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Antimicrobial Effect of Different Sizes of Nano Zinc Oxide on Oral Microorganisms

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A B S T R A C T

Objectives: The purpose of the present study was to evaluate the antimicrobial effect of various sizes and concentrations of zinc oxide (ZnO) nanoparticles on *Streptococcus mutans* (*S. mutans*), *Enterococcus faecalis* (*E. faecalis*), *Lactobacillus fermentum* (*L. fermentum*), and *Candida albicans* (*C. albicans*).

Materials and Methods: Solutions at the concentration of 10 µg/ml were prepared using 20-nm, 40-nm, and 140-nm nano ZnO (nZnO) powder. The antimicrobial effect of nZnO was determined using the disk diffusion method. The inhibition zone (mm) was measured using a ruler. Data were analyzed by analysis of variance (ANOVA) and the Bonferroni correction. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of nZnO were determined using the broth microdilution method in Mueller-Hinton Agar (MHA) for *S. mutans* and *E. faecalis*, De Man, Rogosa, and Sharpe (MRS) agar, and Sabouraud Dextrose Agar (SDA).

Results: The greatest inhibition zones were observed against *S. mutans* with 20-nm and 40-nm nZnO, while 140-nm nZnO formed the greatest inhibition zones against *S. mutans* and *E. faecalis*. The smallest inhibition zones were observed against *C. albicans* with the three nZnO particle sizes. The MICs for *C. albicans* with 40-nm and 140-nm particles and for *L. fermentum* with 140-nm particles were higher than 10 µg/ml. A significant correlation was found between the particle size and the antibacterial activity against *S. mutans* (P=0.00), *L. fermentum*, and *E. faecalis* (P<0.02).

Conclusion: The antimicrobial activity of nZnO increases with decreasing the particle size. The greatest antimicrobial effect was observed against *S. mutans* and *E. faecalis*. *S. mutans* is more sensitive to the changes in the particle size compared to other bacteria.

Keywords: Zinc Oxide; Disk Diffusion Antimicrobial Tests; Nanoparticles

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INTRODUCTION

Nanoparticles usually measure between 1 and 100 nm in at least one dimension [1], with properties such as high stability and the ability to change the surface properties [2]. Inorganic antibacterial agents are more durable and less toxic and have better selective performance and resistance to heat in comparison with organic antibacterial agents. Various inorganic antibacterial agents have been used to overcome antimicrobial resistance in pathogens [3]. Zinc oxide (ZnO) and nano ZnO (nZnO) are considered as antibacterial agents with a strong deterrent nature. ZnO has unique optical, electrical, and chemical properties [4]. Tooth decay is the most common infectious disease characterized by the demineralization of dental hard tissues due to acid fermentation of

carbohydrates by bacteria [5]. *Streptococcus mutans* (*S. mutans*) is one of the main bacterial species responsible for tooth decay [6]. *S. mutans* is anaerobic at pH levels lower than 5.5. It is the most common bacterium found in cariogenic plaque, which produces short-chain organic acids that metabolize sucrose and lead to the synthesis of extracellular polysaccharides, promoting bacterial adhesion to dental surfaces and reinforcing the biofilm; moreover, it is the most common bacteria found in cariogenic plaque [7,8]. Several studies have argued that among metallic materials, metallic silver nanoparticles are the most effective nanoparticles in preventing the growth of *S. mutans* [9]. However, the primary problem of silver in restorative materials is related to the discoloration of tooth-colored materials [10].

