RESEARCH PAPER

Evaluation of the antibacterial and cytotoxic activities of Ag/ZnO nanoparticles loaded polycaprolactone/chitosan composites for dental applications

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ABSTRACT

Objective(s): Bacterial adhesion to orthodontic brackets is a significant issue in orthodontic treatment. Most plaque control approaches rely on the patient's cooperation which is not good enough to control the pathogenic oral microorganisms in most cases. Considering the growth rate of antibacterial resistance species, finding new antibacterial agents to control the oral microbial load seems necessary. This study aimed to evaluate the antibacterial and cytotoxic effects of Ag/ZnO NPs loaded PCL/CS composites.

Materials and Methods: Ag/ZnO NPs were synthesized and characterized using sol-gel and DLS, respectively. After preparing three concentrations of Ag/ZnO NPs, they were loaded on the scaffolds. The release of NPs was measured in the artificial saliva. The antibacterial activities of NPs were evaluated on the medium plates of *S. aureus* and *S. mutants* using the inhibition zone method and compared to the control group (scaffolds without Ag/ZnO NPs). The cytotoxic effects of NPs were assessed using fibroblasts with MTT assay and compared to the control group.

Results: The results showed that Ag/ZnO nanoparticles have antibacterial properties that increase over time. The 25 µg/mL concentration of these NPs had the least effect on L929 fibroblasts.

Conclusion: The Ag/ZnO NPs loaded the PCL/CS scaffolds have controlled slow-released properties. These NPs have antibacterial effects on oral microfilms and can be used to control pathogenic oral microorganisms. Moreover, they are safe with no cytotoxic effects the fibroblast cells and can be used in the oral cavity and skin.

Keywords: *Ag/ZnO Nanoparticles, Chitosan, Cytotoxic activity, Polycaprolactone*

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INTRODUCTION

The oral cavity is a complicated ecosystem and the habitat of more than 300 bacterial species, protozoa, mycoplasmas, and yeast. Any intervention in this environment can result in the distribution of its well-balanced microflora and can cause an increase in the number of pathogenic microorganisms [1-3]. Orthodontic procedures, as an example of these interventions, lead to minor or major changes in the oral cavity environment. Gingivitis, dental caries, and halitosis are more likely to be observed in patients undergoing orthodontic treatments [4]. Applying brackets includes etching the enamel, which supports the attachment and proliferation of *Streptococcus mutans*, *Veillonella spp.*, and *Actinomyces viscosus* [5]. A metallic orthodontic bracket can cause ecological inconsistencies in the oral environment, such

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as decreased pH and plaque formation. These brackets attract bacterial biofilm because of their high surface energy and tension [6]. Most changes in the microbial plaque of orthodontic patients occur during the first week after fixing the brackets. Orange species colonization becomes more stable after 3 months, which is followed by the stabilization of red species [7]. After 6 months of fixed orthodontic treatment, the prevalence of dental caries can rise to 33%, and after 12 months, it can increase to 61% [8, 9].

There are different methods for controlling and removing plaque, but patient cooperation is critical in most of these methods. Finding a new approach to effectively disinfect the oral environment without much need for the patient's cooperation seems necessary.

It is found that nanoparticles of zinc oxide (ZnO) loaded into dental composites at a fraction of 10% (w/w) are antimicrobial and inhibit the growth of bacteria biofilms by about 80% [10]. Silver nanoparticles showed antibacterial features against *Streptococcus mutans*, one of the most important microorganisms in dental caries biofilm [11].

Polycaprolactone (PCL) is a semi-crystalline polyester that degrades very slowly. When PCL is used as a composite with other polymers, the degradation speed and adhesion strength to cells are improved [12]. PCL is biodegradable, compatible with many other polymers, thermally stable, and relatively affordable [13].

Chitosan is a characteristic antibacterial substance that is biodegradable and derived from glucan with repeating units of chitin [14]. The increased chitosan content in anti-adhesive membranes decreased the contact angle, which hints that chitosan plays a role in increasing the hydrophilic properties [15].

Current data about the safety of using nanoparticles (NPs) in the oral cavity are not conclusive and comprehensive; Although the studies point to the promising application of NPs in dentistry [16], some aspects of the application of these materials, including their cytotoxicity, need to be more investigated. Ag and ZnO NPs have been used in different studies [10, 11, 17-20]. However, the evaluation of a combination of these two NPs has not been studied properly. So, we decided to evaluate the antibacterial and cytotoxic effects Ag/ZnO NPs loaded PCL/CS on the oral environment. In this study, PCL/CS diblock and Ag/ZnO NPs were prepared at different concentrations, and the antibacterial effects of each concentration were evaluated on *Streptococcus mutans* and *Staphylococcus aureus* by measuring zone inhibit methods. Meanwhile, the viability of fibroblast cells in the presence of NPs was assessed.

