Original Article / Özgün Araştırma

# Effect of Pediatric Toothpastes Based on 500 to 1450 ppm Sodium Fluoride and Amine Fluoride with Different Detergents on Oxidative Stress and Cell Viability

## *500-1450 ppm Sodyum Florür ve Amin Florür Bazlı, Farklı Deterjan İçerikli Pediatrik Diş Macunlarının Oksidatif Stres ve Hücre Canlılığı Üzerine Etkisi*

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## Abstract

**Objective:** This study evaluated the effect of sodium fluoride and amine fluoride pediatric toothpastes with different detergents on oxidative stress and cell viability.
**Materials and Methods:** Pediatric toothpastes containing sodium fluoride (Sensodyne Pronamel-SP; Ipana Kids-IK; Signal Kids-SK; Oral B-OB) and amine fluoride (Elmex-EL; Elmex Junior-EJ) were obtained. While SP, IK, EL contained cocamidopropyl betaine detergent, SK, OB sodium lauryl sulfate and EJ had olaflur detergents. Toothpaste samples were diluted with medium at different predetermined concentrations. L929 fibroblast cells were exposed to toothpaste extracts at 37 °C for 2 min. Cell viability was tested using methyl tetrazolium test, while the formation of reactive oxygen species (ROS) was detected using flow cytometry.
**Results:** The decreasing concentration ratio from 1:1 to 1:32 decreased the cytotoxicity ($p<0.05$) except EL. The cytotoxicity of original pediatric toothpaste extracts (1:1) were significantly different, compared to the negative control group ($p<0.05$), except IK and EL ($p>0.05$). All toothpastes tested increased the number of ROS in L929 cells ($p<0.05$).
**Conclusion:** Pediatric toothpastes containing sodium fluoride with sodium lauryl sulfate presented more cytotoxic effect.

## Öz

**Amaç:** Bu çalışmada, sodyum florür ve amin florür içeren pediatrik diş macunlarının farklı deterjan içeriklerinin oksidatif stres ve hücre canlılığı üzerine etkisi değerlendirilmiştir.
**Gereç ve Yöntemler:** Sodyum florür (Sensodyne Pronamel-SP; Ipana Kids-IK; Signal Kids-SK; Oral B-OB) ve amin florür (Elmex-EL; Elmex Junior-EJ) içeren pediatrik diş macunları kullanıldı. SP, IK, EL kokamidopropil betain deterjan içerirken, SK, OB sodyum lauril sülfat ve EJ olaflur deterjan içerikliydi. Diş macunu numuneleri önceden belirlenmiş farklı konsantrasyonlarda medyum ile seyreltildi ve L929 fibroblast hücreleri, diş macunu ekstraktlarına 37 °C'de 2 dakika maruz bırakıldı.

Hücre canlılığı metil tetrazolyum testi kullanılarak test edilirken, reaktif oksijen türlerinin oluşumu (ROS) flow sitometrisi kullanılarak tespit edildi.
**Bulgular:** 1:1'den 1:32'ye azalan konsantrasyon oranı, EL haricinde sitotoksisiteyi azalttı ($p<0,05$). Orijinal pediatrik diş macunu ekstraktlarının (1:1) sitotoksisitesi, negatif kontrol grubuna ($p<0,05$) kıyasla, IK ve EL haricinde ($p>0,05$) önemli ölçüde farklıydı. Test edilen tüm diş macunları, L929 hücrelerindeki ROS miktarını artırdı ($p<0,05$).
**Sonuç:** Sodyum florür içeren ve sodyum lauril sülfat içeren pediatrik diş macunları daha fazla sitotoksik etki göstermiştir.

## Introduction

Tooth brushing using a toothpaste is an important oral hygiene practice that is beneficial for dental and gingival health, aids dental plaque removal, and prevents dental caries, especially in children (1). Toothpastes generally comprise abrasive, surface-active, moisturizing, gelling and/or binding, flavoring, preservative, and staining agents; sweeteners; and fluorides (2). The ideal toothpaste for children should comprise ingredients compatible with fluoride delivery to ensure adequate fluoride availability, minimal abrasivity, and consequently, a pleasant brushing experience (1,2).

