

## Original Article

# The antimicrobial sensitivity of *Streptococcus mutans* and *Streptococcus sangius* to colloidal solutions of different nanoparticles applied as mouthwashes

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## ABSTRACT

**Background:** Metal nanoparticles have been recently applied in dentistry because of their antibacterial properties. This study aimed to evaluate antibacterial effects of colloidal solutions containing zinc oxide (ZnO), copper oxide (CuO), titanium dioxide (TiO<sub>2</sub>) and silver (Ag) nanoparticles on *Streptococcus mutans* and *Streptococcus sangius* and compare the results with those of chlorhexidine and sodium fluoride mouthrinses.

**Materials and Methods:** After adding nanoparticles to a water-based solution, six groups were prepared. Groups I to IV included colloidal solutions containing nanoZnO, nanoCuO, nanoTiO<sub>2</sub> and nanoAg, respectively. Groups V and VI consisted of 2.0% sodium fluoride and 0.2% chlorhexidine mouthwashes, respectively as controls. We used serial dilution method to find minimum inhibitory concentrations (MICs) and with subcultures obtained minimum bactericidal concentrations (MBCs) of the solutions against *S. mutans* and *S. sangius*. The data were analyzed by analysis of variance and Duncan test and  $P < 0.05$  was considered as significant.

**Results:** The sodium fluoride mouthrinse did not show any antibacterial effect. The nanoTiO<sub>2</sub>-containing solution had the lowest MIC against both microorganisms and also displayed the lowest MBC against *S. mutans* ( $P < 0.05$ ). The colloidal solutions containing nanoTiO<sub>2</sub> and nanoZnO showed the lowest MBC against *S. sangius* ( $P < 0.05$ ). On the other hand, chlorhexidine showed the highest MIC and MBC against both *streptococci* ( $P < 0.05$ ).

**Conclusion:** The nanoTiO<sub>2</sub>-containing mouthwash proved to be an effective antimicrobial agent and thus it can be considered as an alternative to chlorhexidine or sodium fluoride mouthrinses in the oral cavity provided the lack of cytotoxic and genotoxic effects on biologic tissues.

**Key Words:** Mouthrinse, nanoparticle, *Streptococcus mutans*, *Streptococcus sangius*

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## INTRODUCTION

Dental caries and periodontal problems are among the prevalent oral diseases throughout the world. Acidogenic bacteria such as *Streptococcus mutans*,

*Streptococcus sangius* and *Lactobacilli* are considered as the contributory factors of dental caries.<sup>[1]</sup> *S. sangius* is also involved in periodontal problems. Mechanical methods such as tooth brushing are effective for plaque removal, but they are directly dependent on personal skills. Furthermore, effective tooth brushing is problematic in disabled or traumatized patients. The use of adjunctive methods such as mouthwashes has been shown to be effective for prevention of plaque accumulation.<sup>[2]</sup> Routine mouthrinses like chlorhexidine, however, are associated with the disadvantages including enamel staining, taste

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disturbances and mucosal irritation.<sup>[3,4]</sup> Therefore, searching for an alternative antimicrobial agent with minimal side effects seems to be quite reasonable.

Metal nanoparticles have long been used in medicine because of their bactericidal and bacteriostatic effects.<sup>[5-7]</sup> Nanotechnology has been introduced to the field of dental materials in recent years and nanoparticles have been inserted into the structure of the dental composites<sup>[8,9]</sup> and disinfection solutions.<sup>[10]</sup> The antibacterial properties of metal ions depend on their surface contact area. Decreased size of nanoparticles (<100 nm in diameter) leads to increased surface area and thus increased interaction with organic and inorganic molecules. However, many of the properties of metal nanoparticles are still unknown.<sup>[11]</sup> For example, cytotoxic properties of nanoparticles still need further research. Moreover, bioavailability and stability of nanoparticles as therapeutic delivery systems should be investigated. In addition, discoloration effects and cosmetic changes of some nanoparticles need further clarification.

