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LETTER

Investigation of cell damage of periodontopathic bacteria exposed to silver, zirconium oxide, and silicon oxide nanoparticles as antibacterial agents

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Abstract

Antibiotic resistance is one of the biggest public health problems of our time. The nanoparticles are a powerful alternative to these antibiotics. Engineered nanoparticles show toxic effects on bacteria by different mechanisms. The bacteria–cell interaction of engineered nanoparticles exerts their toxic effects through changes in cell wall, cell membrane, and cytoplasm content/density. Thus, death occurs as a result of cell deformation. In this study, the cellular damage of silver nanoparticles, which are known to have strong antibacterial properties, and zirconium oxide and silicon oxide engineering nanoparticles, which are less known, on periodontopathic (*Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans*) bacteria, were investigated by ultrastructural changes. The lysis of the cytoplasm and separation of the membrane cytoplasm were observed. Both types of bacteria treated with Ag ENP show more hollow cytoplasm than bacteria treated with the other two nanoparticles.

1 | INTRODUCTION

Bacteria are single-celled microorganisms that can be spherical, spiral, and rod-shaped, typically a few μm long. Bacteria exist in every environment on earth [1, 2]. The number of bacteria in the human body is about 10 times that of human cells and is especially abundant in the skin and digestive tract [3]. Most of them are harmless with the protective effect of the immune system, some are beneficial (probiotics), and some are pathogenic and harmful [4]. Intensive use of antibiotics in treating bacterial infections in developed countries increases antibiotic resistance and this causes serious problems [5, 6]. A lipid membrane surrounds the bacterial cell called the cell membrane, which keeps nutrients, proteins, and other cytoplasm components inside the cell. Outside the cell membrane is the bacterial cell wall, which consists of peptidoglycan, a polysaccharide chain cross-linked by peptide chains. In most bacteria, a hard S-layer made of proteins covers the outside of the cell, providing chemical and physical protection to the cell surface and a barrier against the diffusion of macromolecules. The cell membrane is a highly dense, flexible structure found around the cytoplasm in all cells. This membrane gives shape to the cell and acts as a selective barrier by defining the cell's border. The cell membrane has a dynamic and complex structure in which many physiological events occur in the cell [7].

Membranes also form specific sections within the cell. Such intracellular membranes form many structurally distinguishable organelles such as mitochondria, endoplasmic reticulum, sarcoplasmic reticulum, golgi apparatus, glandular particles, lysosomes, and nuclear membrane [8, 9]. The cell membrane (membrane) controls the transport of water and water-soluble substances between the extracellular and intracellular environments and provides the necessary chemical environment for cellular activities [10]. Bacteria are divided into two groups, gram positive and gram negative, according to the reaction of their cell wall with the gram stain of the cells. Some bacteria have a gram-negative cell wall [11]. The cell wall is the part of the bacterium that maintains its integrity and enables division and reproduction. It consists of a polymer compound called murein [12]. The bacterial cell wall resists the intracellular osmotic pressure, which is increased by many dissolved substances in water taken from the external environment by active transport, and

protects the integrity of the bacteria and prevents its disintegration. Antibacterials are chemicals that act on bacteria by stopping the growth and development of bacteria or by directly killing the bacteria [13]. Antibiotics that fall into the antibacterial classification are: They are substances that are produced by living microorganisms such as bacteria, fungi, and actinomycetes or prepared by synthesis, which affect the growth of bacteria or kill them even at low concentrations. Antibacterial agents act by some mechanisms: inhibition of cell wall synthesis [14, 15], deterioration of the structure, and function of the bacterial cytoplasm membrane, its effect on protein and nucleic acid synthesis, and its antimetabolic effect [16, 17].

