

INDUCTION OF APOPTOSIS IN HUMAN GINGIVAL EPITHELIAL CELLS BY SODIUM FLUORIDE

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SUMMARY: Fluoride (F) causes not only chronic but also short-term toxic effects, such as apoptosis, in several kinds of human cells. Although human gingival epithelial cells (HGECs) are frequently exposed to topical applications of F to teeth, the apoptosis effects of F on HGECs does not appear to have been assessed. In this study we determined the extent of F-induced apoptosis in HGECs after 24-hr incubation in the presence of various concentrations of NaF. Lactate dehydrogenase activities and reactive oxygen species increased in some of the groups depending on the F concentration. Early apoptosis and caspase-3 activity were significantly increased at 150 and 200 mg/L NaF. An increment of DNA fragmentation was found at 150 mg/L NaF. At 150 and 200 mg/L NaF, caspase-3, -8, and -9 activities, mitochondrial membrane depolarization, and release of cytochrome c were significantly increased, but the expression of Bcl-2 was not suppressed. It was concluded that 150 to 200 mg/L NaF caused apoptosis in HGECs through both mitochondrial-mediated and death ligand–receptor pathways.

Keywords: Bcl-2 expression; Caspase; Fluoride-induced apoptosis; Human gingival epithelial cells; Mitochondria; Reactive oxygen species.

INTRODUCTION

Prolonged excessive intake of fluoride (F) leads to skeletal and/or dental fluorosis.¹ Topical application of F in high concentrations is frequently used for preventing dental caries. Professionally applied F products contain concentrations as high as 9,000 to 22,600 mgF/L, whereas over-the-counter toothpastes and mouthrinses contain 900 to 1,200 mgF/L and 226 mgF/L, respectively. It is important to note, however, that toothpastes and mouthrinses are used far more frequently than professionally applied gels.

Recently, it was reported that routine use of toothpaste may lead to skeletal fluorosis.² Furthermore, it has been shown that F causes certain short-term effects,³⁻⁵ such as apoptosis in several kinds of human cells, including epithelial lung cells.⁵ Once applied, F is easily and rapidly absorbed by and remains in the oral mucosa.^{6,7} Given that apoptosis of gingival crevicular epithelial cells has been shown to be one cause of periodontal pathology⁸, gingivitis could conceivably be a response to F effects. However, it has not yet been determined if F causes apoptosis in human gingival epithelial cells (HGECs). In this work the effect of F on apoptosis in HGECs has been examined. We found that F at higher concentrations than in saliva induced apoptosis in HGECs and activated both mitochondrial and death-ligand receptor pathways.

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MATERIALS AND METHODS

Assessment of cell death and apoptosis: Cells from HGEC, Ca9-22 (Human Science Research Resources Bank, Osaka, Japan), were incubated in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco™, Invitrogen, USA) supplemented with fetal bovine serum (FBS) (HyClone®, Perbio, USA) in an atmosphere of 5% CO₂ in air. After 24 hr, the cells were placed in fresh medium consisting of DMEM with 10% FBS containing varying concentrations of NaF (0, 10, 50, 100, 150, 200, and 250 mg/L) for a second 24-hr period.

After 24 hr the number of dead cells was assessed by staining them with Trypan blue solution in a modified Neubauer hemocytometer. The amount of lactate dehydrogenase (LDH) released from the cytosol into the supernatant was measured using a Cytotoxicity Detection Kit (Roche Applied Science, Roche Diagnostics, Germany).⁹ Apoptosis was evaluated with a dual-color flow cytometer (Guava® EasyCyte™, GE Healthcare Bioscience, Tokyo, Japan) using Annexin-V and 7-Amino Actinomycin D (7-AAD) (Guava PCA Nexin™; GE Healthcare Bioscience).¹⁰ Cytoplasmic histone-associated DNA fragmentation in apoptotic cells also was detected using a cell death detection ELISA (Roche Diagnostics).¹¹