Several methods have been used to prevent the growth of biofilms in resin composite samples using chlorhexidine which successfully inhibits microbial growth [11-14]; however, since chlorhexidine has a high solubility, it has a short-term effect on biofilms [15,16]. Therefore, insoluble materials and tooth-colored and colorless metal oxide powders such as silica, zirconia, and ZnO may be more effective [17]. Lactobacilli are isolated from deep carious lesions in the oral cavity and are the dominant flora in deep cavities [18]. These bacteria are considered as the pioneer in the development of caries, especially in dentin, and have an antagonistic effect against periodontal pathogens. The inhibition of their growth is through the production of acids (mainly lactic acid) which lowers the environment's pH and release hydrogen peroxide (H2O2) and bacteriocins, thereby playing a key role in maintaining the balance of antimicrobials in the mouth and the digestive system [18-29].

Enterococcus faecalis (*E. faecalis*) is an optionally anaerobic gram-positive coccus; it is the most dominant species in endodontically treated teeth. *E. faecalis* has the ability to adhere to teeth and to form biofilms under harsh environmental conditions [30-36]. *E. faecalis* and *Candida albicans* (*C. albicans*) are frequently isolated from teeth after endodontic retreatment and from root canals with persistent infections [37,38]. The prevalence of *C. albicans* increases with age; it is associated with dental caries in children and adolescents and has a role in the progression of caries [39,40]. The antimicrobial effects of different concentrations and sizes of nZnO particles have been examined in different studies, but precise information about the most appropriate nontoxic concentration in the oral cavity that is effective against microbes is not available. The aim of the present study was to determine the antimicrobial effect of different concentrations and sizes of nZnO particles on *S. mutans*, *E. faecalis*, *Lactobacillus fermentum* (*L. fermentum*), and *C. albicans.*

MATERIALS AND METHODS

In this in-vitro experimental study, the standard strains of *S. mutans* (ATCC 35668), *E. faecalis* (ATCC 29212), *L. fermentum* (ATCC 14931), and *C. albicans* (ATCC 10231) were examined.

The *S. mutans* and *E. faecalis* strains were obtained from the Department of Microbiology at the School of Medicine of Yazd University of Medical Sciences, Yazd, Iran, and the strains of *C. albicans* and *L. fermentum* were obtained from the Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The nZnO powder (Research Nanomaterials Inc., Houston, TX, USA) was prepared with different sizes of 20 nm, 40 nm, and 140 nm and was dissolved in distilled water; accordingly, a solution at the concentration of 10 µg/ml was prepared [2]. The standard strains of *S. mutans* and *E. faecalis* were cultured on a blood agar medium supplemented with 5% defibrinated sheep blood. For *L. fermentum*, a 24-hour De Man, Rogosa, and Sharpe (MRS) culture medium at 37°C was prepared. In order to obtain 48-hour *C. albicans* cultures at 25°C, Sabouraud Dextrose Agar (SDA) was used. Next, from the cultures of the three bacterial species, suspensions with 0.5 McFarland turbidity, holding 1.5×10^8 colony-forming units (CFU)/ml, were obtained. To prepare the *C. albicans* suspension, some colonies were removed from SDA medium, and a suspension holding 1×10^6 cell/ml was prepared in a microtube containing phosphate buffered saline (PBS). *S. mutans* and *E. faecalis* bacterial suspensions were cultured in plates containing Mueller-Hinton Agar with 5% sheep blood, while *L. fermentum* and *C. albicans* suspensions were cultured in plates containing SDA and MRS agar, respectively. Afterward, a 6-mm-diameter blank disc was impregnated with 0.01 ml of nZnO solution with different particle sizes, dried before testing, and placed on the media such that the distance between the discs was 24

mm, and the distance to the wall of the plate was 15 mm. After 15 minutes, the plates were incubated and cultured, and the inhibition zone (mm) was measured using a ruler. Gentamicin and ampicillin were used as controls for the tested bacteria, while fluconazole was used as a control for *C. albicans*. A plate without nZnO, a plate lacking bacteria, and a sterile plate containing distilled water were also used as controls.

