Original Research

Comparison of the Cytotoxic Effects of Nanoparticulate and Microparticulate Calcium Sodium Phosphosilicate Mouthwashes on Human Gingival Fibroblasts: an in-vitro Study

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Abstract

Introduction: This study sought to assess the cytotoxic effects of nanoparticulate and microparticulate calcium sodium phosphosilicate mouthwashes on human gingival fibroblasts (HGFs). **Methods:** This in vitro study was conducted on HGFs isolated and cultured in a 48-well plate containing standard culture medium for evaluation of four concentrations of the two mouthwashes at two time points plus a positive and a negative control group. The HGFs were exposed to 0.001, 0.01, 0.1 and 1 mg/mL concentrations of mouthwashes for 1 and 24 hours. Positive and negative control cells were exposed to saline and distilled water, respectively. Cell viability was assessed using the methyl thiazolyl tetrazolium (MTT) assay, and the number of viable cells was counted in triplicate using a cell counter after transfection (trypsin-EDTA 0.25%, 20 minutes) and exposure to trypan blue. The optical density (OD) values were read by ELISA reader and analyzed using the Kruskal-Wallis test. **Results:** Number of viable cells was not significantly different between the two mouthwashes at the two time points ($P>0.05$). At one hour, number of viable cells was higher in the nanoparticulate group while the number of viable cells in the microparticulate group was higher at 24 hours. **Conclusion:** Nanoparticulate and microparticulate calcium sodium phosphosilicate mouthwashes have no cytotoxicity against HGFs. Cytotoxicity of the nanoparticulate mouthwash was less than that of the microparticulate mouthwash. Also, increased proliferation of fibroblasts was noted over time in both groups of mouthwashes.

Keywords: Cytotoxicity; Human Gingival Fibroblasts; Mouthwash; Calcium Sodium Phosphosilicate; Nanoparticles.

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Introduction

Several modalities are employed to decrease dentin hypersensitivity. These modalities include over-the-counter products, varnishes, liners, adhesives, and recently laser. Toothpastes and mouthwashes are most commonly used for treatment of dentin hypersensitivity due to easy use and low cost. These products are used aiming to obstruct the dentinal tubules to prevent the hydrodynamic movement of dentinal fluid and treat tooth hypersensitivity (1). Products suggested to decrease dentin hypersensitivity include different combinations of strontium chloride, potassium nitrate, casein phosphopeptide amorphous calcium phosphate and calcium sodium phosphosilicate marketed under the brand name of NovaMin. Calcium sodium phosphosilicate is a bioactive ceramic introduced in the early 1970s for crystallization of bone (2). It can chemically bond to bone and is composed of calcium oxide, sodium oxide, phosphorous oxide and silicon oxide. Under in vivo conditions, these ceramics can form a layer of hydroxyapatite on their surface and integrate with the host tissue (3, 4). Calcium sodium phosphosilicate (NovaMin) is used for treatment of tooth hypersensitivity. It contains amorphous calcium sodium phosphosilicate, which can be suspended in water. In the form of powder and small particles, it can also obstruct the dentinal tubules (5).

Cytotoxic effects of mouthwashes on the gingival tissue have always been a concern. Cytotoxic effects of chlorhexidine on human cells was first reported in 1977 (6). However, Pucher et al. (7) reported their in vitro study in which chlorhexidine gluconate mouthwash had no cytotoxic effects on human gingival fibroblasts. Mouthwashes are composed of strontium chloride, potassium nitrate, sodium citrate and sodium fluoride as desensitizing agents. However, due to higher efficacy, the effects and properties of calcium sodium phosphosilicate have become the recent topic of interest (8-10). Some clinical (11-13) and in vitro (14-16) studies have assessed the efficacy of these compounds for obstruction of dentinal tubules and decreasing dentin hypersensitivity. Pradeep et al. (11) showed higher efficacy of calcium sodium phosphosilicate compared to potassium nitrate in toothpastes. Search of the literature yielded no previous study on the biocompatibility and cytotoxic effects of calcium sodium phosphosilicate mouthwash on human gingival fibroblasts (HGFs).