MATERIALS AND METHODS

Materials

Polycaprolactone, chitosan, tin octanoate (Sn(Oct)2), and bacteria medium culture were purchased from Merck (Germany). Zinc nitrate hexahydrate, silver nitrate, and MMT were purchased from Sigma-Aldrich Chemical Co. (Munich, Germany). Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), antibiotic–antimycotic solution (Anti-Anti 100X), and 0.25% trypsin-EDTA solution were purchased from Gibco (Germany). Artificial saliva samples were purchased from Nick Seram Razi Company (Iran).

Preparation and characterization of Ag/ZnO NPs

In order to synthesize ZnO and Ag/ZnO nanoparticles, the sol-gel process was used [21]. Zinc nitrate hexahydrate (Zn (NO_3)2-6H_2O) and silver nitrate ($AgNO_3$) (Sigma-Aldrich, Germany) were used as precursors, and distilled water as solvent. The prepared NPs were characterized using the dynamic light scattering (DLS) technique with a scanning electron microscope (SEM) [22].

Synthesize three-dimensional porous Ag/ZnO NPs loaded polycaprolactone/Chitosan composites

PCL-PEG copolymer was synthesized using ring-opening polymerization of ε-caprolactone in the presence of PEG as a macroinitiator and tin octanoate (Sn(Oct)2) as a catalyst [23]. 10 gr of polyethylene glycol (PEG) (10 mmol, Mw = 2000) was heated in an 80-centigrade oil bath to melt completely. 9.13 gr (80 mmol, 8.86 mL) of caprolactone and 0.032 gr (0.08 mmol) of Sn(Oct) 2 were added. After 24 h stirring the insulated system at 110 °C, precipitation in cold diethyl ether was done. The product was dissolved in dichloromethane, precipitated by adding slowly to cold diethyl ether, and dried in a vacuum oven at room temperature.

There are different methods to synthesize a 3D biodegradable scaffold. The freeze-drying technique was used in this study. After freezing the solution, the solvent was sublimated using high pressure and vacuum. The sublimation of ice crystals resulted in empty spaces in the scaffold and created a high-porosity sponge.

In order to obtain a 20% (w/v) solution of PCL and a 5% (w/v) solution of CS, PCL and CS were dissolved in pure and 90% acetic acid, respectively. Two different volume concentrations of PCL/CS solution (9:10 and 7:30) were prepared. After

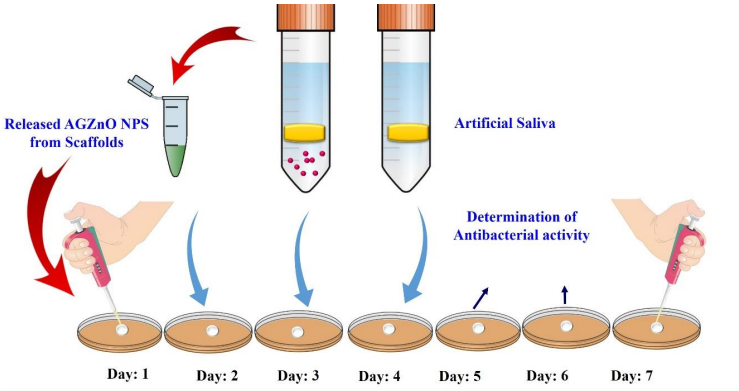

Fig. 1. A schematic of the addition of the artificial saliva to the 5mm-sectioned scaffolds on a lawn of *S. mutans* and *S. aureus* for evaluating the antibacterial activities of Ag/ZnO NPs

freezing the specimens at -80 °C for 24 h, they were freeze-dried at -80 °C for 24 h in a freeze-drying machine (Christ Alpha 2-4 LSD). The 70:30 specimens had a more substantial structure after freeze-drying; therefore, their solution was chosen for producing the scaffolds. This solution was frozen in a 1 mL insulin syringe before freeze-drying and cutting into the same size of each well of 96-well plates (Fig. 4B).

Ag/ZnO NP release study

In order to evaluate the release of Ag/ZnO NPs, AgZnO/PCL/CS (70:30) scaffolds were cut into 5 mm pieces and placed in a Falcon tube containing 10 ml of artificial saliva. These scaffolds are put in a water bath at 37 °C. The concentration of Ag/ZnO in the PBS was measured after 1,3, and 7 days by atomic absorption spectroscopy (AAS).

FTIR spectroscopy of the PCL-PEG-Chitosan scaffold

In order to characterize PCL-PEG-Chitosan scaffold, Fourier-transform infrared (FTIR) spectroscopy (Shimadzu 8400S, Japan) was used to determine whether they had been chemically modified. A range of IR spectra between 4000 and 400 cm^{-1} was recorded. Mixtures of samples with KBr were pressed into discs for IR analysis [24].