It is thought that nanostructures such as carbon nanodots [18], tin oxide [19], nanotruffles [20], bimetallic [21], bismuth [22], arsenic [23],tungsten oxide [24], nickel nanowire [25, 26], silver [27, 28], metal-organic framework [29, 30], zinc oxide [31, 32], and dendrons [33] show cytotoxicity [34, 35], antioxidant [36], photocatalyst [37], antifungal [38, 39], and antibacterial properties [40–42]. In particular, the antibacterial properties of silver (Ag) ENPs have proven themselves in various medical fields [43] and especially in various application areas of dentistry such as endodontics, implantology, and prosthetics [44–46]. Silicon dioxide (SiO_2) ENP, on the other hand, has increased the performance of dental composites with its mesoporous structure and large surface area properties [47, 48]. Zirconium oxide (ZrO_2) ENP has wide applications as a biomaterial due to its high biocompatibility, strength, and low wear properties [49–51]. Compared to other nanoparticles, metabolic interactions, toxicity, and antimicrobial properties of ZrO_2 ENP have been less studied. For these reasons, ENP, known to have strong antibacterial properties in previous studies, and ZrO_2, SiO_2 ENPs, which were less studied, were chosen. In order to observe the antibacterial effects of these MNPs against periodontopathic bacteria such as *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans* and the mechanisms by which these effects are produced, transmission electron microscopy and cell damage were compared and compared.

2 | MATERIALS AND METHODS

2.1 | Engineering nanoparticles

In this study, zirconium oxide (ZrO_2) 40 nm, silicon oxide (SiO_2) 15–25 nm, and Ag 28–48 nm nanopowders with 99.5%+ purity were purchased from Nanography (Ankara, Turkey). Solutions of these nanoparticles were prepared in deionized water (DI-water) at a concentration of 10 μg/ml. Before the experiment, the nanoparticles were kept in an ultrasonic water bath for 20 min to prevent aggregation.

2.2 | Strains and growth conditions

Gram-negative oral bacteria *Prevotella intermedia* (DSMZ catalog no. 20706) and *Aggregatibacter actinomycetemcomitans* (DSM catalog no. 11123) were used in this study. These bacteria were commercially available in lyophilized form from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Freeze-dried bacteria were cultured under anaerobic conditions. *A. actinomycetemcomitans* were inoculated at Caso-bouillon (pH 7.0) for 48 h at 37°C under anaerobic standard conditions (5% CO_2). The inoculum suspension was suspended in Schaedler liquid medium according to a 0.5 McFarland (1.5×10^8 CFU mL^{-1}) turbidity standard. *P. intermedia*, the periodontopic bacteria used in that study, was added to a fastidious anaerobic agar medium with 10% sheep blood, 10% vitamin K, and hemin solution and incubated at 37°C for approximately 5 days under anaerobic conditions.

2.3 | Antibacterial susceptibility assay

The agar well diffusion method was used to determine the antibacterial activity of engineered nanoparticles on anaerobic periodontopathic bacteria. In this method, circular wells of 5-mm diameter and 2-mm depth were drilled on 5% defibrinated sheep blood agar. Then, the prepared bacterial suspensions were dropped onto the medium with the help of a swab. After that, prepared 50-µl nanoparticle solutions were poured into the wells. Then, *P. intermedia* was incubated in an oven at 37°C for 1 week under anaerobic conditions, and *A. actinomycetemcomitans* was incubated for 24–48 h in a 10% CO_2 incubator. The resulting inhibition zone diameters were measured. All experiments were repeated three times and the measurements were averaged.

