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Cytotoxicity of the Ingredients of Commonly Used Toothpastes and Mouthwashes on Human Gingival Fibroblasts

Masoumeh Hasani Tabatabaei[1,2], Farzaneh Sadeghi Mahounak[2], Nafiseh Asgari[3], Zohreh Moradi[1,2*]

1. Dental Research Center, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran
2. Department of Restorative Dentistry, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran
3. Department of Prosthodontics, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran

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**** Corresponding author:***
Dental Research Center, Dentistry Research Institute, Tehran University of Medical Sciences, Tehran, Iran

Email: zohrehmoradi2003@yahoo.com

A B S T R A C T

Objectives: Toothpastes and mouthwashes contain ingredients that may be toxic for oral mucosal tissues. This study aimed to assess the cytotoxicity of the ingredients of commonly used toothpastes and mouthwashes.

Materials and Methods: This experimental study was performed on 16 toothpastes and four mouthwashes widely available in the Iranian market. First, the concentration of six main ingredients of these products, namely sodium fluoride (NaF), sodium lauryl sulfate, cocamidopropyl betaine, zinc lactate, paraben, and sodium benzoate, was determined. The methyl thiazolyl tetrazolium (MTT) assay was used to assess the cytotoxicity of these materials for human gingival fibroblasts (HGFs). The MTT assay was performed at 1, 15, and 30 minutes following exposure to five concentrations of each material in triplicate (according to the concentrations obtained in the isolation step). Data were analyzed using three-way analysis of variance (ANOVA).

Results: The difference in the cytotoxicity of the materials was statistically significant ($P<0.001$). Cytotoxicity was time- and concentration-dependent; by an increase in the concentration of the materials, their cytotoxicity increased over time. The cytotoxicity of sodium lauryl sulfate and cocamidopropyl betaine was >90%. The cytotoxicity of NaF varied from 25% to 70%, and the cytotoxicity of all concentrations of zinc lactate and sodium benzoate was <50% for HGFs.

Conclusion: To decrease the cytotoxic effects of toothpastes, sodium lauryl sulfate and cocamidopropyl betaine should be replaced with safer detergents, and the concentration of fluoride should be decreased to 400 parts per million (ppm). Alternatively, fluoride may be replaced with other antibacterial and cariostatic agents.

Keywords: Cytotoxicity Tests, Immunologic; Sodium Fluoride; Sodium Dodecyl Sulfate; cocamidopropyl betaine; Zinc; Parabens; Sodium Benzoate

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INTRODUCTION

Mechanical plaque removal is the most commonly used method for the promotion of oral hygiene and gingival health. Toothpastes

and mouthwashes are commonly used as adjuncts to mechanical plaque removal [1].

Oral health significantly affects the quality of life. Thus, the need for cost-effective, safe, and efficient oral hygiene measures has been highly emphasized. Toothbrushing along with the use of a suitable toothpaste is the most effective method for the promotion of oral health, elimination of biofilms, and decreasing the risk of gingivitis [1].

Toothpastes have antibacterial properties and decrease the occurrence of plaque-related conditions. However, they have additives with possible toxic effects on the oral mucosa [2, 3]. Toothpastes contain three main ingredients of fluoride, abrasives, and detergents [4]. Fluoride is added to toothpastes to confer cariostatic properties; however, it has toxic effects on all cell types. These effects vary on different cell types depending on the duration of exposure and the concentration of fluoride. Necrosis, as the primary mechanism of cell death, has been reported following exposure to relatively high concentrations of fluoride [5]. Also, exposure to high concentrations of fluoride [10 parts per million (ppm) and higher] often leads to fluorosis, which is characterized by brownish discoloration of the enamel, yielding the appearance of mottled enamel [6]. Abrasives, such as silica, aluminum hydroxide, and calcium carbonate, are also added to toothpastes to confer plaque removal properties. Sodium benzoate and paraben are used as preservatives in toothpastes [4].