Apoptotic pathway: Oxidative stress markers, such as reactive oxygen species (ROS), were measured by means of a fluorescent marker (MitoSOX™, Invitrogen).¹² Mitochondrial membrane potential was measured by MitoPotential™ (GE Healthcare Bioscience).¹³ Cytochrome *c* released into cytosol was determined by Cytochrome *c* ELISA (Calbiochem®, San Diego, CA). Bcl-2, one of the factors that works as an inhibitor of apoptosis, was also measured by ELISA (Bcl-2 ELISA Kit, Calbiochem®). Caspase-3 was measured using Human Active Caspase-3 Immunoassay (R&D Systems, USA).¹⁴ In the groups with 150 and 200 mg/L NaF as well as the control group, caspase-8 and -9 were measured by flow cytometry using fluorescein isothiocyanate (FITC) fluorescent markers (Caspase-8 and Caspase-9 Detection Kits, Calbiochem®).

Statistical analysis: One-way analysis of variance (ANOVA) tests using the Bonferroni method were performed to analyze differences between each group (HALWIN Version 6.24, Gendaisugakusya, Japan). Correlation coefficients between NaF concentrations and each variable were also determined to examine dose-dependent changes (HALWIN Version 6.24). P values of less than 0.05 were considered statistically significant. The data were expressed as mean ± SD.

RESULTS

As seen in Figure 1, both apoptosis or necrosis/late apoptosis increased in a dose-dependent manner with NaF ($p < 0.01$, respectively), whereas the viable cell numbers similarly decreased ($p < 0.01$). LDH release also increased with NaF dose ($p < 0.01$) (Figure 2A), and the number of viable cells detected by trypan blue staining was significantly reduced at 50, 150 and 250 mg/L NaF groups ($p < 0.05$) (Figure 2B). The rate of DNA fragmentation increased significantly in the 150 and 200 mg/L NaF groups compared to 0 mg/L NaF group ($p < 0.01$) (Figure 2C). Moreover, DNA fragmentation increased dose-dependently among the 0 to 200

mg/L NaF groups ($p < 0.05$) (Figure 2C). Because the 150 and 200 mg/L NaF groups showed lower incidences of necrosis/late apoptosis compared to the 250 mg/L group ($p < 0.01$, respectively) (Figure 1B), ROS values were assessed at both concentrations of NaF, and significant increments were found ($p < 0.01$, respectively) (Table 1).

