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Antibacterial Activity of a Series Engineering Nanoparticles Against Oral Anaerobic Periodontal Pathogens Species in Vitro

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Abstract

Objective: Periodontal disease is an essential phenomenon in human health. Oral pathogens can cause severe break which may show the way to serious issues in human disease like chronic obstructive pulmonary disease and cardiovascular diseases. Therefore, the aim of this study is to evaluate the antibacterial effect of a series nanoparticles on oral pathogens.

Methods: In this study, antibacterial activity of a series of nanoparticles such as MWCNT, CuO2, CaCO3, SiO2, Al2O3, MgO and ZrO2 against oral pathogens such as *Porphyromonas gingivalis* (Pg) and *Aggregatibacter actinomycetemcomitans* (Aa) was demonstrated. We evaluated the bactericidal effect of the nanoparticles to perio pathogens by measuring the inhibitor zone region. Antimicrobial experiments were conducted in five replicates.

Results: As a result, we confirmed that engineering nanoparticles exhibited good bactericidal activity. SiO2 nanoparticle was the most effective on Pg. CaCO3 nanoparticle was the most effective on Aa. The order of the nanoparticle types in which the Pg is most sensitive is SiO2> MgO> Al2O3> ZrO2> CuO> MWCNT> CaCO3. For Aa order is CaCO3> SiO2>MgO> ZrO2> CuO> MWCNT> Al2O3.

Conclusion: Our results suggest that engineering nanoparticles have a significant inhibitory effect on Aa and Pg. And, these effects are increased with increasing concentrations of nanoparticles. These results can be further clarified with new studies.

Keywords: Antibacterial activity, *Aggregatibacter actinomycetemcomitans*, engineering nanoparticles, *Porphyromonas gingivalis*

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INTRODUCTION

Recently, nanotechnology offers superior advantages in various fields of science and technology. Pharmaceutical nanotechnology with these advantages has begun to attract the attention of many researchers (1,2). Because highly ionic nanoparticulate metal oxides are particularly interesting as antimicrobial agents, they can be prepared with unusual crystal morphologies that have a large number of surfaces and corners and other potentially reactive sites (3,4). Thus, metal-containing nanomaterials have the potential to be used for infection control in dentistry, but little is known about their antibacterial properties. (5,6). In particular, the molecular mechanisms of the inhibitory effect of silver ions in silver nanoparticles on microorganisms have been described. Accordingly, the expression of DNA loses its ability to proliferate, and that other ribosomal subunit proteins and other cellular proteins and enzymes required for it are lost and ATP production is disabled (4,7,8). Therefore, it is foreseen that metal oxide nanoparticles with antimicrobial activity (nanoanthibiotics) may provide a good alternative to reduce and / or control the growth of bacteria in the oral cavity (4,9). Gingivitis is a gingival inflammation due to microbial dental plaque accumulation in gingival margin. Periodontitis is the most common chronic inflammatory disease in the society characterized by loss of gums and bone, which can develop when gingivitis is not treated (10). Bacteria in the microbial dental plaque on one hand directly damage the host tissue with the products they secrete, while at the same time, they induce tissue destruction by activating the host tissue immune system (11). In this respect, the quantitative and qualitative reduction of the bacteria in the plaque is essential for the health of the periodontal tissues (12). In addition, proliferation of pathogenic bacteria in the mouth leads to periodontitis, an inflammatory disease that is a risk factor for other systemic diseases such as chronic obstructive pulmonary disease and cardiovascular diseases (9,13).

In vitro studies have shown that certain metal nanoparticles inhibit some microbial species. Various nanoparticles, composites and derivatives have attracted great interest for their potential antimicrobial effects. In particular, metal nanoparticles such as silver, silver oxide (Ag2O), titanium dioxide (TiO2), silicon (Si), copper oxide (CuO), zinc oxide (ZnO), gold (Au), calcium oxide (CaO) and magnesium oxide (MgO), have been shown to exhibit antimicrobial activity (14). The two most important parameters affecting the antimicrobial effect of nanoparticles are the type and size of materials used (15,16). The smaller the size of the nanoparticles, the stronger the bactericidal effect is known (14). The size of the nanoparticles is related to the surface / volume ratio and has different properties than the larger size of the same particle with the reduction in particle size (17). The reason for this is that as the size of the nanoparticles decreases, the fraction of the surface molecule significantly increases, improving the properties of the nanoparticles such as heat treatment, mass transfer, dissolution rate, catalytic activity (18). However, although the mechanisms of antibacterial action of the nanoparticles are still not fully elucidated, free metal ion toxicity and reactive oxygen species (ROS) formation and morphological (shape) and