The efficacy of fluoride-containing toothpastes depends on their fluoride concentration, frequency of use, volume of toothpaste used, and rinsing habits after brushing (3). Children between the ages of two-four and five-seven years swallow 34% and 13% toothpastes, respectively, while older children and adults ingest only 6% (4). Therefore, dentists should be aware of the potential adverse effects of toothpastes and fluorides and counsel patients and/or parents accordingly. Since many decades, prescription of fluoride toothpastes in carefully selected patients has been effective in preventing dental caries (5). However, some toothpaste components such as detergents may be harmful because of their foaming ability (6). Sodium lauryl sulfate (SLS), which is the most commonly used detergent in mouthwashes and toothpastes, is capable of denaturing proteins (7). Short-term use of toothpastes with ≤2% SLS content is considered harmless (5). Cocamidopropyl betaine (CAPB) is another commonly used detergent in toothpastes. CAPB-containing toothpastes are believed to be less irritating and can alleviate symptoms of dry mouth (8). Amine fluoride (AF) in toothpastes can also act as a detergent and affects L929 fibroblast cells (9).

Limited information is available in the literature on commercially available pediatric toothpastes for children ≤12 years old regarding the cytotoxic effects on gingival fibroblasts and L929 mouse fibroblasts and potential for causing oral squamous cell carcinoma (9,10).

Reactive oxygen species (ROS) are generated during mitochondrial oxidative metabolism. Oxidative stress refers to the instability caused by excess ROS or oxidant production. Various chemicals can disrupt the stable cellular redox balance, resulting in increased levels of ROS and subsequent cell death through apoptosis (11).

Therefore, in this study, we evaluated the effects of sodium fluoride- and AF-based pediatric toothpastes containing different detergents on oxidative stress and cell viability. The null hypothesis was that the type of detergent in sodium fluoride- and AF-based toothpastes in different dilutions would not have a significant effect on oxidative stress and cell viability of L929 murine fibroblast cells.

## Materials and Methods

This study was approved by the Selçuk University Faculty of Dentistry Ethics Committee (decision no: 2015/01, date: 08.10.2015). Commercial pediatric toothpastes containing sodium fluoride (Sensodyne Pronamel-(SP), 1450 ppm; Ipana Kids-IK, 500 ppm; Signal Kids-(SK), 500 ppm; Oral B-OB, 500 ppm) and AF (Elmex-EL, 500 ppm; Elmex Junior-EJ, 1400 ppm) were obtained. SP, IK, and EL comprised CAPB; SK and OB contained SLS, and EJ comprised olaflur (Table 1).

The toothpastes were diluted in a medium (50 w/v%), homogenized using a vortex (WisemixVM-10; Daihan Scientific Co., Ltd., Seoul, South Korea), centrifuged (Hettich 320R Centrifuge, maximum speed: 15000 rpm, Germany), filtered, and immediately used in experiments. The original extracts (1:1) were diluted at 1:2, 1:4, 1:8, 1:16, and 1:32 in the medium.

### Cytotoxicity Testing

L929 cells were seeded on each well of a 96-well plate at a density of $2\times10^4$ cells/well and incubated at 37 °C for 24h cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine

**Table 1. Chemical composition of toothpastes used in the present study**

| Group | Detergent content | Fluoride content | Composition |
|---|---|---|---|
| SP | Cocamidopropyl betaine | Sodium fluoride 1450 ppm | Aqua, sorbitol, hydrated silica, glycerin, PEG-6, cocamidopropyl betaine, xanthan gum, aroma, sodium fluoride, sodium saccharin, sucralose, titanium dioxide, sodium hydroxide, limonene |
| IK | Cocamidopropyl betaine | Sodium fluoride 500 ppm | Sorbitol, aqua, hydrated silica, aroma, cocamidopropyl betaine, benzyl alcohol, carbomer, mica, sodium chloride, sodium fluoride, sodium phosphate, sodium saccharin, trisodium phosphate, xanthan gum, Cl16255, Cl77891 |
| SK | Sodium lauryl sulfate | Sodium fluoride 500 ppm | Sorbitol, aqua, hydrated silica, PEG-32, sodium lauryl sulfate, cellulose gum, sodium saccharin, sodium fluoride, mica, calcium gluconate, tocopheryl acetate, glycerin, limonene, phenylcarbinol, Cl12490, Cl77891 |
| OB | Sodium lauryl sulfate | Sodium fluoride 500 ppm | Aqua, sorbitol, hydrated silica, sodium lauryl sulfate, cellulose gum, aroma, sodium saccharin, carbomer, trisodium phosphate, sodium fluoride, limonene, Cl42090 |
| EL | Cocamidopropyl betaine, olaflur | Aminfluorid 500 ppm | Aqua, sorbitol, hydrated silica, hydroxyethylcellulose, titaniumdioxide, cocamidopropyl betaine, olaflur, aroma, limonene, sodium saccharin, hydrochloric acid |
| EJ | Olaflur | Aminfluorid 1400 ppm | Aqua, hydrated silica, sorbitol, olaflur, hydroxyethylcellulose, aroma, limonene, PEG-40 hydrogenated, castoroil, titaniumdioxide, saccharin, hydrochloric acid |

