05$).

Discussion

Fluoride is an essential trace element, and its insufficiency can cause decayed teeth. However, ingestion of excessive amounts of fluoride leads to skeletal and dental fluorosis. Moreover, high levels of fluoride also lead to an increasing risk of hair loss. Following the investigation of 330 residents who lived in areas with high levels of fluoride, Liu et al. found an alopecia rate of 83.6% for them [16]. To further study how fluoride affected hair growth and apoptosis, we isolated and cultured hair follicles in vitro and treated them with different concentrations of NaF. Although low NaF (0.1 mmol/L) had little effects on hair follicle growth, middle (1.0 mmol/L) and high (10 mmol/L) NaF significantly inhibited hair follicle growth. Moreover, high NaF resulted in an almost complete suppression of hair follicle growth. Besides the inhibition of hair elongation, middle and high NaF also caused an increased apoptosis in the areas where terminal differentiation never takes place, i.e., in the outer layer of the ORS, the epithelial strand, and the lower bulb matrix surrounding the DP. The formation of apoptotic body was confirmed by TEM. This apoptosis triggered by fluoride is similar to that in catagen hair, suggesting that high amounts of fluoride may promote anagen hair to enter into a catagen-like state. Together, these data demonstrate that high levels (e.g., >1.0 mmol/L) of fluoride compromise hair growth and accelerate hair follicle apoptosis, thereby contributing to hair loss.

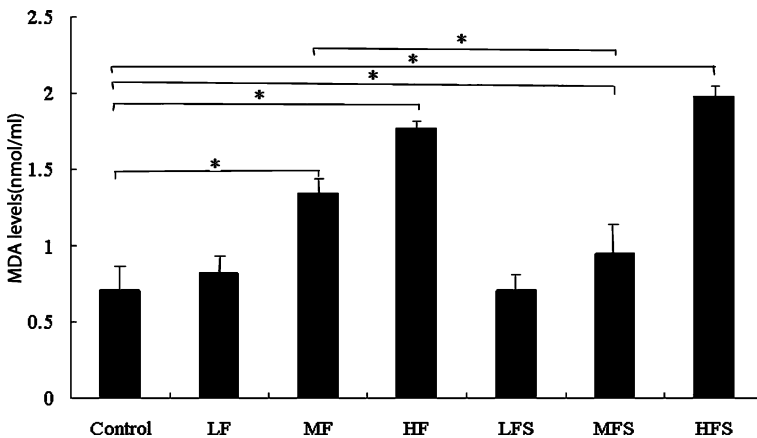


Fig. 4 Analysis of the MDA (nmol/ml) levels. The MDA levels were quantified in hair follicles treated with different concentrations of NaF with or without Na_2SeO_3 . Control no treatment; LF, MF, and HF treatment with 0.1, 1.0, and 10.0 mmol/L NaF, respectively; LFS, MFS and HFS: treatment with 0.1, 1.0, and 10.0 mmol/L NaF in the presence of 0.01 mmol/L Na_2SeO_3 , respectively. * $P < 0.05$ obtained using the one-way ANOVA test

Oxidative stress has been well accepted to be a common trigger of apoptosis [25–27]. Many studies have documented that fluoride can induce oxidative stress and generation of reactive oxygen species [28–31]. Here, we examined the effects of NaF on intracellular oxidative stress. Our results showed that middle and high NaF remarkably increased oxidative stress, as evidenced by an elevation in the formation of MDA, a reliable indicator of lipid peroxidation [32]. Moreover, the MDA level was positively correlated with the apoptotic index of hair follicles; higher MDA level was coupled with higher apoptotic index. These observations suggest that the promotion of hair follicle apoptosis by high fluoride may be mediated at least partially through the induction of oxidative stress. However, the exact molecular mechanism(s) for how fluoride initiates oxidative stress in hair follicles still needs to be further addressed.