Until now, there are only few studies that have determined the antimicrobial effects of nanoparticles against cariogenic and periodontal disease bacteria in simulated oral conditions.<sup>[9,12]</sup> The present study aimed to investigate the bactericidal and bacteriostatic effects of colloidal solutions containing zinc oxide (ZnO), copper oxide (CuO), titanium dioxide (TiO<sub>2</sub>) and silver (Ag) nanoparticles on *S. mutans* and *S. sanguis* and to compare the results with those of chlorhexidine and sodium fluoride mouthrinses.

## MATERIALS AND METHODS

Nanoparticles including ZnO (nanoZnO), CuO (nanoCuO), TiO<sub>2</sub> (nanoTiO<sub>2</sub>) and Ag (nanoAg) were purchased from Fanavaran Araz Tajhiz Co., Iran. According to the supplier, nanoparticles were more than 98% pure after ignition. The nanoparticles were added to a water based-solution in pharmaceuticals laboratory of Faculty of Pharmacy of Mashhad University of Medical Sciences, Mashhad, Iran. The nanoparticles were characterized by ultraviolet-visible spectroscopy (Shimatzu) and further examined by a particle — size analyzer (Zetasizer [Nano-zs] by Malvern) to find out their size distribution. Mean size of the nanoparticles ranged from 40 to 60 nm for nanoTiO<sub>2</sub> and nanoCuO, 50-60 nm for nanoAg and 20 nm for nanoZnO. Colloidal solutions containing nanoparticles were prepared with initial concentration

of 25 ppm and were sterilized in gravity autoclave before antimicrobial tests.

The study included six groups of mouthwashes. Groups I to IV included colloidal solutions containing nanoZnO, nanoCuO, nanoTiO<sub>2</sub> and nanoAg, respectively. Groups V and VI consisted of 2.0% sodium fluoride and 0.2% chlorhexidine mouthwashes, respectively, which served as controls.

### Preparation of bacterial suspensions

Antimicrobial experiments were carried out with *S. mutans* (PTC 1683) and *S. sanguis* (PTCC 1449) procured from BuAli Research Institute, Mashhad, Iran. They were subculture in 5% sheep's blood agar. At first, 5-6 colonies from an overnight culture were diluted in brain heart infusion broth and were incubated in an aerobic environmental condition for 1-2 h at 35°C to reach the concentration of  $1.5 \times 10^8$  CFU/ml. The colonies were then diluted with saline solution to a final concentration of  $1.5 \times 10^6$  CFU/ml.

The lowest concentration of each antimicrobial agent that inhibits the growth of the microorganisms being tested is known as minimum inhibitory concentration (MIC) and is detected by lack of turbidity matching with a negative control. Furthermore, the minimum bactericidal concentration (MBC) is defined as the lowest concentration of an agent killing the majority of bacterial inoculums.<sup>[12,13]</sup>

The MICs and with subcultures the MBCs of *S. mutans* and *S. sanguis* were determined from a known concentration of nanoparticles or mouthwashes in micrograms per milliliter, using the liquid microdilution method. In order to mimic the clinical conditions, artificial saliva was used for serial dilution. The cutoff points were compared to those described by the National Committee for Clinical Laboratory Standards, at the Clinical and Laboratory Standards Institutes.

For antibacterial experiments, 0.5 ml of diluted microorganisms was placed in tubes containing different concentrations of each nanoparticle and was incubated overnight at 35-37°C in a closed environment. Determination of MIC was based on the turbidity measured by spectrophotometer (Eppendorf AG, Hamburg, Germany). After determining the MIC, 50 ml of the corresponding bacterial suspension was spread in sheep's blood agar and was incubated at 37°C for 24 h. The numbers of colonies growing from each of the test tubes were counted and the

number of colonies corresponding to a thousand-fold reduction was recorded as the MBC. All experiments were conducted in triplicate for each concentration.