2.4 | Cell culture TEM procedure

After the incubation, the cells are removed with trypsin enzyme and the falcon is taken into tubes and centrifuged with PBS (0.5 mL) for 10 min at 1000 g. After centrifugation, the supernatant is removed and recentrifuged at 1000 g for 15 min two times by adding PBS. After centrifugation, 2.5% Glutaraldehyde (2 mL) in 0.1 M PBS was added to the pellet and the cells were left to fixate at +4°C for one night. After primary fixation, cells were washed two times with PBS and centrifuged at 1000 g for 5 min. Washed cells were treated with 1% osmium tetroxide (2 mL) and secondary fixation was carried out for 1 h at +4°C. After the second fixation, the cells were washed two times with PBS and centrifuged at 1000 g for 5 min. Rapidly, 5% molten agar was added to the cells in the falcon tube with the help of a pasteur pipette and a homogeneous cell–agar mixture was obtained. The homogeneous cell agar mixture was dropped on the slide with the help of a pipette in tiny drops and allowed to freeze. The agar pieces on the slide were cut into small pieces with the help of a scalpel tip and the cut pieces were gently taken into brown bottles. Uranyl acetate was added to the cells in the flask and incubated at +4°C for 15 min. After this procedure, cells embedded in agar were passed through an alcohol series. At +4°C for 10 min, this series is 30% ethyl alcohol, 50% ethyl alcohol, 70% ethyl alcohol, 90% ethyl alcohol and 90% ethyl alcohol. After this process, it was kept in propylene oxide at +4°C for 10 min. Pure propylene oxide was pipetted

TABLE 1 Mean values of the inhibition zone of periodontopathic bacteria measured by the agar well method.

ENPs	*A. actinomycescomitans*	*P. intermedia*
10 μg ZrO_2	27	21
10 μg SiO_2	23	19
10 μg Ag	21	16

and a 1:1 ratio of propylene oxide/epon mixture was added and incubated at +4°C for 10 min. Afterwards, a pure epon mixture was added and cells were kept at +4°C for 48 h. After treatment, cells were embedded in araldite and polymerized at 60°C for 48 h. Then, 60-nm-thick thin sections were taken from the buried samples with an ultramicrotome (Leica Ultracut R). These samples on copper grids were stained with uranyl acetate-lead citrate. Then, ultrastructural findings were examined under a transmission electron microscope (Hitachi HT7800, Japan).

3 | RESULTS AND DISCUSSION

A. actinomycetemcomitans and *P. intermedia* are bacteria that often cause periodontitis. Periodontitis is an infectious disease that often does not respond to conventional antibiotic therapy. For this reason, ENPs, which have been very popular in medical applications such as medicine and dentistry in recent years, have been remarkable alternative adjuvants to antibiotic therapy. In many studies, the antimicrobial activity of ENPs has been tested in periodontitis-causative microorganisms.

In this study, 10 μg was determined as the equal and lowest concentration of nanoparticles that inhibit the growth of microorganisms tested by disk diffusion test since it allows a more convenient comparison of the antibacterial effect of different ENPs on bacteria at the same concentration. ZrO2, SiO2, and Ag ENP indicated antimicrobial activity against the tested periodontopathic bacteria, and Table 1 presents the zone diameter obtained for each tested bacteria. Among the tested ENP species, silver ENP has the lowest antibacterial activity and toxicity. However, it is also the most preferred in dentistry applications because silver ENP is frequently used in dental composites due to its anti-inflammatory effects and biocompatible properties.

In previous studies, ENPs were applied against various periodontitis-causative periodontopathic bacteria. In these applications, the antibacterial effects of the ENPs are proportional to the ion release. Therefore, as the concentration of nanoparticles increases, the amount of ions released increases, increasing bacterial cell toxicity. For this reason, the lowest concentration of applied nanoparticles allowing comparison was applied to bacterial cells. In addition, toxicity in bacteria manifests itself through different mechanisms. When ENPs interact with the bacterial cell membrane, cell functions are disrupted, and then changes occur that cause the death of the bacteria.