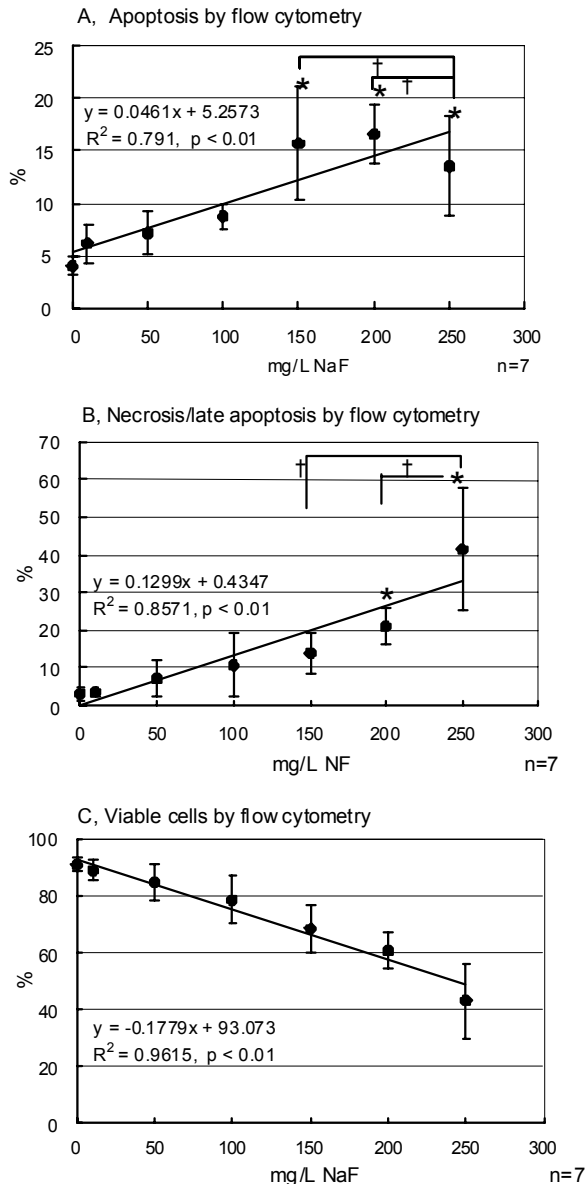


Figure 1. Statistically significant differences between the test and control (0 mg/L) groups are shown by * $p < 0.05$; † $p < 0.01$, respectively). Both apoptosis or necrosis/late apoptosis increased NaF dose-dependently, whereas the viable cell numbers decreased dose-dependently (Figure 1A, B, and C). In Figure 1A, the percentage of apoptotic cells in 250 mg/L group was significantly decreased in comparison to 150 and 200 mg/L groups († $p < 0.01$). In Fig. 1B, the percentage of necrosis/late apoptotic cells in 250 mg/L group was significantly increased in comparison to 150 and 200 mg/L groups († $p < 0.01$).

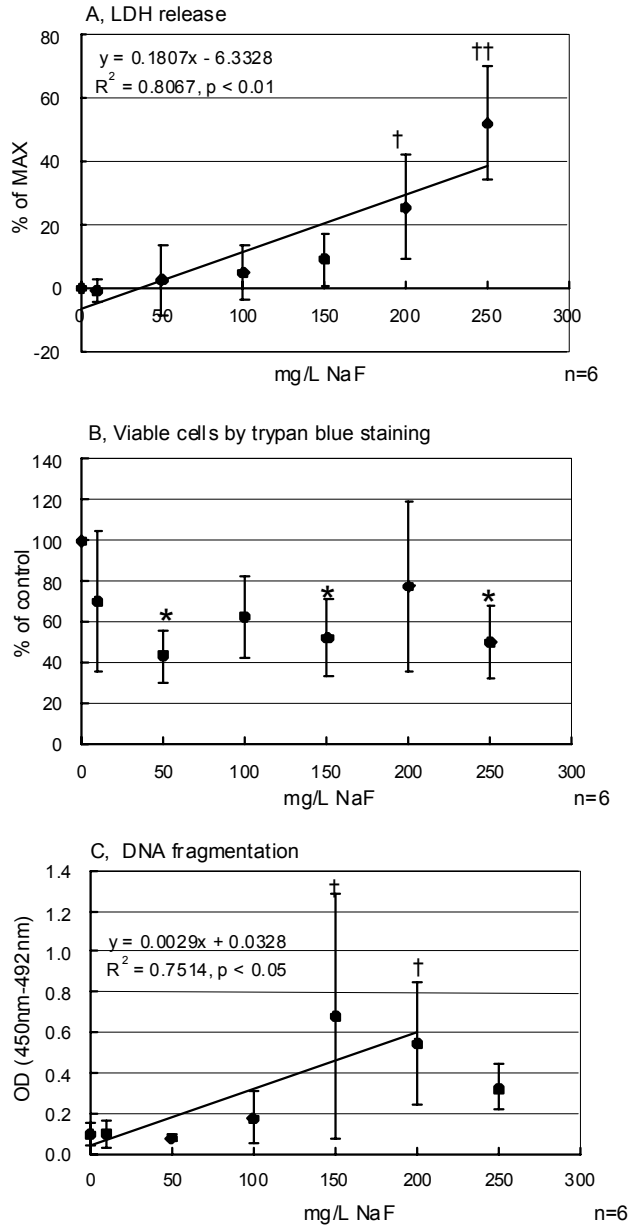


Figure 2. Statistically significant differences between the test and control (0 mg/L) groups are shown by *p<0.05, †p<0.01, ‡p<0.001, respectively). LDH release increased NaF dose-dependently (Figure 2A). However, no correlation between the number of viable cells and NaF concentrations were found (Figure 2B). In Figure 2C, significant correlation was found only among 0 to 200 mg/L (p<0.05).

Mitochondrial membrane depolarization also increased at these concentrations (p < 0.01, respectively), as did cytochrome c release (p < 0.05, respectively) (Table 1). Caspase-3 was strongly activated in the 150 mg/L NaF group (p<0.01), while caspase-8 and -9 also increased in the 150 and 200 mg/L NaF groups (p < 0.01,

respectively) (Table 2). However, expression of Bcl-2 was not suppressed (Table 2).