To determine the required time before initiating bactericidal effect, 50 ml of each test specimen was mixed with 50 ml of the bacterial suspensions (containing  $5 \times 10^3$  colonies). After 1 and 5 min, it was cultured on blood agar. Following overnight incubation at 37°C, the remaining colonies were counted. One-way analysis of variance (ANOVA) was run to determine any significant differences in MIC and MBC of the study groups, followed by Duncan multiple range test for pairwise comparisons. The statistical analysis was performed through SPSS (Statistical Package for Social Sciences, version 16, Chicago, IL, USA) and the significance level was determined at  $P < 0.05$ .

## RESULTS

Table 1 presents the means and standard deviations regarding MICs and MBCs of the study groups

against *S. mutans* and *S. sanguis*. The sodium fluoride mouthrinse did not show antibacterial effects against any of the microorganisms. The colloidal solution containing TiO<sub>2</sub> nanoparticles had the lowest MIC against *S. mutans* and *S. sanguis* and also displayed the lowest MBC against *S. mutans*. Furthermore, the solutions containing nanoTiO<sub>2</sub> and nanoZnO showed the lowest MBC against *S. sanguis*. On the other hand, the highest MIC and MBC against both *streptococci* pertained to the 0.2% chlorhexidine mouthrinse [Table 1].

ANOVA revealed a significant difference between MICs and MBCs of the study groups against *S. mutans* and *S. sanguis* [Table 1]. Between-group comparisons by Duncan test demonstrated that the MICs and MBCs of the colloidal solutions containing nanoparticles were comparable to each other and all were significantly lower than that of the chlorhexidine mouthrinse against both microorganisms [Table 1].

Table 2 demonstrates the number of colonies of *S. mutans* and *S. sanguis* after 1 and 5 min of

**Table 1: Mean MIC and MBC (µg/ml) of the test groups against *S. mutans* and *S. sanguis* and the results of statistical analysis for comparison between groups**

Group	<i>S. sanguis</i>				<i>S. mutans</i>			
	MBC	Pairwise comparisons	MIC	Pairwise comparisons	MBC	Pairwise comparisons	MIC	Pairwise comparisons
nanoZnO	0.0976	a	0.0976	a	3.1250	a	0.3906	a
nanoCuO	0.1627	a	0.0976	a	25	a	12.5	a
nanoTiO <sub>2</sub>	0.0976	a	0.0488	a	0.1953	a	0.0976	a
nanoAg	0.1302	a	0.0976	a	25	a	25	a
Chlorhexidine	2.438	b	0.9765	b	83.33	b	62.5	b
P value	<0.001		<0.001		<0.001		<0.001	

*S. mutans*: *Streptococcus mutans*; *S. sanguis*: *Streptococcus sanguinis*; MBC: Minimum bactericidal concentration; MIC: Minimum inhibitory concentration; nanoZnO: Nanoparticles including zinc oxide; nanoCuO: Nanoparticles copper oxide; nanoTiO<sub>2</sub>: Nanoparticles titanium dioxide; nanoAg: Nanoparticles silver; a, b- Different letters shows significant difference between two groups revealed by Duncan test

**Table 2: The number of colonies of *S. mutans* and *S. sanguis* after 1 and 5 min of exposure to each solution and the results of statistical analysis for comparison between groups**

Group	<i>S. sanguis</i>				<i>S. mutans</i>			
	1 min		5 min		1 min		5 min	
	Mean ± SD	Pairwise comparisons	Mean ± SD	Pairwise comparisons	Mean ± SD	Pairwise comparisons	Mean ± SD	Pairwise comparisons
nanoZnO	1000±200	b	566±404	a	2900±655	b	1153±799	b
nanoCuO	1833±288	b	1066±115	a	2400±529	b	1533±757	b
nanoTiO <sub>2</sub>	406±53	a	206±24	a	3033±550	b	1366±57	b
nanoAg	1933±503	b	1433±208	a	4333±1154	c	3000±25	c
Sodium fluoride	3666±330	c	3666±239	b	4666±577	c	4666±577	c
Chlorhexidine	0	a	0	a	0	a	0	a
P value	<0.001		0.001		<0.001		<0.001	