physicochemical properties of the nanoparticles have proven to have an effect on their antimicrobial activities (15). Few studies have been carried out on gram negative anaerobic pathogenic bacteria which are the periodontitis agents of nanoparticles. Although studies focused mainly on the bacterial species found in the oral cavity, gram positive facultative anaerobic bacteria such as Streptococcus mutans were chosen for ease of use. Unfortunately, antibiotic treatments have made these bacteria resistant to conventional antibiotics.

Therefore, the aim of this study was to investigate the effects of various engineering nanoparticles on *Porphyromonas gingivalis* (Pg) and *Aggregatibacter actinomycetemcomitans* (Aa), which are pathogenic bacteria for periodontal diseases.

METHODS

Nanoparticles materials

In this study, multi-walled carbon nanotubes (MWCNT) 90+% purity, 5-10 nm and metal oxide powders (in 99.5+% purity, zirconium oxide (ZrO2) 40 nm, alumina oxide (Al2O3) 20 nm, copper oxide (CuO) 25-55 nm, calcium carbonate (CaCO3) 50 nm, silicon oxide (SiO2) 15-25 nm, magnesium oxide (MgO) 20 nm nanopowders were purchased from Nanography (Ankara, Turkey) to investigate the concentration dependence of the antibacterial effect of engineering nanoparticles.

Strains and growth conditions

The lyophilized gram negative anaerobic species (Aa DSM catolog no. 11123 and Pg DSMZ catolog no. 20709) used in this research were obtained from German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Braunschweig, Germany). Each inactive bacteria for reactivation were grown under anaerobic conditions and then stored in a bacterial suspension. Briefly, the Aa starin were incubated in CaSo Bouillon (Carl Roth) for 24-48 hours at 37 °C in a 5 % CO2 medium under anaerobic standard conditions. After incubation, the Aa were suspended in Schaedler liquid medium to provide a turbidity equivalent to the 108 CFU/mL-1 McFarland standard. Pg strain was cultured under anaerobic conditions 10% C02, 5% H2 and 85% N2 on Columbia blood agar plates containing 5% sheep blood and 0.5% K vitamin at 37 °C for at least 48 hours.

Characterization and Preparation of Engineering nanoparticles

MWCNT and nanoparticle powders were suspended in chlorhexidine (CHX) prepared with 2% by ddH2O to interact with bacteria. The nanoparticle was left to sonication for 30 minutes to ensure homogenization and dispersion of the suspension. Then, from these stock solutions, test solutions were prepared at concentrations of 1.5, 3 and 6 mg/L for CuO, 4, 8 and 16 mg/L for ZrO2, 0.05, 0.1 and 10 m /L for CaCO3, 5, 10 and 20 mg/L for MWCNT, 12.5, 25 ve 50 mg/L SiO2, 25, 50 ve 100 mg/L for Al2O3 and MgO. The nanoparticle powders were characterized by SEM. These solutions were then autoclaved to eliminate naturally occurring microorganisms and was then sonicated for 20 min. and immediately used for disk diffusion tests.

Antibacterial activity assay

Agar disc diffusion method was used to determine the antibacterial activity of nanoparticles solutions with CHX. 100 µL of the bacterial suspension adjusted according to Mcfarland 0.5 at the

spectrophotometer (0.5 McFarland standard) were impregnated uniformly on the surface of solids containing nutrient agar solid medium for Aa and solid blood agar for Pg. 20 µL of the nanoparticle solutions previously prepared with CHX were impregnated on sterile empty discs (6 mm diameter) and placed at equal distances to the petri dishes. 20 µL 0.2 % CHX was used as positive control and ethanol was used as negative control. The petri dishes were incubated at anaerobically for 72 hours at 37°C. After incubation, the average diameters of the bacterial inhibition growth zones formed around the discs in petri dishes were measured. For each nanoparticle solutions and for each bacterial strain, the mean and standard deviation values were obtained from six replicates.

Statistical analysis

Antimicrobial experiments were conducted in five replicates. Data points were expressed as the mean ± SD. Data were analyzed using analysis of variance (ANOVA) from SAS version 9.1 software (SAS Inst., Inc., Cary, N.C., U.S.A.). Duncan's multiple range tests were used to determine the significant difference of mean values. Unless stated otherwise, significance was expressed at 5% level.