Detergents present in the composition of toothpastes include sodium lauryl sulfate and cocamidopropyl betaine. Sodium lauryl sulfate has shown significant toxic effects in vitro [4]. It can alter the oral mucosal proteins in vitro [7] and increase the blood flow in the gingiva [8]. Zinc lactate is another ingredient of toothpastes with antimicrobial properties [9]. Desensitizers, anti-plaque agents, anti-inflammatory agents, anti-odor agents, preservatives, artificial colors, and essences are also added to toothpastes, which may have toxic effects [4]. For instance, paraben affects the endocrine system and impairs the production of hormones. It is also carcinogenic [10]. Sodium benzoate can cause anaphylactic shock, swelling of the nasal mucosa, and dyspnea [10]. Zinc lactate is also used in the composition of toothpastes and can negatively affect the respiratory system [10]. Cocamidopropyl betaine is used in the formulation of toothpastes as a foaming agent and can cause allergic reactions [10].

Previous studies have theoretically expressed the toxicity of these materials; however, no specific concentration has been determined as the threshold of cytotoxicity of these agents. This study aimed to assess the cytotoxicity of the main ingredients of toothpastes and mouthwashes commonly available in the Iranian market. The cytotoxicity of different ingredients was compared to find the material with the highest cytotoxicity.

MATERIALS AND METHODS

In this in-vitro experimental study, sixteen toothpastes, namely Sanino kids (Evyap, Istanbul, Turkey), Sehat (Sehat Co., Tehran, Iran), Oral-B PRO-EXPERT (Procter & Gamble Co., Cincinnati, OH, USA), Crest 7 Complete (Procter & Gamble Co., Cincinnati, OH, USA), Colgate (Colgate Palmolive Co., Piscataway, NJ, USA), Crend 3 (Pakrokh Cosmetic and Hygienic Co., Tehran, Iran), Pooneh (Paksan, Tehran, Iran), Parodontax GUM CARE (GlaxoSmithKline, s.r.o., London, UK), Close Up (Unilever, Jersey City, NJ, USA), Nasim (Paksan, Tehran, Iran), Sensodyne (GlaxoSmithKline, s.r.o., London, UK), Bath (Iran Avandfar Co. Ltd., Alborz, Iran), Signal (Unilever, Jersey City, NJ, USA), Himalaya Complete Care (Himalaya, Bengaluru, Karnataka, India), Himalaya Sparkling White (Himalaya, Bengaluru, Karnataka, India) and 4 mouthwashes, namely Irsha (Shafa cosmetic laboratories Co., Tehran, Iran), Oral-B (Grossgerau Co., Hessen, Germany), Vi-One (Rojn Cosmetic Lab. Co., Tabriz, Iran), and Listerine (Pfizer Inc., Morris Plains, NJ, USA), commonly available in the Iranian market, were evaluated to determine the concentration of six main ingredients in their composition, namely sodium fluoride (NaF), sodium lauryl sulfate, cocamidopropyl betaine, zinc lactate, paraben, and sodium benzoate.

The concentration of NaF in the composition of toothpastes and mouthwashes was

determined by potentiometric titration.

The isolation of sodium lauryl sulfate was performed using the active anionic test, which is based on stoichiometric reactions between the anionic surfactant and a standard amine solution, leading to the formation of the related complex in the organic phase.

The biphasic (acid-base) titration test with litmus papers (Whatman indicator paper, Camlab, Cambridge, UK) was used to measure the concentration of cocamidopropyl betaine.

The isolation of paraben from toothpastes and mouthwashes was performed using gas chromatography (Shimadzu, Markham, Ontario, Canada). Zinc lactate and sodium benzoate were isolated using high-performance liquid chromatography (HPLC; Agilent Technologies 1200 Infinity series, Santa Clara, CA, USA). In addition to the upper and lower limits of the extracted concentrations of the six ingredients, the median, a higher concentration, and a lower concentration (for further accuracy) were used for cytotoxicity assessment of each experimental material.

Cells used for the cytotoxicity test:

HuGu cell line of human gingival fibroblasts (HGFs; Cell No. IBRC C10459), established from a biopsy of the gingiva of a 45-year-old homo-sapien female, were obtained from the cell bank of the Iranian Biological Resource Center (IBRC). The culture medium composed of Dulbecco's Modified Eagle Medium (DMEM), 2mM of L-Glutamine, and 10% Fetal Bovine Serum (FBS). The storage media included 90% FBS and 10% dimethyl sulfoxide (DMSO) at about 1×10^6 cells/vial incubated at 37°C with 5% carbon dioxide (CO_2). HGFs adhered to the bottom of the culture plate. Thus, trypsin was used to detach the cells. In each well of a 96-well plate, 10,000 cells per 100 µl (about one million cells) were required.