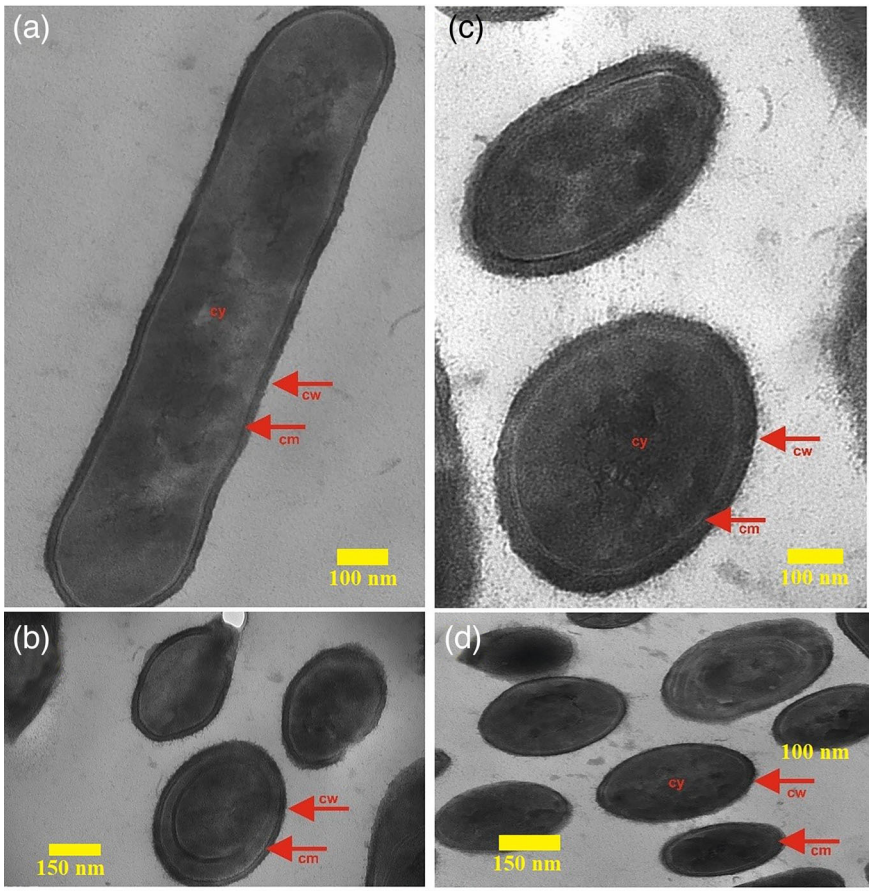


FIGURE 1 TEM micrographs of periodontopathic bacteria not exposed to ENPs (A) *Prevotella intermedia* and (B–D) *Aggregatibacter actinomycetemcomitans*. (cm: cell membrane, cw: cell wall, cy: cytoplasm).

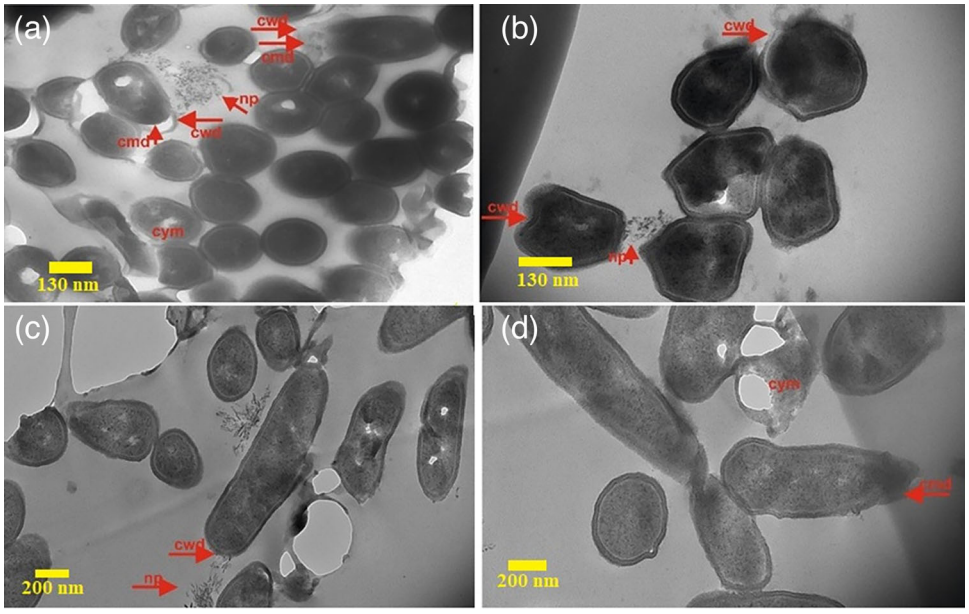


FIGURE 2 TEM micrographs of periodontopathic bacteria exposed to ZrO_2 ENPs (A, B) *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* (C, D). (cym: cytoplasmic melting, cmd: cell membrane damage, cwd: cell wall damage, np: nanoparticles).