RESULTS

Table 1 shows the mean values of bacterial growth inhibition zone diameters for two bacterial species exposed to multi-walled carbon nanotubes (MWCNT) and nanoparticles (CuO, SiO2, Al2O3, CaCO3, MgO, ZrO2) prepared with CHX based on six repetitive determinations. Disc diffusion test reveals the differences in sensitivity of nanoparticles (CuO, CaCO3, SiO2, Al2O3, MgO and ZrO2) and MWCNT for Pg (DSMZ 20709) and Aa (DSMZ 11123). Aa selected for this study were noted to be the most sensitive species for all nanoparticles and MWCNT. The order of the nanoparticle types in which the Pg is most sensitive is SiO2> MgO> Al2O3> ZrO2> CuO> MWCNT> CaCO3. For Aa order is CaCO3> SiO2>MgO> ZrO2> CuO> MWCNT> Al2O3. In addition, both bacterial species are more susceptible to SiO2 nanoparticles. This means that the nanoparticle type which increases the antibacterial property of CHX is SiO2. In contrast, both bacterial species have shown low sensitivity to MWCNT. When compared CHX (control group) and nanoparticle concentrations, significant differences were observed in all concentration groups of Pg and Aa bacteria ($P < 0.01$). The comparison of all nanoparticle concentrations with CHX resulted in significant differences in the highest concentrations ($P < 0.01$). Generally, the antibacterial effect of the lowest nanoparticle concentrations was lower than the control group (CHX). This means that the addition of nanoparticles to CHX increases the antibacterial activity at high doses and has no effect on low concentrations. In fact, it was observed that the addition of low concentrations of CHX to the nanoparticle reduced the antibacterial activity of CHX. For example, the addition of MgO nanoparticle to CHX at concentrations of 100 and 50 mg/L increased the antibacterial activity of CHX. Another result is stronger antibacterial effect on CHX and nanoparticles than Aa bacteria Pg. Unlike other nanoparticles, ZrO2 and SiO2 nanoparticles increased the antibacterial activity of the control group (CHX) even at the lowest concentrations. This means that even the lowest concentrations of these

nanoparticles to CHX has increased the antibacterial activity of CHX.