The methyl thiazolyl tetrazolium (MTT) assay:

The MTT assay was used to assess the cytotoxicity of the materials. One-hundred µl of each of the cell suspensions was added to a 96-well plate and incubated at 37°C with 5% CO_2 and 100% humidity for 12 to 24 hours to ensure cell attachment. Then, pure materials (Merck, Rahway, NJ, USA) with the respective concentrations plus 10 µl of the MTT dye were added to each well after the designated period. Three wells containing 100 µl of culture medium plus 10 µl of MTT were considered as blank. The plate was covered with aluminum foil and incubated at 37°C with 5% CO_2 and 100% humidity for four hours. The presence of purple deposits was evaluated under an inverted microscope. Afterwards, the culture medium was removed from the wells (containing attached cells), and 100 µl of DMSO was added to each well. Then, the plates were placed in the dark for two to four hours, and the optical density (OD) of the wells was read (for both experimental and blank wells) using a spectrophotometer (X-Rite, Grand Rapids, MI, USA) at a 570-nm wavelength. The results were recorded for the quantitative comparison of cell proliferation. All the above-mentioned procedures were repeated three times. This was done for all the six ingredients at the designated concentrations at 1, 15, and 30 minutes following exposure. Three well plates were used for this purpose for the three repetitions. For instance, for a material with five concentrations, 15 wells were allocated. When the material was added to the well, the time was recorded, and the MTT was added one minute later. After one minute, 10 µl of MTT at a final concentration of 5 mg/ml was added to each well. The remaining steps were the same as those mentioned earlier. The same was done for assessments at 15 and 30 minutes with the difference that the MTT was added to the wells 15 and 30 minutes after exposure.

Statistical analysis:

Three-way analysis of variance (ANOVA) was used to assess the effect of time, concentration, and type of ingredient on cytotoxicity. $P<0.05$ was considered statistically significant.

RESULTS

The concentrations of the extracted ingredients obtained using relevant methods were as follows (%: mg/100 ml of distilled water):

NaF: 160-1400 ppm

Sodium lauryl sulfate: 0.75-2.75 %

Cocamidopropyl betaine: 1.5-4 %

Zinc lactate: 0.1-0.4 %

Paraben: 0.01-0.1 %

Sodium benzoate: 0.1-0.3 %

Table 1. Concentrations of the six ingredients used for cytotoxicity assessment

Materials	Concentrations used for cytotoxicity assessment				
Sodium fluoride	2800 ppm	1400 ppm	1000 ppm	400 ppm	160 ppm
Sodium lauryl sulfate (%)	5.5	2.75	1.5	0.75	0.37
Cocamidopropyl betaine (%)	8	4	2	1.5	1
Zinc lactate (%)	0.6	0.4	0.2	0.1	0.05
Paraben (%)	0.2	0.1	0.02	0.01	0.005
Sodium benzoate (%)	0.6	0.3	0.15	0.1	0.03

ppm: parts per million

The concentrations used for the MTT assay are presented in Table 1. Three-way ANOVA showed a significant difference in the cytotoxicity of paraben, zinc lactate, and sodium benzoate (P<0.001). Also, the difference in the cytotoxicity of each material at 1, 15, and 30 minutes following exposure was statistically significant (P<0.001). Significant differences were noted in the cytotoxicity of sodium lauryl sulfate, cocamidopropyl betaine, and NaF at all concentrations at 1, 15, and 30 minutes (P<0.001).

The MTT assay results for the six ingredients were as follows: At 1, 15, and 30 minutes, a significant difference was noted in the cytotoxicity of sodium benzoate, paraben, and zinc lactate (P<0.001). At all three time points, zinc lactate showed high and paraben showed low cytotoxicity. Sodium benzoate showed cytotoxicity higher than that of paraben and less than that of zinc lactate. The cytotoxicity of NaF, sodium lauryl sulfate, and cocamidopropyl betaine was also significantly different at 1, 15, and 30 minutes (P<0.001).