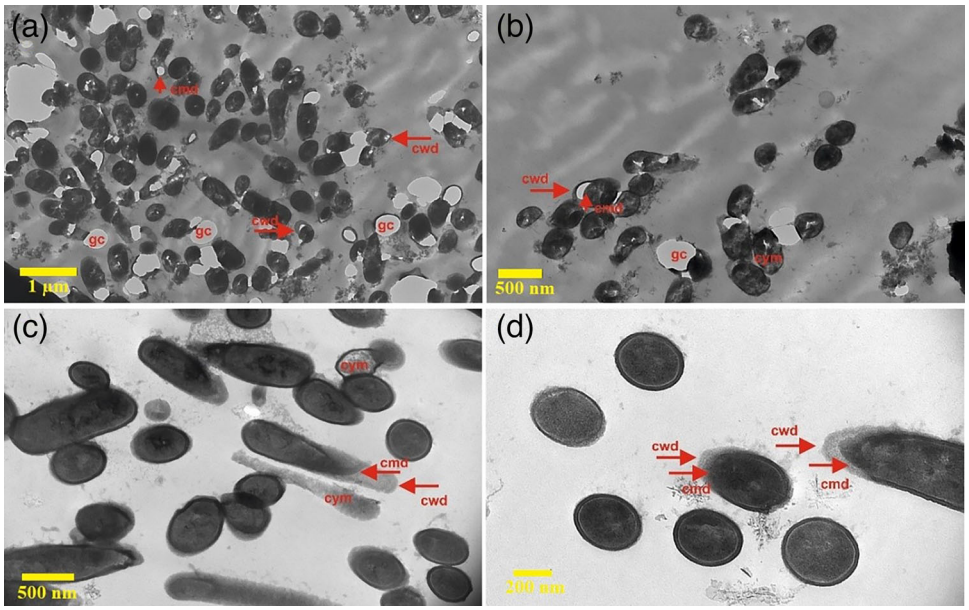


FIGURE 3 TEM micrographs of periodontopathic bacteria exposed to SiO_2 ENPs (A, B) *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* (C, D). (cym: cytoplasmic melting, cmd: cell membrane damage, cwd: cell wall damage, gc: ghost cell np: nanoparticles).

The other mechanism is the deterioration of protein function due to excessive production of reactive oxygen species by bacterial cells. Figures 2–4 exhibit the toxic effects of NPs through these mechanisms. However, the mechanism that causes these toxic effects has yet to be demonstrated.

Figure 1 indicates the cell morphologies of the control group bacteria. The cell wall and cell membrane of these cells are flat and discrete. Cell shapes are uniform, and there is no membrane deformation. The cytoplasm and cellular integrity of the cells are ensured.

In Figures 2A and 2B, cells of *P. intermedia* exposed to ZrO_2 ENP lysed, the contents of the cytoplasm contents leaked out in some cells, and membrane and wall damage occurred in some cells. In several *P. intermedia* cells, bleb formations out of the membrane were observed. Morphological deformities and outward bending of the cell membrane and cell wall structure were observed in the cells that came into contact with ZrO_2 ENP. It has been observed that some ZrO_2 ENPs can penetrate the cell membrane and wall and enter the cytoplasm. Few intact cells were observed from *P. intermedia* cells exposed to ZrO_2 ENP. In