Introduction of nanotechnology enabled the production of materials with totally different properties by manipulating their atomic and molecular dimensions. Evidence shows favorable changes in properties of materials when manufactured in nanoscale because the surface area increases in such circumstances. Nanoparticulate toothpastes were recently introduced to the market with optimal efficacy for reduction of dentin hypersensitivity (17). However, to the best of authors' knowledge, no previous study has evaluated the cytotoxic effects of nanoparticulate calcium sodium phosphosilicate mouthwash. Considering the gap of information in this respect, this study aimed to determine the cytotoxic effects of nanoparticulate and microparticulate calcium sodium phosphosilicate mouthwashes, prescribed for elimination of dentin hypersensitivity, on HGFs.

Materials and Methods

In this in vitro study, HGFs in standard culture medium were exposed to four different concentrations of the two mouthwashes and their viability was evaluated at two time points (one hour and 24 hours) (7).

HGFs were obtained from the Pasteur Institute of Iran and cultured in 48-well cell culture plates (Nunc, Denmark) containing Dulbecco's modified Eagle's medium (Sigma, USA) supplemented with 7% fetal bovine serum (Gibco, Germany), 0.14% (v/v) sodium bicarbonate, 100 u/mL penicillin, 100 g/mL streptomycin sulfate and 0.25 g/mL amphotericin B. The plates were then incubated at 37°C under CO2 for 48 hours (7).

The fibroblasts were allowed to adhere to the 48-well plate for 24 hours.

Nanoparticulate calcium sodium phosphosilicate powder (NovaMin) was purchased from Denfotex. A ball mill grinder operating at 250 rpm was used to powder 2.5 weight ratio of NovaMin. The milling time was 210 minutes. To ensure the optimal dimensions of the nanoparticles, dimensions and diameter of the particles were evaluated under a scanning electron microscope (XL 30, Philips). The dispersed solutions of calcium sodium phosphosilicate were prepared using double distilled water and sonicated in an ultrasonic bath for 15 minutes. The 1, 0.1, 0.01 and 0.001 mg/mL aqueous solutions of calcium sodium phosphosilicate, containing initial powder or nanopowder, were used for in vitro

comparison of the cytotoxic effects of calcium sodium phosphosilicate mouthwashes on HGFs.

Before rinsing with fetal bovine serum and complete culture medium, fibroblasts were exposed to 0.001, 0.01, 0.1 and 1 mg/mL concentrations of nanoparticulate (24 wells) and microparticulate (24 wells) NovaMin mouthwashes (18). To prepare 1 mg/mL concentration of NovaMin mouthwash (NovaMin Technology Inc., PA, USA), 1 mg of the mouthwash was weighed and dissolved in 1 mL of saline. To prepare other concentrations, 0.1, 0.01 and 0.001 mg of the mouthwashes were dissolved in 1 mL of saline.

Positive control cells (24 wells) were exposed to saline (standard culture medium), while negative control cells (24 wells) were exposed to water (which is 100% toxic for fibroblasts).

Number of viable HGFs was counted with a cell counter after transfection (trypsin-EDTA, 20 minutes 0.25 %) and staining with trypan blue at both time points (7). Each test was repeated in triplicate (7).

The methyl thiazolyl tetrazolium (MTT) assay was used to assess cytotoxicity at both time points. For this purpose, 5 mg/mL of MTT salt (Sigma Aldrich, USA) was dissolved in phosphate buffered saline plus Dulbecco's modified Eagle's medium in 1:10 ratio. The optical density (OD) was read using ELISA Reader (Anthus, Australia). The OD data were divided by the OD of the negative controls to assess cell viability (19).

Normal distribution of OD data was first checked using the Kolmogorov-Smirnov test, which showed that data were not normally distributed. Thus, the non-parametric Kruskal-Wallis test was used for the comparison of groups. Pairwise comparisons were performed using t-test. All statistical analyses were performed using SPSS version 20 (SPSS Inc., IL, USA).