Mechanical properties and stability of the PCL-PEG-Chitosan scaffold

Mechanical properties and hardness of the scaffolds were evaluated using UVI universal testing machine (Koopa, Iran), and their stability was evaluated using dental orthodontic typodont.

Evaluation of the antibacterial effects of

The inhibition zone method was used to evaluate the antibacterial activity based on the Li proposed method [25]. The PLC/CS discs were used as the control group. The scaffolds were sectioned into 5-mm disks and placed on a lawn of *Streptococcus mutans* and *Staphylococcus aureus* on an agar medium plate. Then, the artificial saliva was added to them, and after 24h incubation at 37 °C, the inhabitation zone was measured (Fig. 1). This evaluation continued for up to 7 days, and each plate's inhibition zone was measured three times.

L929 fibroblast cells culture

The stative cell culture was used to culture the L929 fibroblasts. The scaffolds were cut in the same size as 96-well plates and were sterilized using either 1 h UV irradiation or 24 h soaking in 70% ethanol. After sterilization, they were washed with PBS 3 times. Seeding of the L929 cells was performed on the scaffolds. 100 µL of the medium and 1000 cells were added to each well of the 96-well plates. Then, the plates were put in a 37-centigrade incubator with a concentration of 5% CO_2 [26, 27].

The evaluation of cytotoxicity using MMT *assay*

The cytotoxicity effects were analyzed with MMT [28]. DMEM with 10% FBS was used for the culture of all L929 fibroblast cells in 96-well plates at 10,000 cells/well for 24 h. AgZnO NPs were prepared at 25, 50, and 100 µg/mL concentrations. 100 µL of MTT was added to each well. The existing medium was replaced with 100 mL of the fresh medium after 4 h of incubation at 37 °C. 20 µL of MTT (5 mg/ml in PBS) was added to each well before 2 h incubation at 37 °C. After removing the medium, 100 µL DMSO was added to dissolve the crystals of formazan. A microplate reader (infinite NanoQuant M 200), Tecan (Zurich, Switzerland), was

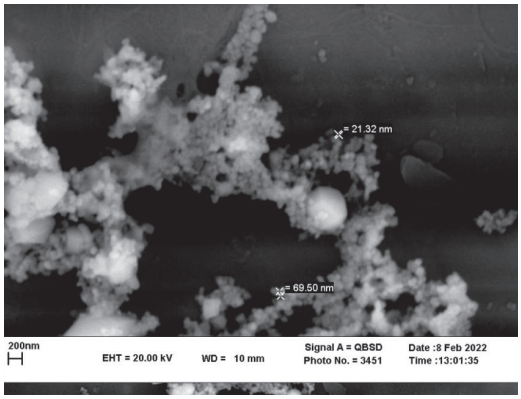

Fig. 2. The NPs' SEM image

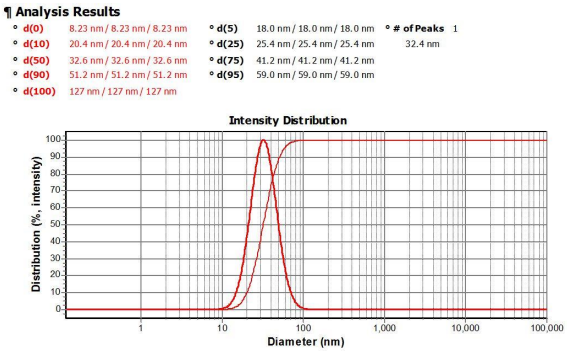

Fig. 3. The analysis results of DLS

used to measure absorbance at 570 nm (reference wavelength 630 nm). Treated cells were compared to control cells (Cell viability = Atreated/Acontrol 100), and the mean ± SD of triplicates was reported [29].

Statistical analysis

All data were reported as the mean ± Standard deviation (SD). The groups of this study were described using descriptive statistics (frequency and distribution, charts, central tendency, and variance-to-mean ratio). Statistical analysis was performed using one-way analysis of variance (ANOVA) in SPSS 20 with a 0.05 level of significance.

RESULTS

Scanning electron microscope (SEM)

Scanning Electron Microscope (SEM) (MIRA3TESCAN-XMU, Czech) was used to analyze the scaffolds' microstructure, including the size of the pores, the uniformity of the distribution of the pores, and whether the cells are open or closed (Fig. 2). Due to the insulation of the specimens, they were covered with a layer of gold before SEM imaging [30].

Particle size measurement

The measurement of Ag/ZnO NPs revealed the particles were uniform and had the optimal size. The estimated average diameters of these NPs were 32.4 nm (Fig. 3).