nanoZnO: Nanoparticles including zinc oxide; nanoCuO: Nanoparticles copper oxide; nanoTiO<sub>2</sub>: Nanoparticles titanium dioxide; nanoAg: Nanoparticles silver; SD: Standard deviation; a, b, c- Different letters shows significant difference between two groups revealed by Duncan test

bacterial exposure to each colloidal solution or mouthwash. Sodium fluoride mouthrinse showed the largest colony count after both 1 and 5 min of bacterial exposure. In contrast, no *S. mutans* and *S. sanguis* colonies were observed after 1 and 5 min of exposure to chlorhexidine mouthwash [Table 2]. There was no significant difference in the number of *S. mutans* colonies between nanoTiO<sub>2</sub>, nanoZnO and nanoCuO mouthwashes after 1 and 5 min. However, significantly fewer *S. mutans* colonies were observed in these groups compared to the sodium fluoride mouthrinse and nanoAg colloidal solution [Table 2].

Regarding *S. sanguis*, significantly fewer colonies were counted in chlorhexidine mouthwash and nanoTiO<sub>2</sub>-containing solution after 1 min compared to the other groups. After 5 min, the number of *S. sanguis* colonies in all nanoparticles groups was comparable to that of chlorhexidine.

## DISCUSSION

*S. mutans* is known as a main etiological factor in dental caries. Also, *S. sanguis* plays a vital role in dental plaque formation and thus dental caries and periodontal problems.<sup>[14]</sup> Application of mouthrinses has been proposed as an adjunct to mechanical methods of plaque removal. Sodium fluoride and chlorhexidine are among the routine mouthwashes used for prevention of dental caries or periodontal problems.<sup>[2]</sup> Discoloration of restorations, unfavorable taste, allergy and xerostomia are some of the disadvantages of chlorhexidine.<sup>[3,4]</sup> Furthermore, it damages the microflora of the oral cavity due to its long-lasting effects.<sup>[15]</sup>

In the present study, the serial dilution method was used to determine the MICs of the test groups. This method is more accurate compared with the disc diffusion test and is more easily interpreted.<sup>[15]</sup> The artificial saliva was used in the test tubes to reveal any probable effect of proteins and other salivary constituents on the antibacterial activity of nanoparticle containing solutions.

Antibacterial properties of some nanoparticles such as silver and gold have been verified in previous studies<sup>[12,16]</sup> and different mechanisms have been proposed for their effects. Nanosilver inhibits the enzymes of the cell respiratory cycle and damages the deoxyribonucleic acid (DNA) synthesis.<sup>[17,18]</sup> Hernández-Sierra *et al.*<sup>[12]</sup> indicated that nanosilver inhibits the growth of *S. mutans* at lower

concentrations compared to nanoZn and nanoAu and thus it may be more effective against dental caries. Although, the antibacterial effect of Zn nanoparticles against *S. mutans* has been demonstrated, its mechanism of action is still unknown.<sup>[19]</sup> It is assumed that the mechanism of action of nanoCu is similar to that of nanosilver. Cu ions adhere to DNA molecules and form cross links within and between nucleic acid chains and thus disrupting the helical structure of the nucleus. Moreover, Cu ions impair the biochemical processes of bacterial cells. Combination of silver and copper nanoparticles may give rise to a more complete bactericidal effect against mixed bacterial populations.<sup>[20]</sup> TiO<sub>2</sub> nanoparticles show photocatalytic characteristics and prevent the accumulation of pathogenic bacteria.<sup>[21]</sup>