Table 1. Oxidative stress, mitochondrial membrane potential and cytochrome c

Sample (Concentration of NaF)	ROS (%)		Depolarized ^a (%)		Cytochrome ^a (OD450-570nm)	
0 mg/L(control)	14.0 ± 2.0	2.0	21.1 ± 3.3	3.3	0.1 ± 0.05	0.05
150 mg/L	33.9 ± 4.8*	4.8*	49.2 ± 12.6*	12.6*	0.3 ± 0.05 [†]	0.05 [†]
200 mg/L	51.0 ± 15.2*	15.2*	66.2 ± 15.7*	15.7*	0.3 ± 0.11 [†]	0.11 [†]

(Values are mean ± SD, n=7, ^an=5); *p<0.01, [†]p<0.05.

Table 2. Caspase -3, -8, -9 activities and Bcl-2 levels

Sample (Concentration of NaF)	Caspase-3 (OD 450-570 nm)		Caspase-8 (%)		Caspase-9 (%)		Bcl-2 (U/mL)	
0 mg/L(control)	0.01 ± 0.00	0.00	7.7 ± 1.1	1.1	7.0 ± 1.4	1.4	8.3 ± 6.4	6.4
150 mg/L	0.31 ± 0.06*	0.06*	42.0 ± 11.4*	11.4*	37.2 ± 14.7*	14.7*	6.8 ± 2.2	2.2
200 mg/L	0.29 ± 0.08 *	0.08 *	46.2 ± 8.6*	8.6*	38.7 ± 16.3*	16.3*	6.0 ± 3.3	3.3

(Values are mean ± SD, n=5); *p<0.01.

DISCUSSION

In this study, we sought to determine the concentration of NaF that would trigger apoptosis in HGECS. We found that apoptosis significantly increased at concentrations of 150, 200, and 250 mg/L NaF, but especially at 150 mg/L NaF due to the increased fragmentation of DNA caused by apoptosis. The ratio of necrotic/late apoptotic cells and the LDH release increased at 250 mg/L NaF; this concentration of NaF turned “apoptotic effects” into “necrotic effects” in HGECS. The concentrations of F used in this study were higher than the levels normally found in blood or saliva. However, various F treatments such as topical application conventionally use a much range of F concentrations, ranging from 900 to 22,600 mg/L, so it is conceivable that HGECS exposure to fluoride can occur at much higher concentrations than that in saliva, albeit for short periods of time.

Our observations are comparable to the results obtained by Jeng et al., who reported that 4 mM NaF (75.6 mg/L) or higher showed cytotoxicity to human oral mucosal fibroblasts.⁵ While the concentration of NaF was lower than that used in our study, it is conceivable that HGECS may develop greater resistance to NaF toxicity than fibroblasts.

In this study, we also determined the apoptotic pathway. The increases in caspase-3, -8, -9, ROS, mitochondrial membrane depolarization, and cytochrome *c* imply that one of the pathways of apoptosis caused by NaF is mitochondria mediated. Nevertheless, Bcl-2 expression was not suppressed, although recently Lee et al. reported Bcl-2 suppression in apoptosis caused by NaF in gingival fibroblasts.¹⁵ We therefore postulate that caspase-3 was directly activated by caspase-8 as well as caspase-9,¹⁶ which is involved in a death ligand-receptor pathway. Furthermore, because Bcl-2 was not suppressed, the apoptotic process caused by NaF may be independent from the p53 pathway.¹⁷ Future work will examine this possibility in greater detail by studying the expression of the Bax family and/or Fas induced apoptosis.

Conditions in which 150 mg/L NaF or greater is maintained for 24 hr may not occur in oral tissues when the F topical applications, except a F-varnish, are carried out. If a F-varnish comes in contact with the gingiva, it might stay on the gingiva for 24 hours because of its strong adhesive glue-like properties. Nevertheless, our results suggest that F might involve periodontal pathologic changes, since F sensitivity is different within different cells or different ages of the subjects,^{18,19} and as very low concentration of NaF (5 mg/L) caused apoptosis in osteoblasts, which also play an important role in periodontal pathology.²⁰

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