Results

Table I shows the mean OD in presence of different concentrations of nanoparticulate and microparticulate mouthwashes. Table 2 shows the mean OD of the nanoparticulate mouthwash, microparticulate mouthwash, positive control and negative control groups at different time points. As shown, at one hour, nanoparticulate mouthwash group had higher number of viable cells compared to the microparticulate group but this difference was not statistically significant (Table II).

The Kruskal-Wallis test showed significant differences in cell viability of 0.1 mg/mL microparticulate mouthwash and the negative control group at one hour (P=0.002), 0.001 mg micro-particulate mouthwash and the negative control group at one hour (P=0.002) and the negative control group at one hour and negative control group at 24 hours (P=0.0001).

At 24 hours, number of viable HGFs counted following the MTT assay showed higher number of viable cells in microparticulate mouthwash compared to nanoparticulate mouthwash group (P=0.0001). Also, both mouthwash groups showed higher number of viable cells compared to the positive control group (Table II). The Kruskal Wallis test showed that 0.1 mg microparticulate group had a significant difference with the negative control group in terms of cell viability at 24 hours (P=0.024).

Comparison of different concentrations of the nanoparticulate and microparticulate mouthwash groups at one hour revealed that 0.01 mg concentration of nanoparticulate mouthwash had the highest cytotoxicity among different concentrations; 0.001 mg concentration of nanoparticulate mouthwash had less cytotoxicity than 0.01 mg group. By an increase in concentration, the cytotoxic effects decreased (P=0.0001; Table I, Fig. 1).

In the microparticulate group, 0.01 mg concentration had the highest cytotoxicity at one hour and 0.001 and 0.1 mg concentrations of this mouthwash were equally less cytotoxic (P=0.0001). Also, 1 mg concentration of this mouthwash had the lowest cytotoxicity at one hour (Table I, Fig. 1).

The lowest cytotoxicity among different concentrations of the two mouthwashes at one hour belonged to 1 mg concentration of nanoparticulate mouthwash (P=0.0001, Table I, Fig. 1). The highest cytotoxicity at one hour among different concentrations of the two mouthwashes belonged to the 0.01 mg concentration of micro-particulate group (Table I, Fig. 1).

Comparison of different concentrations of the two mouthwashes at 24 hours revealed that in nanoparticulate mouthwash, 0.001 mg concentration had the highest cytotoxicity among all concentrations. By an increase in concentration, cytotoxicity against HGFs decreased (Table I, Fig. 2).

For the microparticulate mouthwash, 0.01 mg concentration had the highest cytotoxicity among different concentrations at 24 hours. Also, 0.001 mg concentration had less cytotoxicity than the afore-mentioned concentration. For other concentrations,

increase in concentration decreased cytotoxicity (P=0.0001).

The lowest cytotoxicity at 24 hours among different concentrations of the two mouthwashes belonged to 1 mg concentration of microparticulate group (Table I, Fig. 2). The highest cytotoxicity at 24 hours among different concentrations of the two mouthwashes belonged to 0.001 mg concentration of nanoparticulate group (Table I, Fig. 2).

In the nanoparticulate group at both time points, the highest cytotoxicity belonged to 0.01 mg concentration at one hour while the lowest cytotoxicity belonged to 1 mg concentration at 24 hours (Table I, Fig. 3). The cytotoxicity of different concentrations of nanoparticulate mouthwash at one hour was higher than that of similar concentrations of this mouthwash at 24 hours; however, these differences were not statistically significant (P>0.05, Table I, Fig. 3).

For the microparticulate mouthwash at both time points, the highest cytotoxicity belonged to 0.01 mg concentration at one hour while the lowest cytotoxicity belonged to 1 mg concentration at 24 hours (Table I, Figure 4). The cytotoxicity of different concentrations of micro-particulate mouthwash at one hour was higher than that of similar concentrations of this mouthwash at 24 hours (P=0.0001, Table I).