Of interest, selenium was found to be able to counteract the effects of middle NaF on hair follicles; i.e., the presence of Na_2SeO_3 considerably diminished the suppression of hair elongation and induction of hair follicle apoptosis by middle NaF. Moreover, the middle-NaF-induced MDA formation was significantly suppressed by Na_2SeO_3 treatment, suggesting that selenium could protect cells from oxidative stress and consequent apoptosis. The protective role of selenium in fluorosis has also been demonstrated by several earlier studies [33, 34]. Chen et al. reported that high selenium increases the expression of heat shock protein (HSP70) and the activities of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, and catalase, which subsequently contribute to the prevention of oxidative damage in Fincoal-type fluorosis [33].

In conclusion, our data demonstrate that high amounts of fluoride can inhibit hair follicle elongation and induce intracellular oxidative stress and cell apoptosis *in vitro*. The presence of selenium is able to antagonize the toxicity of high levels of fluoride, which may be partially mediated through the suppression of excessive oxidative stress. Nevertheless, more studies regarding the exact mechanism by which selenite improves the F-induced apoptosis in hair follicle are required.

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References

1. Bartlett JD, Dwyer SE, Beniash E, Skobe Z, Payne-Ferreira TL (2005) Fluorosis: a new model and new insights. *J Dent Res* 84:832–836
2. Reddy GB, Arjun LK, Reddy PY, Rao GS, Balakrishna N, Srivalli I (2003) Antioxidant defense system and lipid peroxidation in patients with skeletal fluorosis and in fluoride-intoxicated rabbits. *Toxicol Sci* 72:363–368
3. Zager RA, Iwata M (1997) Inorganic fluoride. Divergent effects on human proximal tubular cell viability. *Am J Pathol* 150:735–745
4. Ghosh D, Das Sarkar S, Maiti R, Jana D, Das UB (2002) Testicular toxicity in sodium fluoride treated rats: association with oxidative stress. *Reprod Toxicol* 16:385–390
5. Levy SM (2003) An update on fluorides and fluorosis. *J Can Dent Assoc* 69:286–291
6. Zeiger E, Shelby MD, Witt KL (1993) Genetic toxicity of fluoride. *Environ Mol Mutagen* 21:309–318
7. Anna M, Anna MM, Wojciech M, Iwona S, Boguslaw M (2001) NaF-induced apoptosis in human bone marrow and cord blood CD34 positive cells. *Fluoride* 34:258–263
8. Anuradha CD, Kanno S, 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 Biol Med* 31:367–373
9. He LF, Chen JG (2006) DNA damage, apoptosis and cell cycle changes induced by fluoride in rat oral mucosal cells and hepatocytes. *World J Gastroenterol* 12:1144–1148
10. Wang AG, Xia T, Chu QL, Zhang M, Liu F, Chen XM, Yang KD (2004) Effects of fluoride on lipid peroxidation, DNA damage and apoptosis in human embryo hepatocytes. *Biomed Environ Sci* 17:217–222