SP: Sensodyne pronamel, IK: Ipana kids, SK: Signal kids, OB: Oral B, EL: Elmex, EJ: Elmex junior

serum, penicillin (150 IU/mL), and streptomycin (150 µg/mL). The cells were then exposed to 100 µL of toothpaste extracts, while the cell culture medium was used as a negative control. After 24h, cell survival was evaluated by assessing enzyme activity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The cells were exposed to 200 µL MTT solution (0.5 mg/mL) for 2h at 37 °C. The blue formazan precipitate was then dissolved by shaking for 30 min at room temperature in 200 µL dimethyl sulfoxide. Absorption was assessed using a spectrophotometer at 540 nm (BioTek Epoch, BioTek Instruments, Inc., Winooski, VT, USA). Three independent experiments were performed in four wells (n=12 per group). Optical density readings from cultures exposed to extracts were compared with untreated control cells (100%).

**Statistical Analysis**

The Shapiro-Wilk test was used to test the normal distribution of data. One-Way analysis of variance and Tukey's post-hoc test were used to compare cell survival ($\alpha$=0.05).

**Reactive Oxygen Species Measurements**

ROS levels were monitored using the oxidation-sensitive fluorescent probe 2'7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Invitrogen Molecular Probes, Karlsruhe, Germany). Dichlorofluorescein, a non-fluorescent compound, is formed as a result of intracellular esterase activity. When oxidized, it converts to dichlorodihydrofluorescein diacetate (DCF-DA) and becomes fluorescent. L929 cells were seeded on each well of a 6-well plate at a density of $2\times10^5$ cells/well and incubated at 37 °C for 24h. Thereafter, the cells were exposed to toothpaste extracts for 2 min, with cells exposed to triethyleneglycol dimethacrylate (1 mmol/L TEGDMA, Sigma-Aldrich, Taufkirchen, Germany) as the positive control. Subsequently, L929 cells were incubated with 10 µM H2DCF-DA at 37 °C for 30 min. The cells were seperated using 0.05% trypsin/ethylenediamine tetraacetic acid and aggregated by centrifugation and washed with PBS. DCF fluorescence values were determined using flow cytometry (FACS Aria III, BD Biosciences, San Jose, CA, USA) at an emission wavelength of 519 nm and an excitation wavelength of 488 nm (FITC-1). The mean fluorescence values were obtained using histogram statistics (FACSDiva v6.1.3, BD Biosciences). Two wells were used in two independent experiments (n=4 per group), and the results were averaged. All further analyses were performed using the averaged values. Differences in ROS levels were statistically evaluated

**Table 2. Effect of the original extracts and concentrations of pediatric toothpastes on the viability of L929 cells**

| Test groups | Toothpaste concentration (Mean ± SD) | | | | | |
|---|---|---|---|---|---|---|
| | 1:1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 |
| SP | 63.9±10.9$^{aA}$ | 81.1±7.9$^{A}$ | 82±5.6$^{A}$ | 103.4±51$^{B}$ | 107.6±4.3$^{B}$ | 111.9±10.2$^{B}$ |
| IK | 84.3±14.2 | 84.1±10.8 | 89.7±15.3 | 88.5±24.2 | 90.9±14.6 | 88.7±27.7 |
| SK | 7.6±1.1$^{a}$ | 8.0±0.6$^{a}$ | 8.3±1.1$^{a}$ | 8.4±1.5$^{a}$ | 10.8±1.9$^{a}$ | 26.2±5$^{a}$ |
| OB | 10.8±1.3$^{aA}$ | 10.1±0.8$^{aA}$ | 17.1±3.1$^{aA}$ | 59.4±11.7$^{aB}$ | 93.2±8.4$^{B}$ | 96.9±4.4$^{B}$ |
| EL | 102.7±4.0 | 102.8±11.3 | 96.9±10.0 | 104.2±5.5 | 85.0±7.4 | 87.1±4.7 |
| EJ | 18.0±1.6$^{aA}$ | 40.4±7.2$^{a}$ | 52.1±8.8$^{aB}$ | 56.2±9.9$^{aB}$ | 61.1±18$^{aB}$ | 85.4±9.4$^{B}$ |

SP: Sensodyne pronamel, IK: Ipana kids, SK: Signal kids, OB: Oral B, EL: Elmex, EJ: Elmex junior, SD: Standard deviation. Two min post-exposure was evaluated using the standard MTT assay. $^{A,B}$: Different upper-case superscript letters indicate a statistically significant (p<0.01), $^{a,b}$: Different lower-case superscript letters indicate a statistically significant (p<0.01)