The minimum inhibitory concentration (MIC) of nZnO was determined using microdilution methods. Different sizes of nZnO were first dissolved in distilled water to prepare solutions at the concentration of 10 µg/ml. After mixing for 5 seconds and transferring to a bath sonicator (D-78224, Elma Schmidbauer GmbH, Singen, Germany) for 30 minutes at 0ºC, a homogeneous solution was obtained. The solution was mixed further using the IKA Vortex Mixer (type VF2; Janke & Kunkel, Germany).

Suspensions at concentrations ranging from 0.156-10µg/ml were prepared from different sizes of nZnO. Bacterial suspensions with 0.5 McFarland turbidity and 1.5×10^5CFU/ml were obtained.

The *C. albicans* suspension was also prepared by following the procedures described above 100 µl of the broth, 50 µl of the microbial suspensions, and 50 µl of different concentrations of nZnO were poured into each well of 96-well microplates. The microplates were incubated for 24 hours at 37°C for the bacteria, and for 48 hours at 25°C for *C. albicans*. The microbial growth was checked using a spectrophotometer (UV-150-02; Shimadzu Co., Tokyo, Japan) at a 540-nm wavelength, and the concentration of nZnO that prevented the growth of bacteria and fungi was considered as the MIC

For determining the MIC, Mueller-Hinton Agar liquid medium, MRS, and SDA were used.

To determine the minimum bactericidal concentration (MBC), 0.1 ml of the media in the MIC wells with no microbial growth was inoculated into each medium containing bacteria and fungi, and after incubation, the plates containing the lowest concentration resulting in no microbial growth were indicative of the MBC and the minimum fungicidal concentration (MFC) for the bacteria and *C. albicans*, respectively. Due to the fact that the study was performed in vitro, at least three replicates were necessary [41]. Three sizes of nZnO and four microorganisms were examined (a total of 36 bacterial and fungal plates). Data were entered into SPSS 16 software (SPSS Inc., Chicago, IL, USA). To test the normality of the data, Kolmogorov-Smirnov test was used. To compare the mean of the groups, we used analysis of variance (ANOVA), while the Bonferroni correction was used for pairwise comparisons.

RESULTS

The largest inhibition zones against *S. mutans* were observed with 20-nm and 40-nm nZnO, while the largest inhibition zones against *S. mutans* and *E. faecalis* were observed with 140-nm nZnO. The smallest inhibition zones were noted against *C. albicans* with the three sizes of nZnO (Fig. 1). According to ANOVA, the different sizes of nZnO had no statistically significant differences in terms of the size of the inhibition zone against *C. albicans* (P=0.226). According to the Bonferroni test, the three sizes of nZnO showed statistically significant differences in terms of the size of the inhibition zone against *S. mutans* (P<0.001).

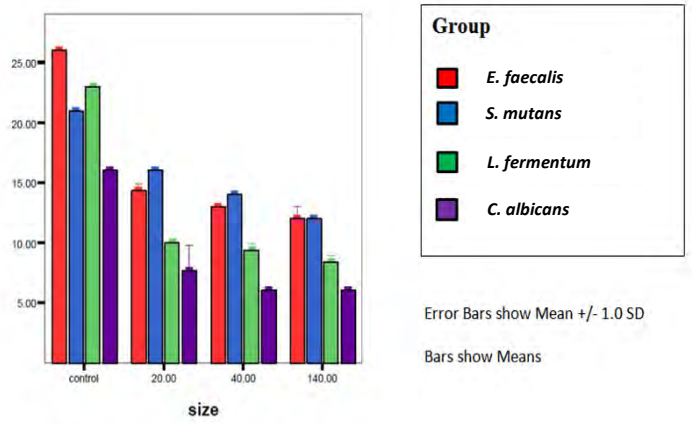

Fig. 1: Error bar of means and standard deviations of microbial inhibition zone diameter (mm) formed by 20-nm, 40-nm, and 140-nm nano zinc oxide and the controls against the studied microorganisms; SD: Standard Deviation

Regarding *L. fermentum* (P=0.015) and *E. faecalis*, only 20-nm and 140-nm particles showed statistically significant differences (P=0.016). The difference between the antibiotics (the control) and the three sizes of nZnO was significant with regard to each microorganism (P<0.001; Table 1). Considering the resulting MIC and MBC, the antimicrobials had the greatest impact on *E. faecalis* and *S. mutans*, respectively. The MICs for *C. albicans* with 40-nm and 140-nm particles and for *L. fermentum* with 140-nm particles were higher than 10 µg/ml (Table 2).