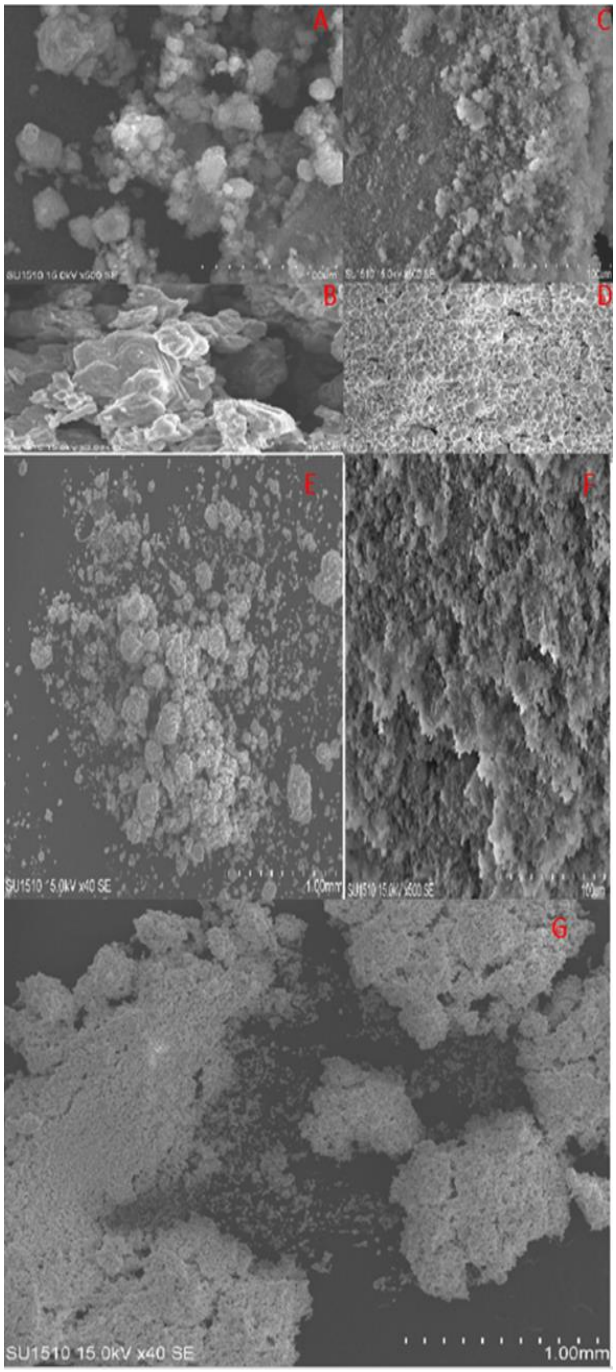

Figure 1. SEM image of nanoparticles, A) MWCNT B) CuO C) SiO2 D) Al2O3 E) CaCO3 F) MgO G) ZrO2

DISCUSSION

Many studies on nanoparticles have increased in recent years. Researchers have focused on environmental impacts or how we can use them in medical studies (19, 20).

Table 1. The mean zone diameters of nanoparticles as measured by disk diffusion method.

Treatments	Pg (inhibition zone) Mean±SD	Aa (inhibition zone) Mean±SD
Control (CHX)	12.00c±0.01	14.35c±0.01
6 mg/L CuO	16.22a±0.01	20.60a±0.01
3 mg/L CuO	12.52b±0.01	18.51b±0.01
1.5 mg/L CuO	10.70d±0.01	14.30d±0.01
Control (CHX)	12.00d±0.01	14.35c±0.01
16 mg/L ZrO2	16.70a±0.01	20.95a±0.01
8 mg/L ZrO2	15.16b±0.01	18.10b±0.01
4 mg/L ZrO2	13.25c±0.01	14.20d±0.01
Control (CHX)	12.00c±0.01	14.35d±0.01
10 mg/L CaCO3	14.11a±0.01	22.33a±0.01
0.1 mg/L CaCO3	12.25b±0.01	19.00b±0.01
0.05 mg/L CaCO3	11.08d±0.01	16.42c±0.01
Control (CHX)	12.00d±0.01	14.35d±0.01
20 mg/L MWCNT	16.26a±0.01	20.55a±0.01
10 mg/L MWCNT	13.72b±0.01	18.80b±0.01
5 mg/L MWCNT	12.85c±0.01	15.00c±0.01
Control (CHX)	12.00d±0.01	14,35d±0.01
50 mg/L SiO2	21.23a±0.01	21.15a±0.01
25 mg/L SiO2	16.83b±0.01	18,45b±0.01
12.5 mg/L SiO2	12.57c±0.01	15.00c±0.01
Control (CHX)	12.00c±0.01	14.35d±0.01
100 mg/L Al2O3	18.25a±0.01	19.75a±0.01
50 mg/L Al2O3	15.00b±0.01	16.00b±0.01
25 mg/L Al2O3	10.48d±0.01	14.78c±0.01
Control (CHX)	12.00c±0.01	14.35d±0.01
100 mg/L MgO	19.00a±0.01	21.00a±0.01
50 mg/L MgO	12.89b±0.01	18.50b±0.01
25 mg/L MgO	10.78d±0.01	16.00c±0.01

**P<0.001
[a,b,c,d] P<0.05. Significant differences between study groups
Each nanoparticle concentration experiment was repeated 5 times.

Major advances in nanotechnology have led to the investigation of nanometer-sized metal oxides as antimicrobial agents. The use of inorganic metal oxide NPs has attracted a great deal of attention due to the biocompatibility of mammalian cells, as well as promising antimicrobial activity, even at low concentrations (21). NPs have been accepted as antibacterial agents and have been used for oral infection control in dentistry (4). The exact mechanisms for the bacterial toxicity of nano-metals have still not been fully elucidated. The possibilities,