**Table 3. Generation of ROS in L929 cells after exposure to pediatric toothpastes**

| | Mean | Standard deviation |
|---|---|---|
| Negative control | 1.00 | 0.00 |
| Positive control | 8.92 | 0.24 |
| SP | 5.29$^{n,p,a}$ | 0.49 |
| IK | 5.23$^{n,p,a}$ | 0.24 |
| SK | 2.90$^{n,p,b}$ | 0.31 |
| OB | 2.42$^{n,p,b}$ | 0.06 |
| EL | 1.95$^{n,p,b}$ | 0.03 |
| EJ | 8.57$^{n,p,b}$ | 0.01 |

SP: Sensodyne pronamel, IK: Ipana kids, SK: Signal kids, OB: Oral B, EL: Elmex, EJ: Elmex junior, p: Significant difference from positive control group, n: Significant difference from negative control group, $^{A,B}$: Different upper-case superscript letters indicate a statistically significant (p<0.01)

using the Mann-Whitney U test (α=0.05). Multiple comparisons were performed using the Bonferroni post-hoc test (p<0.05).

## Results

### Cytotoxicity of Pediatric Toothpastes

Cell viability was significantly affected by the type and concentration (in ppm) of detergent (both p<0.05) in the toothpastes. The decrease in concentration from 1:1 to 1:32 decreased the cytotoxicity of toothpastes significantly (p<0.05), except that of EL (Table 2).

The effects of original extracts (1:1) and concentrations (1:2, 1:4, 1:8, 1:16, and 1:32) of pediatric toothpastes on the viability of L929 cells after 2 min of exposure were evaluated using the standard MTT assay. Varying degrees of cytotoxicity were determined from the experiments (Table 2).

The survival rates were significantly different between L929 cells exposed to 1:1 concentration of SP and cells in the negative control group (p<0.05). There were no significant differences in the survival rates between L929 cells exposed to IK and EL and cells in the negative control group (p>0.05). Significant differences were observed in the survival rates between the L929 cells exposed to all concentrations of SK and cells in the negative control group (p<0.05). However, significant differences were observed between cells in the negative control group and L929 cells exposed to 1:2, 1:4, and 1:8 concentrations of OB (p<0.05). Significant differences were observed in the survival rates between L929 cells exposed to all concentrations of EJ (except 1:32) and cells in the negative control group (p<0.05).

### Production of ROS by Pediatric Toothpastes

ROS production was measured using the oxidation-sensitive fluorescent probe H2DCF-DA. The L929 cell cultures were exposed to the toothpastes in the cell culture medium for 2 min and cell cultures exposed to 1 mmol/L TEGDMA were used as positive control. The mean fluorescence intensities were compared with those of untreated control cultures (n=4). The amount of ROS was increased by approximately eight-fold with 1 mmol/L TEGDMA. All toothpastes showed significant increase in the amount of ROS in L929 cells (p<0.05) (Table 3). ROS values were significantly increased with SP, IK, SK, OB, EL, and EJ by 5.29, 5.23, 2.90, 2.42, 8.57, and 1.95-fold, respectively (p<0.05).

## Discussion

Biocompatibility is a unique property exhibited by a substance that interacts with its environment. The biological response of substances varies with changes

in the host, method of substance application, or substance itself (12). When a specific biocompatible substance is applied to the host, the corresponding effect may be either cell damage or stimulation of the cellular synthesis of some proteins, leading to inflammation. The results of this study indicate the possibility of the contents of pediatric toothpastes to show toxic effects, which may increase in the presence of some ingredients, specifically SLS. Additionally, fluoride-rich toothpastes have toxic effects. In the current study, only SP and IK were not cytotoxic; other pediatric toothpastes showed cytotoxic effects on the L929 cells, partially proving our hypothesis.

Toothpastes and mouthwashes have been used for more than 3000 years and formulated to conceal malodor, remove tooth stains, and treat or prevent diseases of the teeth. The toothpastes generally contain detergents, foaming agents, preservatives, antimicrobial agents, moisturizers, and homogenizers. Each component has a specific function and provides different characteristics to the toothpaste (2,13,14). The complete removal of products designed to expectorate after use is not possible, even for adults. Generally, it is more difficult in young children. Therefore, the frequent/daily use of toothpastes increases the necessity to investigate the potential systemic toxicity of their components (5).