DISCUSSION

The inherent properties of metal nanoparticles, such as ZnO, are primarily determined according to the size, composition, and morphology. Reducing the size of a nanoscale particle can change its chemical, mechanical, electrical, structural, and optical properties.

Nano-particles modified with biomolecules have a high surface area to volume ratio; the physical delivery of nanoparticles into the internal structures of the cells has been facilitated [42,43], which features the antibacterial and antifungal properties of nZnO, production of reactive oxygen species (ROS), such as H2O2 and superoxide ions (-O2), which have important biological applications [44]. The size and concentration of nZnO play an important role in the antibacterial activity. H_2O_2 production mainly depends on the surface area of nZnO; larger surface areas and higher concentrations of smaller particles may provide additional antibacterial activity [45]. In the current study, the smallest size (20 nm) of nZnO particles exhibited the greatest antimicrobial effect. However, the four microorganism species were less sensitive to nZnO in all three sizes than to the control antibiotics, which is consistent with the results of previous studies [45-50].

S. mutans, in comparison with other bacteria, is more sensitive to the changes in the size of nZnO; this may be due to the ability of microorganisms to produce active species such as H_2O_2 and to the differences in their intrinsic resistance and infectivity [49,51].

Kasraei et al [52] tested the antimicrobial properties of composites containing 50-nm nZn (1 wt%) and 20-nm nanosilver (1 wt%) against lactobacilli and *S. mutans*. The results showed that the antibacterial effect of nZn against *S. mutans* was significantly greater than that of nanosilver, while no significant differences were observed between nanosilver and nZn against lactobacilli [52]. In our study, full-size nZn had a significantly greater antimicrobial effect on *S. mutans* than on *L. fermentum*.

Mirhashemi et al [7] also reviewed the antimicrobial effect of chitosan in combination with dental composites and nZnO at the concentrations of 1, 5, 10, and 0 wt% in the control group against *S. mutans*, *Lactobacillus acidophilus*, and *Streptococcus sanguinis*. The

Table 1: Comparison of the mean±standard deviation (SD) diameter (mm) of the inhibition zones formed by different sizes of nano zinc oxide (nZnO) and the controls against each microorganism

Microorganism	Inhibition zone diameter				P value
	20-nm nZnO	40-nm nZnO	140-nm nZnO	Control	
Streptococcus mutans	16±0.00 [a*]	14±0.00 [b]	12.03±0.57 [c]	21±0.00 [d]	<0.001
Lactobacillus fermentum	10±0.00 [a]	9.33±0.57	8.33±0.57 [b]	23±0.00 [d]	<0.001
Enterococcus faecalis	14.33±0.57 [a]	13±0.00	12±1.00 [b]	26±0.00 [d]	<0.001
Candida albicans	7.66±2.08 [a]	6±0.00 [a]	6±0.00 [a]	16±0.00 [d]	<0.001

*Different letters indicate that the significant differences in each row are small; SD: Standard Deviation

Table 2: Mean minimum inhibitory concentration (MIC; µg/ml) and minimum bactericidal (or fungicidal) concentration (MBC or MFC; µg/ml) of different sizes of nano zinc oxide (nZnO) in the studied microorganisms