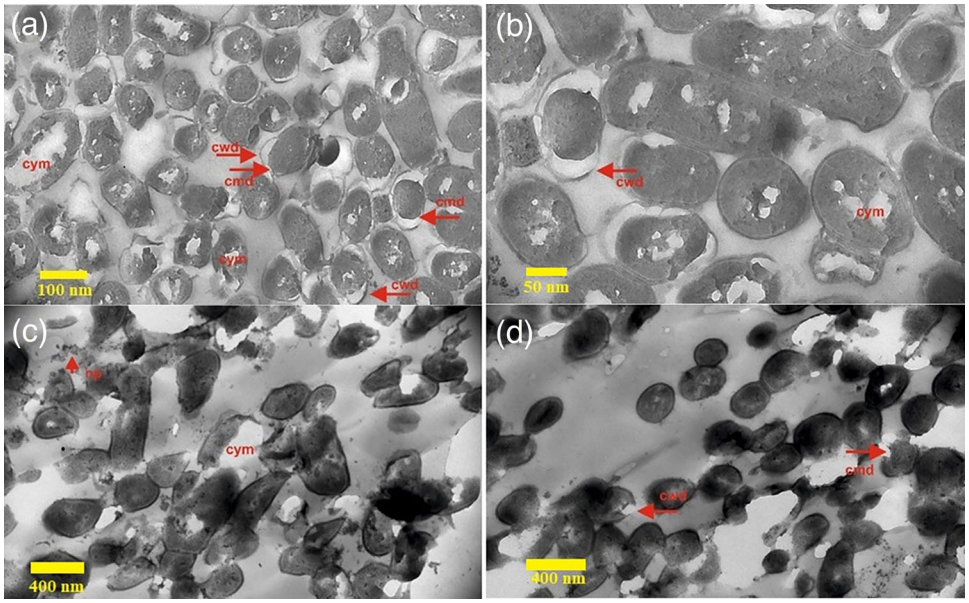
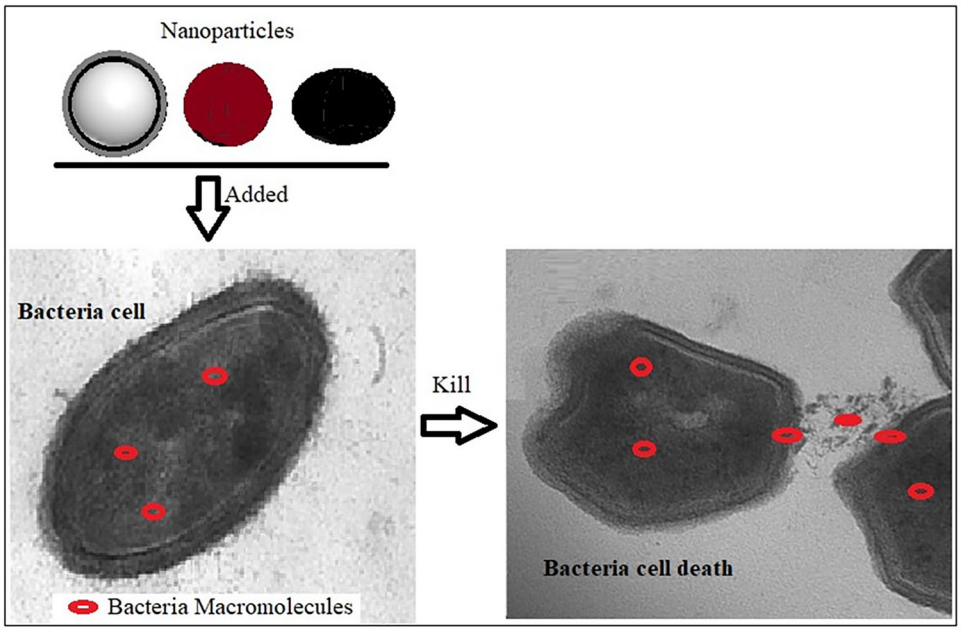


FIGURE 4 TEM micrographs of periodontopathic bacteria exposed to Ag ENPs (A, B) *Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans* (C, D). (cym: cytoplasmic melting, cmd: cell membrane damage, cwd: cell wall damage, np: nanoparticles).