Most of the previous studies investigated the antibacterial properties of nanosilver and there is limited data available on the bactericidal properties of other nanoparticles, especially when they are prepared in colloidal solutions as mouthwashes. Jung *et al.*<sup>[22]</sup> obtained an average MIC of 50 µg/ml against *S. mutans* for silver nanoparticles which were twice that of our finding. This difference can be attributed to the method of disc diffusion test that they used to find the MIC. The contact area of nanoparticles with bacterial microorganisms is higher in serial dilution method compared to the culture media, thus increasing their antibacterial effect. Hernández-Sierra *et al.*<sup>[12]</sup> in their study have reported an average MIC of 4.86 ± 2.71 mg/ml against *S. mutans* for nanoZnO, which was somewhat higher than that achieved in this study (3.12 ± 0.390 µg/ml). Sadeghi *et al.*<sup>[10]</sup> evaluated the antimicrobial effect of chlorhexidine against *S. sanguis* and found an average MIC of 256 µg/ml, which was higher compared to that found in the present study (83.33 µg/ml) possibly due to the different concentration of chlorhexidine (0.12%) they employed.

In this study, the nanoTiO<sub>2</sub>-containing solution resulted in less number of *S. sanguis* after 1 min of exposure compared to other nanoparticle-containing solutions and its antibacterial effect was comparable to that of chlorhexidine. The solutions containing nanoCuO, nanoZnO and nanoTiO<sub>2</sub> resulted in less number of *S. mutans* colonies after 1 and 5 min of bacterial exposure in comparison to the solution including silver nanoparticles. However, the antibacterial effects of all the nanoparticle groups were significantly lower than that of the 0.2% chlorhexidine mouthrinse

against *S. mutans*. This is in contrary to the results of Sadeghi *et al.*<sup>[10]</sup> who showed that nanosilver had bactericidal effects against *S. mutans* after 30 s, which was comparable to that of chlorhexidine.

In the present study, the antibacterial effect of silver nanoparticles was not desirable against *S. mutans*. It is possible that nanoAg particles adhere to each other and form micrometer particles at high concentrations, which leads to less antimicrobial activity. The solutions containing TiO<sub>2</sub> and ZnO nanoparticles inhibited the development of the *S. sangius* strain at a lower concentration than other test groups. In general, development of *S. sangius* was inhibited at lower concentrations of antimicrobial agents compared to *S. mutans* colonies. The MIC and MBC of the nanoTiO<sub>2</sub> colloidal solution against *S. mutans* was found to be 640 and 427 times lower than that of the chlorhexidine. In other words, nanoTiO<sub>2</sub> colloidal solution affected *S. mutans* at significantly lower concentrations, which may allow achieving clinical effects with reduced side effects. Therefore, TiO<sub>2</sub> nanoparticles are promising as antimicrobial agents to be inserted in mouthrinses and be used as an alternative to routine antibacterial mouthwashes. However, detailed research and comparative study of strain specific variability is required to determine its bactericidal efficiency. Furthermore, its biocompatibility should be further investigated before commercialization.

It should be noted that complete simulation of the oral cavity is not possible in the laboratory conditions. The incubator cannot completely resemble the mouth temperature. Furthermore, the antibacterial agents contact constantly with bacterial microorganisms in the culture media or test tubes, but the contents of mouthwashes are diluted and neutralized immediately in the oral cavity.

Further studies are warranted to elucidate the antimicrobial effects of nanoparticle solutions when used as mouthwashes under *in vivo* conditions and any possible side-effects of these solutions on oral microflora. Future investigations can be designed to reveal the bactericidal properties of combinations of various nanoparticles on different microorganisms. Furthermore, bioavailability of nanoparticle containing mouthwashes compared to commercially available ones need more investigations.

## CONCLUSION

The solution containing TiO<sub>2</sub> nanoparticles showed the lowest inhibitory concentration against *S.*

*mutans* and *S. sangius* compared to those of other nanoparticle containing solutions and chlorhexidine mouthwash and thus it may be further investigated as an alternative to chlorhexidine.

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