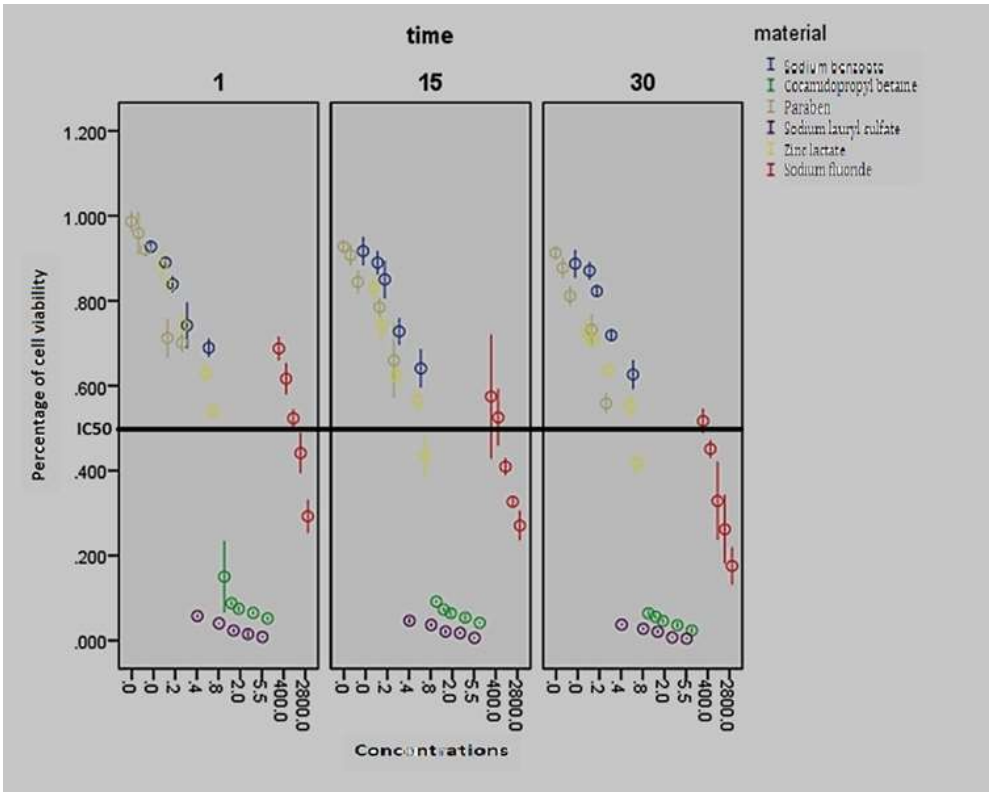

Fig. 1. The error bar of means and 95% confidence interval (CI) of the mean cell viability percentages for different concentrations of the studied materials at 1, 15, and 30 minutes.

At all three time points, zinc lactate showed high and paraben showed low cytotoxicity. Sodium benzoate showed cytotoxicity higher than that of paraben and less than that of zinc lactate. The cytotoxicity of NaF, sodium lauryl sulfate, and cocamidopropyl betaine was also significantly different at 1, 15, and 30 minutes ($P<0.001$). At all three time points, sodium lauryl sulfate showed the highest and NaF showed the lowest cytotoxicity.

Sodium benzoate, paraben, and zinc lactate had the lowest cytotoxicity at all three time points. The cytotoxicity of all three ingredients at all concentrations was less than 50%. In other words, sodium benzoate, paraben, and zinc lactate eliminated less than 50% of HGFs.

Sodium lauryl sulfate and cocamidopropyl betaine, at all time points, resulted in the elimination of a considerable percentage of cells and had the highest cytotoxicity. The cytotoxicity threshold of NaF at one minute was 400 ppm, which means that at the 400-ppm concentration, NaF eliminates 50% of HGFs.

The comparison of the cytotoxicity of different concentrations of the six main ingredients at three time intervals is presented in Figure 1.

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DISCUSSION

All chemicals used in the oral cavity must be evaluated in terms of cytotoxicity. Dental materials undergo different tests by the American Dental Association (ADA), the Food and Drug Administration (FDA), and the International Organization for Standardization (ISO). One of the primary tests involves the use of cell culture to determine the cytotoxic effects of these materials. The MTT test is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan crystals by viable cells. Since in most cell populations, the overall mitochondrial activity depends on the number of viable cells, this test is used to assess the toxic effects of medications on cells in vitro [2].

Toothpastes are used for dental care every day; however, their effects on oral mucosal cells have not been well evaluated. Since these materials are in direct contact with the mucosa, all of their negative effects must be investigated [2]. Some previous studies have assessed the cytotoxicity of toothpastes and mouthwashes, and some of them have reported the toxicity of toothpastes for oral mucosal cells [2,4].