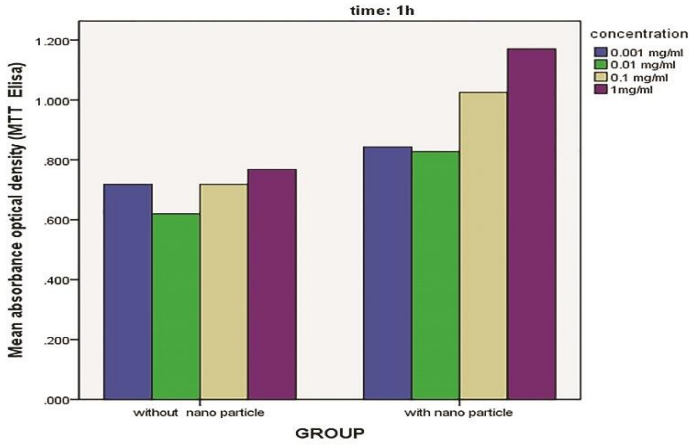

Figure 1. Mean optical density in different concentrations of the two mouthwashes at one hour

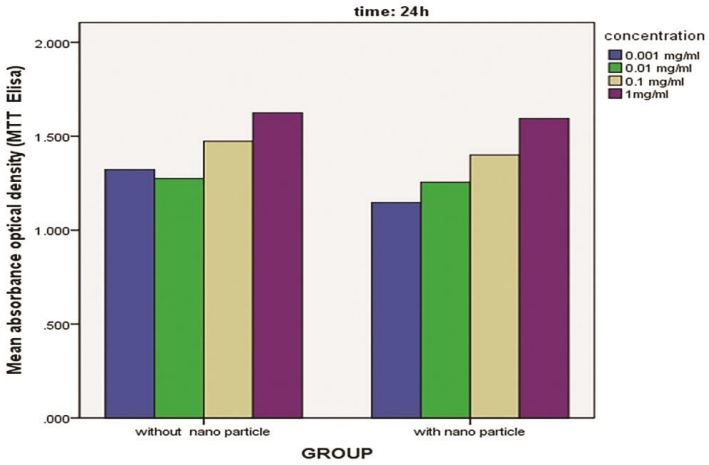

Figure 2. Mean optical density in different concentrations of the two mouthwashes at 24 hours