There are many studies in the literature on the cytotoxicity of toothpastes (9,15-17). Detergents, particularly SLS, have cytotoxic effects (9). The study of Cvikl et al. (9) was that SLS-and AF-containing toothpastes were more cytotoxic for L929 fibroblast cells than CAPB and Steareth-20-containing toothpastes. In our study, all concentrations of SK containing SLS were cytotoxic to L929 cells. However, the 1:16 and 1:32 dilutions of OB containing SLS had no cytotoxic effects. Both these groups showed similar levels of ROS production. This result shows that cytotoxicity is affected by concentration.

Moore et al. (13) observed that detergents are associated with cell membrane disruption *in vitro*, a finding consistent with other *in vitro* studies; Cell incubation with SLS for 2 minutes reduces TERT-1 keratinocyte viability. Ghapanchi et al. (15) found that toothpastes containing SLS had various toxic effects on the primary epithelium and HeLa cells of the oral cavity. SLS showed the highest cytotoxicity at all time-points in a study by Tabatabaei et al. (18).

Elevated ROS levels affect the redox biological signals that maintain the physiological functions (19). SLS may accelerate cell death and rapidly eliminate deleterious microorganisms from the population (20). SLS initially interacts with cell membranes, causing an increase in intracellular Ca2+. Ca2+ stimulates the secretion of IL-1α due to calpain activation. IL-1α also stimulates ROS formation (21).

ROS production in cells is an important determinant of cell damage or redox signaling (19). ROS production was high in non-cytotoxic groups showing that cell damage occurs even when the cell does not lose its viability. In this study, CAPB containing SP, IK, and EL showed high ROS levels. All these toothpastes contain different concentrations and types of CAPB and fluoride. SP and IK contain 1450 ppm and 500 ppm sodium fluoride, respectively, and even 1:1 concentration of SP was cytotoxic. The difference between these two materials suggests that high doses of fluoride affect cell viability. Tabatabaei et al. (18) stated that the cytotoxicity of sodium fluoride was significantly correlated with time and increased over time. In other words, high concentrations for lesser durations and low concentrations for greater durations showed similar results.

The highest ROS level was found with EL containing 500 ppm AF, but EL containing 1400 ppm AF showed the lowest levels. This shows that low concentrations of AF leads to cell damage, even if the cell is alive. AF is also used as a detergent in toothpastes. Our results showed that all concentrations of EL and the 1:32 concentration of EJ were nontoxic. The determination of the most appropriate fluoride concentration for pediatric toothpastes requires comprehensive personalized evaluation after meticulous risk assessment. Toothpastes with lower fluoride concentrations can be recommended for children at risk of high fluoride toothpaste sensitivity (22). In general, children should brush their teeth twice a day using a fluoride and age-appropriate amount of toothpaste (23).

CAPB is also used as a detergent in toothpastes. No toxic effects were observed with any concentration of IK. Corroborating the results of our study, Cvikl et al. (10) proposed that children should only use CAPB-containing dentifrices that are specially formulated for them. Despite its foaming ability, CAPB is less harmful than SLS (24).

Recent studies on cell viability, cytotoxicity, and genotoxicity have revealed the potential adverse effects of toothpaste ingredients (9,14,16). However, the conducive environment of the oral cavity differs from *in vivo* conditions, and many factors, such as saliva, mucus layer, creatinine levels, blood flow, and normal flora, can protect the oral environment from harmful effects (15,16). Children older than six years have mixed dentition, and usually use adult dentifrices with higher fluoride concentrations and higher foaming properties, attributed to higher surfactant concentrations (1). This might explain the differing results for cell viability at varying ages after stimulation with toothpastes from the same manufacturer (10).

Further studies are needed to investigate the suitability of the oral cavity as a target tissue. Nonetheless, cell culture is an excellent method for assessing the mechanisms of incompatibility reactions. Alternatively, data from cytotoxicity tests, implantation studies, or cell culture models should be used to evaluate the biocompatibility of toothpastes.

## Conclusion

Pediatric toothpastes containing sodium fluoride with SLS are more cytotoxic. AF-containing toothpastes with 1400 ppm fluoride are more cytotoxic than with 500 ppm fluoride, but ROS levels are greater at 500 ppm fluoride. Toothpastes containing CAPB are less toxic.

### Ethics

**Ethics Committee Approval:** This study was approved by the Selçuk University Faculty of Dentistry Ethics Committee (decision no: 2015/01, date: 08.10.2015).

**Informed Consent:** This study does not require patient consent.

**Peer-review:** Externally peer-reviewed.

### Authorship Contributions

Design: F.K., H.E.Ü., Data Collection or Processing: F.K., Analysis or Interpretation: H.E.Ü., G.T., M.Ö., Literature Search: F.K., Writing: F.K., H.E.Ü., G.T., M.Ö.

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