Toothpastes have three main components, including (I) detergents, such as sodium lauryl sulfate and cocamidopropyl betaine, (II) insoluble abrasives for plaque removal, such as silica, aluminum hydroxide, and calcium carbonate, and (III) cariostatic agents, such as fluoride compounds (NaF and sodium monofluorophosphate). Sodium lauryl sulfate and cocamidopropyl betaine are among the most toxic components in the composition of toothpastes [2]. Other substances added to toothpastes for different functions include desensitizers, anti-plaque agents, anti-inflammatory agents, anti-odor agents, preservatives, artificial colors, and essences; each of these agents can have toxic effects [4].

In addition to toothpastes, zinc lactate, paraben, and sodium benzoate are also added as antimicrobial agents and food preservatives. Some studies have evaluated the carcinogenicity of these materials, especially paraben [11,12].

Since any of toothpaste ingredients can have toxic effects on oral mucosal cells, and no previous study has compared the cytotoxicity of toothpaste and mouthwash ingredients at their standard concentrations, this study assessed the cytotoxicity of six main ingredients of toothpastes and mouthwashes against HGFs, which are the most resistant cells to apoptosis and mutation.

Cvikl et al [4] evaluated the effect of toothpaste ingredients on cell viability. They evaluated nine toothpastes with different detergents by exposing HGFs to 1% concentration of these toothpastes for two minutes. They showed that toothpastes containing sodium lauryl sulfate and amine fluoride strongly compromised cell

viability while toothpastes containing cocamidopropyl betaine had less effect on cell viability [4]. One shortcoming of the cited study was the lack of attention to time as an influential factor in cytotoxicity since all tests were performed after two minutes, and all materials were found to be cytotoxic. In our study, time was found to have a significant effect on cytotoxicity, and the cytotoxicity of sodium lauryl sulfate and cocamidopropyl betaine was evaluated at three time points and five concentrations.

In our study, the cytotoxicity of sodium lauryl sulfate and cocamidopropyl betaine was the highest, which was similar to the results reported by Cvikl et al [4]. These two detergents are highly cytotoxic shortly after exposure and even at concentrations much lower than the safe threshold for use in toothpastes. In our study, the cytotoxicity of cocamidopropyl betaine was slightly less than that of sodium lauryl sulfate.

Jeng et al [13] evaluated the cytotoxicity of NaF against human oral mucosal fibroblasts. The results showed that NaF at concentrations of four mmol/l (80 ppm) and higher was toxic for human oral mucosal fibroblasts. NaF at concentrations of 4, 8, and 12 mmol/l after two hours of incubation inhibited mitochondrial activity by 31%, 56%, and 57%, respectively [13].

Their results were different from ours. In the present study, the cytotoxicity of NaF had a significant correlation with time and increased over time. In other words, the 1000-ppm concentration of NaF at one minute, 400 ppm at 15 minutes, and 160 ppm at 30 minutes were non-toxic while an increase in time from one minute to 15 minutes for the 1000-ppm concentration and from 15 minutes to 30 minutes for the 400-ppm concentration increased the cytotoxicity against HGFs. This can be due to the different methodologies.

Torrado et al [3] evaluated the cytotoxicity of Crest Extra Whitening and NMTD toothpastes on mouse fibroblasts using the MTT assay. They reported that none of the toothpastes had any significant effect on cell viability, and by an increase in incubation time, the cytotoxicity significantly increased [3].

All toothpastes, including Crest Extra Whitening, have considerable amounts of detergents. In the current study, even the lowest concentration of detergents used in toothpastes was found to be cytotoxic for HGFs. Moreover, this cytotoxicity increased with time. The main shortcoming of the study by Torrado et al [3] was that they evaluated the cytotoxicity of toothpastes using mouse fibroblasts, which may be responsible for the difference in the results.

Camargo et al [14] compared the cytotoxicity (the MTT assay), evaluated the genetic toxicity (by observing the changes in the nuclei of hamster fibroblasts), and assessed changes in enamel surface roughness caused by whitening toothpastes (Colgate and Oral-B) and non-whitening toothpastes. The cytotoxicity, genetic toxicity, and surface roughening caused by the two whitening toothpastes were greater than that of other toothpastes. Also, the cytotoxicity of Colgate Whitening was higher than that of Oral-B toothpaste [14]. Their findings regarding higher cytotoxicity of whitening compared to non-whitening toothpastes agreed with our results. Whitening toothpastes have higher amounts of sodium lauryl sulfate, and according to our results, sodium lauryl sulfate has the highest cytotoxicity among the six main ingredients of toothpastes and is responsible for higher cytotoxicity of whitening toothpastes.