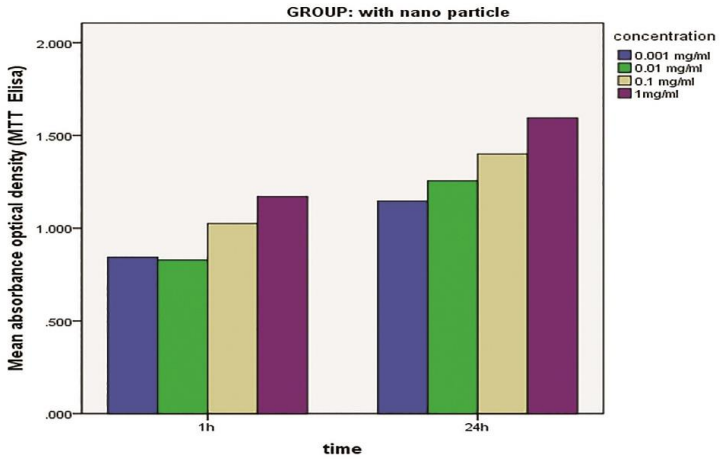

Figure 3. Mean optical density in the nanoparticulate group at one and 24 hours

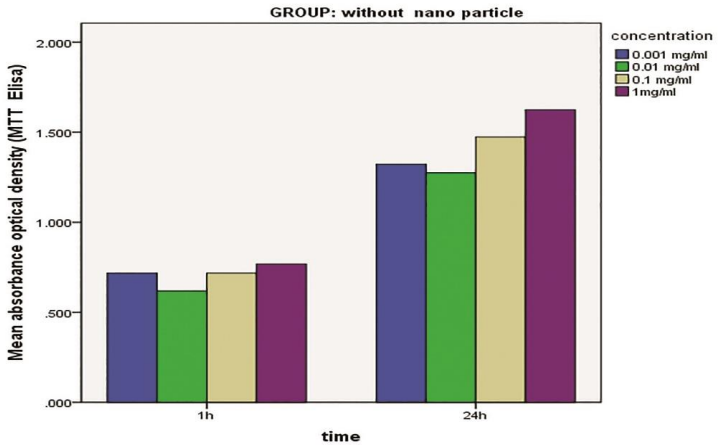

Figure 4. Mean optical density in the micro-particulate group at one and 24 hours

Table I. Mean OD in presence of different concentrations of nanoparticulate and microparticulate mouthwashes

					Optical density			P-value
					Mean and SD	Minimum	Maximum	
Group	Microparticulate	One hour	Concentration	0.001 mg/mL	0.72±0.05	0.67	0.77	0.0001
				0.01 mg/mL	0.62±0.04	0.59	0.67	
				0.1 mg/mL	0.72±0.06	0.67	0.79	
				1 mg/mL	0.77±0.06	0.72	0.84	
		24 hours	Concentration	0.001 mg/mL	1.32±0.18	1.15	1.51	
				0.01 mg/mL	1.27±0.13	1.19	1.42	
				0.1 mg/mL	1.47±0.06	1.40	1.52	
				1 mg/mL	1.62±0.19	1.46	1.83	
	Nanoparticulate	One hour	Concentration	0.001 mg/mL	0.84±0.07	0.79	0.92	
				0.01 mg/mL	0.83±0.02	0.81	0.84	
				0.1 mg/mL	1.03±0.07	0.98	1.11	
				1 mg/mL	1.17±0.02	1.15	1.18	
		24 hours	Concentration	0.001 mg/mL	1.15±0.18	0.95	1.30	
				0.01 mg/mL	1.26±0.15	1.11	1.40	
				0.1 mg/mL	1.40±0.04	1.36	1.44	
				1 mg/mL	1.59±0.10	1.53	1.71	

SD: Standard deviation

Table II. Mean OD of the nanoparticulate mouthwash, microparticulate mouthwash, positive control and negative control groups at one and 24 hours

				OD Mean and SD
Group	Microparticulate	Time	One hour	0.073±0.706
			24 hours	1.424±0.192
	Nanoparticulate	Time	One hour	0.967±0.153
			24 hours	1.349±0.206
	Negative control	Time	One hour	0.042±0.008
			24 hours	0.043±0.009
	Positive control	Time	One hour	1.476±0.043
			24 hours	1.340±0.023

SD: Standard deviation; OD: Optical density

Discussion

Cytotoxicity of dental materials and oral hygiene products has always been a concern for dental clinicians. These products should be biocompatible; otherwise, they would cause inflammatory reactions (6).

At present, dentin hypersensitivity following periodontal treatment is a common complaint of most patients. Finding an effective mouthwash to decrease dentin hypersensitivity would be highly cost-effective. The efficacy of many new products has been evaluated for elimination of dentin hypersensitivity. Calcium sodium phosphosilicate under the brand name NovaMin has shown significant efficacy for this purpose. (1, 12, 15, 20-25).

Introduction of nanotechnology favorably changed the properties of many dental materials. Thus, this study evaluated the cytotoxicity of nanoparticulate calcium sodium phosphosilicate mouthwash compared to its microparticulate (regular) form. The results showed that the tested concentrations of the two mouthwashes did not have cytotoxic effects on HGFs at one or 24 hours. Although cell viability was lower in the nanoparticulate mouthwash group compared to the microparticulate group, this difference did not reach statistical significance. Reduction in cytotoxicity by an increase in concentration (noted in 0.1 and 1 mg concentrations of both mouthwashes at both time points) was probably due to the agglomeration of micro- and nano-particles and formation of larger particles, which could not penetrate into the cells.