SCHEME 1 Schematic diagram illustrating the antibacterial mechanism of nanoparticles with resulting in a change in membrane surface penetrability.

Figures 2C and 2D, the cells of *A. Actinomycetemcomitans* exposed to ZrO_2 ENP have regular oval-round wall and membrane structures. However, the cells were relatively preserved with a homogeneous cytoplasm appearance; damage in the form of cell walls and membrane lysis was observed where the cells in contact with ZrO_2 ENP came into contact. A complete lysis event was observed in a small number of cells.

In Figures 3A and 3B, severe damage occurred in *P. intermedia* cells exposed to SiO_2 ENPs. These damages are manifested in the cell wall, membrane and cytoplasm. The cytoplasm was observed to be electron-dense and locally melted. SiO_2 ENPs penetrated the bacterial cell and caused lysis in the cytoplasm. In addition, lysed cells were found and bleb formation increased in the cells. In Figures 3C and 3D, *A. actinomycetemcomitans* exposed to SiO_2 ENPs also have normal healthy cells. In cells exposed to SiO_2 ENPs, membrane-cytoplasmic separations are evident, especially in the apical part. However, cell wall membrane lysis and cytoplasm leaks are also present in these damaged apical parts of the cells. In a small number of cells, the cytoplasm has leaked out and almost completely emptied of its contents.

However, intact bacterial cells are seen in bacteria exposed to SiO2 ENPs.

In Figures 4A and 4B, severe damage and lysis were observed in *P. intermedia* cells exposed to Ag ENPs. These damage occurred in the form of wall and membrane damages. In addition, lysis of the cytoplasm and separation of the membrane cytoplasm were observed. In general, all cells of *P. intermedia* exposed to Ag ENPs are damaged. In Figures 4C and 4D, severe damage is seen in *A. actinomycetemcomitans* cells exposed to Ag ENPs. These damages were seen as membrane and wall damage in cells. Mainly, excessive lysis was observed with membrane and wall fragmentation. Both types of bacteria treated with Ag ENP show more hollow cytoplasm than bacteria treated with the other two nanoparticles.

The exact antibacterial mechanisms of NPs are not understood yet, but the currently robust suggested mechanisms include oxidative ion release, stress induction and non-oxidative mechanisms [52]. The NPs lead to neutralizing the bacterial membrane surface electric charge, resulting in a change in stability and penetrability (Scheme 1).

The production of reactive oxygen species (ROS) led to damage to the cell membrane [53]. Also, NPs can interact with DNA and proteins, changing their function [54]. In previous studies, silver NPs have proven to be highly reactive in the ionized state. Thanks to this reactivity, silver ions interact with the peptidoglycan of bacterial cells, plasma membranes, cell walls, bacterial proteins, and bacterial DNA [55]. Thanks to this reactivity, silver ions interact with the peptidoglycan, plasma membrane, cell wall, bacterial proteins, and bacterial DNA of bacterial cells and cause structural changes that result in the death of bacterial cells [56].

4 | CONCLUSION

The antibacterial effects of silver, zirconium oxide, and silicon oxide nanoparticles against periodontopathic bacteria (*Prevotella intermedia* and *Aggregatibacter actinomycetemcomitans*) are studied. The cell damage of bacteria cell is monitored by transmission electron microscopy. The lysis of the cytoplasm and separation of the membrane cytoplasm were observed. Both types of bacteria treated with Ag ENP show more hollow cytoplasm than bacteria treated with the other two nanoparticles. Compared to the other antibacterial agents, antibacterial nanomaterials either as delivery systems have lower side effects and higher efficacy.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: **Yeşim D.** Performed the experiments: Methodology: Mustafa C; Omer E: Writing-review & editing: All authors; Supervision and Funding acquisition: Fuad A.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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