Tsay et al [15] evaluated the effect of sodium benzoate on larvae of a type of fish and reported that the 2000-ppm concentration of sodium benzoate was highly toxic for the larvae; 1400-1500 ppm sodium benzoate caused the death of over 50% of the larvae. They also showed that sodium benzoate was more toxic for muscle cells and neurons [15].

Sodium benzoate is used in foods, cosmetics, and hygienic products as a preservative. The above-mentioned study highlighted the cytotoxicity of >1400-ppm concentrations of sodium benzoate for muscle cells and neurons, irrespective of its safe concentration for use in foods, cosmetics, and hygienic products [15]. However, our results showed that sodium benzoate at the standard concentrations used in toothpastes and mouthwashes was not toxic for HGFs.

Harvey and Everett [16] evaluated the role of paraben in the development of breast cancer. They found high amounts of paraben in resected tumoral tissues and stated that paraben may play a role in increasing the rate of breast cancer [16].

The negative and carcinogenic effects of preservatives, such as paraben, in foods, cosmetics, and hygienic products, have been previously documented. These effects are mainly due to long-term contact (over the years) with substances containing paraben and its accumulative effect, which eventually causes cancer in humans [16].

In the current study, paraben at the concentrations used in toothpastes and mouthwashes did not have any toxic effect on HGFs at 1, 15, and 30 minutes. However, further studies are required on adverse systemic effects and carcinogenesis of other concentrations of paraben in longer exposure times. Ng et al [17] evaluated the cytotoxicity and genetic toxicity of zinc oxide for human lung fibroblasts and fruit fly in vitro and in vivo. They observed that zinc oxide caused the death of human lung fibroblasts. Moreover, oxidative stress caused DNA damage in these cells. Zinc oxide significantly increased the death rate of fruit flies during their development from hatching to maturity [17].

In the current study, the cytotoxicity of zinc lactate was evaluated instead of zinc oxide due to the important role of zinc lactate and its antibacterial activity in toothpastes. The study by Ng et al [17] did not consider the concentration of zinc oxide or the exposure time in the assessment of toxicity. Also, despite the highly similar chemical activity and toxic effects of zinc oxide and zinc lactate, the cytotoxic effects of zinc oxide reported in the cited study were much higher than those of zinc lactate in our study, which may be because they did not consider the concentration of the material and the duration of exposure. Moreover, different cell types were evaluated in the two studies (human lung fibroblasts versus HGFs).

CONCLUSION

Within the limitations of this study, the following conclusions can be drawn:

1. The cytotoxicity of NaF at the 1400-ppm concentration (maximum concentration used in toothpastes) at one minute was 53% while its cytotoxicity at the 160-ppm concentration (minimum concentration used in toothpastes and mouthwashes) was 25%. The cytotoxicity of NaF had a significant correlation with time; at 30 minutes, the cytotoxicity of the 160-ppm concentration of NaF increased to 50%. The highest cytotoxicity of NaF was 71% (1400 ppm at 30 minutes).
2. The concentration threshold of fluoride is 400 ppm; it was not toxic for HGFs after 1, 15, and 30 minutes of exposure.
3. Sodium lauryl sulfate had the highest cytotoxicity at all the time points, and it showed >90% toxicity at all concentrations.
4. Cocamidopropyl betaine had cytotoxicity ranging from 85% to 97%. It had the highest cytotoxicity after sodium lauryl sulfate. Its cytotoxicity had a significant correlation with time and concentration; by an increase in concentration and time, the cytotoxicity increased.
5. By an increase in the concentration of sodium lauryl sulfate and cocamidopropyl betaine, the cytotoxicity of toothpastes and mouthwashes increased.
6. Sodium benzoate and paraben showed the lowest cytotoxicity. Also, 0.6% concentration of sodium benzoate at 30 minutes (30% cytotoxicity) and 0.2% concentration of paraben at 30 minutes (40% cytotoxicity) showed the highest cytotoxicity.

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CONFLICT OF INTEREST STATEMENT

None declared.

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