Search of the literature by the authors did not yield any study on the cytotoxic effects of NovaMin on HGFs to compare our results with. Thus, we compared our findings with those of studies on the cytotoxic effects of other mouthwashes.

Bansal et al. (26) in 2017 compared the efficacy of NovaMin, arginine and an herbal paste for treatment of tooth hypersensitivity. The results showed that NovaMin caused a greater improvement in tooth hypersensitivity in long-term. This finding, similar to our study, suggested that NovaMin can be effectively used for management of tooth hypersensitivity.

Balloni et al. (23) in 2016 compared the cytotoxicity of essential oils, chlorhexidine and amine fluoride/stannous fluoride mouthwashes against HGFs. The results showed that amine fluoride/stannous fluoride mouthwash had less cytotoxicity than the other two but reduction in cell proliferation was noted in all groups. However, our study showed increased proliferation of cells 24 hours after exposure to both microparticulate and nanoparticulate mouthwashes compared to the control groups. This indicates that calcium sodium phosphosilicate probably has less cytotoxicity than other commercially available mouthwashes evaluated by Balloni et al. (23). Also, they used Invitrogen cell counter with trypan blue staining for assessment of cell viability while we used ELISA reader to measure the OD of viable cells. The latter method is faster and more accurate than the Invitrogen cell counter. The MTT assay is extensively used for assessment of cell viability in studies on cytotoxicity of mouthwashes (25).

Song et al. (27) in 2013 evaluated the cytotoxicity of different concentrations of SiF for HGFs. Using MTT assay, they showed that exposure to 0.01% concentration

of ammonium fluorosilicate for less than 5 minutes had no cytotoxic effects. Their study was different from ours in terms of type of mouthwash used and assessment time points. However, their study was similar to ours in terms of concentrations of the mouthwash tested and using the MTT assay for evaluation of cell viability. In their study, 0.1 and 1 mg/mL concentrations showed higher cytotoxicity than 0.01 and 0.001 mg/mL while in our study, 0.01 and 0.001 mg concentrations indicated greater cytotoxicity than 0.1 and 1 mg concentrations. This difference is probably due to the agglomeration of calcium sodium phosphosilicate microparticles and nanoparticles in their respective groups. As the result, the larger particles could not penetrate into the cells. In our study, nanoparticulate calcium sodium phosphosilicate mouthwash was significantly superior to the microparticulate type in terms of obstruction of dentinal tubules, which may be attributed to the smaller size of particles (10^{-9} compared to 10^{-6}). Thus, particles can easily penetrate into the dentinal tubules, which have a diameter of about 0.9-1.1 µm at the dentinoenamel junction, obstruct them and prevent the movement of intratubular fluid, which would consequently decrease dentin hypersensitivity. On the other hand, smaller diameter of particles and their further penetration into dentinal tubules prevent their quick washout by the saliva compared to microparticles. Thus, nanoparticulate mouthwashes are superior in terms of function and obstruct a higher number of dentinal tubules. Moreover, nanoparticulate mouthwashes have greater substantivity in the oral environment compared to the microparticulate type.

Gopinath NM et al.(28) evaluated calcium sodium phosphosilicate particles of different sizes and concluded that the smaller the size of particles, the faster and more effectively they would release calcium and phosphorous ions. Since they used macro-scale particles in their study, converting macro-particles to nano- and smaller particles obviously enhanced their chemical properties.

Future clinical studies are required to assess the efficacy of these mouthwashes. Furthermore, cytotoxic effects of these mouthwashes on other cell lines should be evaluated in future studies.

Conclusion

Nanoparticulate and microparticulate calcium sodium phosphosilicate mouthwashes have no cytotoxicity against HGFs. Also, increased proliferation of fibroblasts was noted in both groups of mouthwashes over time.

Conflicts of interest

This article was extracted from a thesis. There is no conflict of interest to declare by all authors.

Acknowledgements

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