Microorganism	20-nm nZnO		40-nm nZnO		140-nm nZnO	
	MIC	MBC	MIC	MBC	MIC	MBC
Streptococcus mutans	0.625	1.25	1.04	2.08	1.66	3.33
Enterococcus faecalis	0.312	0.625	0.625	1.25	1.25	2.5
Lactobacillus fermentum	5	10	10	>10	>10	>10
Candida albicans	10	MFC>10	>10	MFC>10	>10	MFC>10

results showed a decrease in the number of *S. mutans* colonies at the concentrations of 5 and 10 wt%, whereas the number of lactobacilli only decreased at the concentration of 10 wt% [7]. In the present study, based on the results of the MIC, a greater number of *S. mutans* bacteria were eliminated, in comparison with *L. fermentum*, at lower concentrations of nZnO (in all sizes).

In the present study, *E. faecalis*, in comparison with other microorganisms, was eliminated by lower concentrations of nZnO (in all sizes). The high antibacterial effect of nZnO on *E. faecalis* and *S. mutans* can be attributed to the production of active oxygen species (AOS) which adhere to the cell surface or accumulate in the cell cytoplasm.

Ghaderian et al [53] examined the effect of nZnO on Escherichia coli (E. coli) and *E. faecalis* and found no difference between the elimination of these two bacteria by 100-nm and 5-nm particles. E. coli was more sensitive to both sizes of nanoparticles and was eliminated even at lower concentrations of nZnO. The reason for this difference can be attributed to the type of *E. faecalis* coverage as gram-positive cocci have multiple peptidoglycan layers and are more intrinsically resistant than gram-negative *E. coli* [53].

It is likely that nZnO disturbs a two-layered lipid membrane in fungal organisms, thereby interfering with the cellular functions and destroying the fungal hyphae to prevent the growth of the fungus [54,55].

In our study, the 20-nm nZnO exhibited a very narrow inhibition zone against *C. albicans*, and the MIC for nanoparticles in this size was equal to 10 µg/ml.

The difference between the different sizes of nZnO in terms of the inhibition zone diameter against this fungus was not significant; this can be attributed to the low concentration of the used nZnO. In spite of the fact that Palanikumar et al [41] used the sizes of 15 nm and 25 nm and the density of 38 (200 µg/ml) of nZnO against *C. albicans* in their study, the results related to inhibition zones showed that *C. albicans* is less sensitive toward nZnO than toward the control antibiotics. In addition, the MIC of nZnO against *C. albicans* in all nanoparticle sizes was reported to be 200 µg/ml [41].

Yousef and Danial [2] reviewed the anti-microbial activity of ZnO and nZnO against multiple strains of pathogens including *C. albicans*, Aspergillus niger, and E. coli, and reported the MIC of nZnO against *C. albicans* to be 10 µg/ml, whereas the lowest antimicrobial effect was found against *C. albicans* and Aspergillus niger [2]. The difference in the reported MIC among various articles may be due to the difference in the methods of preparing the nZnO and the differences in the size of the particles.

In the present study, the sensitivity of *C. albicans* toward nZnO was much lower than to the control antibiotic; this is likely due to the use of Zn metal ions as a coenzyme in the regulation of the metabolic functions and the stability of the structure of the fungal enzymes [45]. However, the presence of too many metal ions can cause cytotoxicity. The findings of the present study suggest the usage of 20nm nZnO at concentrations in the range of 0.312 to 10 µg/ml to control the spread of oral bacterial infections. However, the toxicity to the environment and human cells should be precisely evaluated.

Further studies on high concentrations of nZnO

are suggested in order to investigate the antifungal effects. In addition, the effect of different methods of preparation of nZnO solutions on the antimicrobial activity should be further examined.

CONCLUSION

According to the results, the antimicrobial activity of nZnO increased with a decrease in the size of nanoparticles. *S. mutans* and *E. faecalis* were more sensitive to nZnO in comparison with other microorganisms. *S. mutans* was more sensitive to the changes in the size of nZnO particles.

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CONFLICT OF INTEREST STATEMENT

None declared.

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