11. Refsnes M, Schwarze PE, Holme JA, Låg M (2003) Fluoride-induced apoptosis in human epithelial lung cells (A549 cells): role of different G protein-linked signal systems. *Hum Exp Toxicol* 22:111–123
12. Thrane EV, Refsnes M, Thoresen GH, Låg M, Schwarze PE (2001) Fluoride-induced apoptosis in epithelial lung cells involves activation of MAP kinases p38 and possibly JNK. *Toxicol Sci* 61:83–91
13. Sternweis PC, Gilman AG (1982) Aluminum: a requirement for activation of the regulatory component of adenylate cyclase by fluoride. *Proc Natl Acad Sci USA* 79:4888–4891
14. Heidenreich O, Neining A, Schrott G, Zinck R, Cahill MA, Engel K, Kotlyarov A, Kraft R, Kostka S, Gaestel M, Nordheim A (1999) MAPKAP kinase-2 phosphorylates serum response factor in vitro and in vivo. *J Biol Chem* 274:14434–14443
15. Wergedal JE, Lau KH (1992) Human bone cells contain a fluoride sensitive acid phosphatase: evidence that this enzyme functions at neutral pH as a phosphotyrosyl protein phosphatase. *Clin Biochem* 25:47–53
16. Liu J, Wu YH, Li Y (1993) The alopecia caused by long-term drinking of high fluoride water. *Chin J Endem* 12:168–169
17. Nakayama F, Hagiwara A, Kimura M, Akashi M, Imamura T (2009) Evaluation of radiation-induced hair follicle apoptosis in mice and the preventive effects of fibroblast growth factor-1. *Exp Dermatol* 18:889–892
18. Sharov AA, Li GZ, Palkina TN, Sharova TY, Gilchrest BA, Botchkarev VA (2003) Fas and c-kit are involved in the control of hair follicle melanocyte apoptosis and migration in chemotherapy-induced hair loss. *J Invest Dermatol* 120:27–35
19. Yang K, Chen J, Wang G, Liu S (1998) Study on the antagonistic action of selenite on fluoride-induced lipid peroxidation and on the changes of trace elements in rats. *Wei Sheng Yan Jiu* 27:201–204
20. Zhang Z, Shen X, Xu X (2001) Effects of selenium on the damage of learning-memory ability of mice induced by fluoride. *Wei Sheng Yan Jiu* 30:144–146
21. Yang ZQ, Tu JB, Yao TH, Zhao XG (2004) Effects of NGF and estrogens on human hair follicle in vitro. *Chin J Plast Surg* 20:48–50
22. Tsutomu S, Masashi O, Jun S, Tadahito T, Toshihiko H (1998) Analysis of apoptotic cell death in human hair follicles in vivo and in vitro. *J Invest Dermatol* 111:948–954
23. Guney M, Oral B, Take G, Giray SG, Mungan T (2007) Effect of fluoride intoxication on endometrial apoptosis and lipid peroxidation in rats: role of vitamins E and C. *Toxicology* 231:215–223
24. Paus R, Cotsarelis G (1999) The biology of hair follicles. *N Engl J Med* 341:491–497
25. Zhan AX, Wang M, Xu ZR, Li WF, Li JX (2006) Evaluation of caspase-dependent apoptosis during fluoride-induced liver lesion in pigs. *Arch Toxicol* 80:74–80
26. Ozben T (2007) Oxidative stress and apoptosis: impact on cancer therapy. *J Pharm Sci* 96:2181–2196
27. Shivrajashankara YM, Shivashankara AR (2002) Brain lipid peroxidation and antioxidant systems of young rats in chronic fluoride intoxication. *Fluoride* 35:197–203
28. Zhang M, Wang A, He W, He P, Xu B, Xia T, Chen X, Yang K (2007) Effects of fluoride on the expression of NCAM, oxidative stress, and apoptosis in primary cultured hippocampal neurons. *Toxicology* 236:208–216
29. Guney M, Oral B, Demirin H, Karahan N, Mungan T, Delibas N (2007) Protective effects of vitamins C and E against endometrial damage and oxidative stress in fluoride intoxication. *Clin Exp Pharmacol Physiol* 34:467–474
30. Chouhan S, Flora SJ (2008) Effects of fluoride on the tissue oxidative stress and apoptosis in rats: biochemical assays supported by IR spectroscopy data. *Toxicology* 254:61–67
31. Shanthakumari D, Srinivasalu S, Subramanian S (2004) Effect of fluoride intoxication on lipid peroxidation and antioxidant status in experimental rats. *Toxicology* 204:219–222
32. Serbecic N, Beutelspacher SC (2005) Anti-oxidative vitamins prevent lipid-peroxidation and apoptosis in corneal endothelial cells. *Cell Tissue Res* 320:465–475
33. Chen Q, Wang Z, Xiong Y, Xue W, Kao X, Gao Y, Muhammad N, Song D (2009) Selenium increases expression of HSP70 and antioxidant enzymes to lessen oxidative damage in Fincoal-type fluorosis. *J Toxicol Sci* 34:399–405
34. Yu RA, Xia T, Wang AG, Chen XM (2006) Effects of selenium and zinc on renal oxidative stress and apoptosis induced by fluoride in rats. *Biomed Environ Sci* 19:439–444