however, include free metal ion toxicity resulting from the dissolution of metals from the surface of NPs and the formation of oxidative stress by the production of reactive oxygen species (ROS) on the crystal surfaces of NPs (20). The occurring ROS can then act synergistically in bacteria by attacking polyunsaturated phospholipids and may cause local DNA damage (22). Electrostatic attraction between the negative charge of the bacterial cell membrane and the positively charged NPs showed that it was critical for antimicrobial activity (4). In one study, structural changes resulting in cell death and damage to bacterial membranes have been demonstrated (23). It is estimated that the 3-20-fold decrease in the negatively charged peptidoglycans in the gram-negative species (Pg, Prevotella intermedia, and Aa) will cause differences in sensitivity. Furthermore, the ability of silver, copper and zinc to bind to the basic enzyme sulfhydryl (-SH) groups may produce differences in sensitivity to these metals between anaerobic and aerobic bacteria. For example, the low affinity of zinc for sulfhydryl groups may explain the lack of antimicrobial activity against Pg (4). However, it is still unclear whether NPs have superior antibacterial properties compared to conventional metal salts used in dentistry or other routine antibacterial products for oral cavity such as chlorhexidine used in surgical gargles (4). Most microbial studies with NPs have been made with well-known model organisms such as Escherichia coli (23). However, NP concentration and size are the most important factors affecting the antimicrobial properties of NPs. Therefore, ultrasonic and dispersants are often used to break up NP agglomerates (24). In this study, sonication (20 min) was used to prevent agglomeration of NPs prepared with CHX. In addition, the agar medium is chosen instead of broth, because the NPs in the broth can precipitate and make the estimation of the nominal exposure concentration of NPs difficult (25). NPs greater than 10 nm accumulate on cell membranes, and may compromise cell permeability. This causes leakage of intracellular components and subsequent cell death (26). The nanoparticles smaller than 10 nm can accumulate in the cell by penetration into the membranes, thereby making an effect on the nucleic acid (27). Another mechanism in which the NPs exhibit antimicrobial activity in the presence of oxygen is ROS production. By disrupting normal cellular functions such as breathing chain, NPs trigger ROS formation such as OH^{-}, O2- and H2O2, and can cause death of bacteria (28).

In this study, anaerobic conditions are important in terms of antibacterial properties of NPs. In addition, the SiO2 NPs having the lowest size as mentioned above has the highest antibacterial activity in both bacterial species. In previous studies, the antimicrobial activity of SiO2 NPs has become more important due to increased surface area (14). Although it does not have a strictly toxic mechanism, silica causes a negative change in the biofilm to reduce adhesion and thus the growth of bacteria (22). Among the NPs tested in our study is the most sensitive nanoparticle SiO2 for Pg. For Aa, the most sensitive nanoparticle after CaCO3 was reported to be SiO2 NPs. The results we have obtained have been confirmed by previous studies. However, the previous studies did not use anaerobic pathogens (29).

In several studies, CaO and MgO NPs have been shown to have strong antibacterial activity related to alkalinity and active oxygen species. The antibacterial mechanism of CaO and MgO NPs has been confirmed to provide an increase in pH by the superoxide production on the surface of these particles and also by hydration of CaO and MgO (14). Thus, MgO NPs damage the cell membrane and then cause the intracellular contents of bacterial cells to be destroyed (30). In our current study, the nanoparticle having the least sensitivity in all the NPs tested for Pg was CaCO3. Interestingly, for Aa the most sensitive is the nanoparticle. For MgO NPs, the sensitivity of both species is almost the same.

There is not much literature study on the species used in our study (Aa and Pg). Moreover, the current studies in the literature differ from the nanoparticles we use. For example, Vargas-Reus et al. (28), Ag, Cu2O, CuO, ZnO, TiO2, tungsten oxide (W03), Ag + CuO composite and The activities of the NPs of the Ag + ZnO composite were evaluated against Prevotella intermedia, Pg, Fusobacterium nucleatum and Aa with minimum inhibitory (bacteriostatic) concentration (MIC) and minimum bactericidal concentration (MBC). The NPs evaluated showed that the antimicrobial properties were different according to the bacterial species. Besinis et al. (22), Ag, TiO2 and SiO2 NPs and routine disinfectant CHX compared the toxicity of Streptococcus mutans against oral pathogenic species. All analyzes showed that Ag NPs had the strongest antibacterial activity among tested NPs, and reported that they were 25 times lower than CHX in bacterial growth.

CONCLUSION

This study confirmed that MWCNT, CuO2, CaCO3, SiO2, Al2O3, MgO and ZrO2 nanoparticles against Pg and Aa have antibacterial effect. The results of this study showed that the antibacterial properties of CHX mouthwash increased with these nanoparticles. It has been noted that these effects are increased with increasing concentrations of nanoparticles. Our results suggest that engineering nanoparticles have a significant inhibitory effect on Aa and Pg. These results can be further clarified with new studies.

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