FTIR spectroscopy

PCL-conjugation to CS was confirmed using FTIR spectroscopy (Fig. 4). The first and second amide carbonyl stretch absorbance peak at 1650 cm^{-1} and 1576 cm^{-1}, respectively, confirmed the presence of amid bonds in PCL-CS derivatives.

Ag/ZnO NPs release study

The diagram of the absorption of Ag/ZnO NPs (Fig. 5A) was used in order to illustrate the line graph of Ag/ZnO NP release according to AgZnO concentration. The NPs loaded PCL/CS scaffolds had properly released over time. The highest

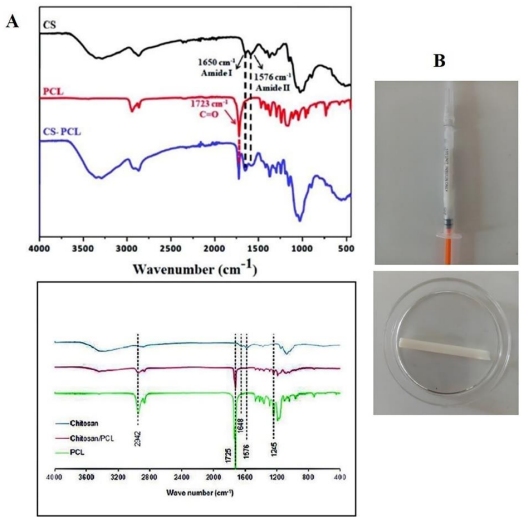

Fig. 4. (A) FTIR spectroscopy of the PCL-PEG-Chitosan scaffolds (B) The frozen 70:30 solution in the 1mL insulin syringe

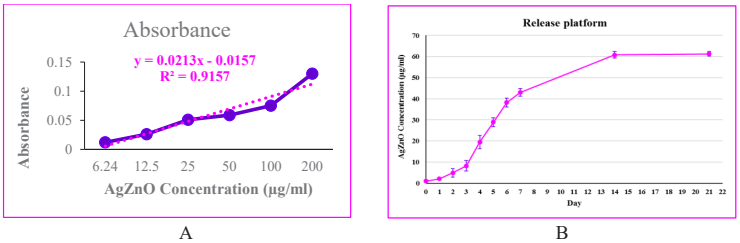

Fig. 5. A) The standard absorption of Ag/ZnO NPs. B) The release platform of Ag/ZnO NPs

concentration of NPs was seen on 14th day (60 µg/mL). They released considerably during the first two weeks before reaching a plateau from 14 to 21 days (Fig. 5B).

Stability of the PCL-PEG-Chitosan scaffold

In order to evaluate the stability of the scaffolds, a round cross-section orthodontic wire was passed through the scaffold, and its stability was evaluated by observation (Fig. 6). The result of this observation confirmed that the PCL-PEG-CS scaffolds had proper stability.

Antibacterial effects of Ag/ZnO

Fig. 7 shows the changes in the diameters of the inhibition zone on the *S. mutans* medium plate during 21 days. The diameter of the inhibition zone had increased until the 21st day. This increase was more significant in the first 14 days and, after that, plateaued at about 2.8 cm.

Fig. 8 shows the changes in the diameters of the inhibition zone on the *S. aureus* medium plate during 21 days. The diameter increased significantly during the first 7 days before reaching a plateau (3.5 cm).

Fig. 9 shows the inhibition zone of Ag/ZnO NPs loaded scaffolds compared to the scaffolds without NPs on both *S. mutans* and *S. aureus* medium plates on the 21st day. The clear ring of the inhibition zone was seen only around

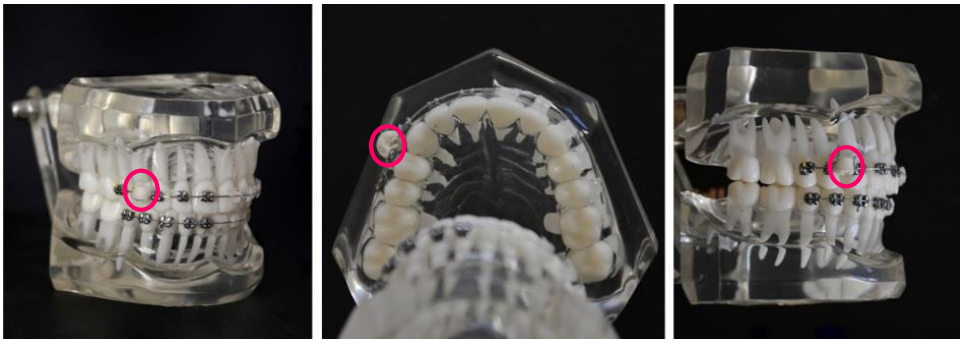

Fig. 6. The dental orthodontic typodont with the PLC-PEG-Chitosan scaffold

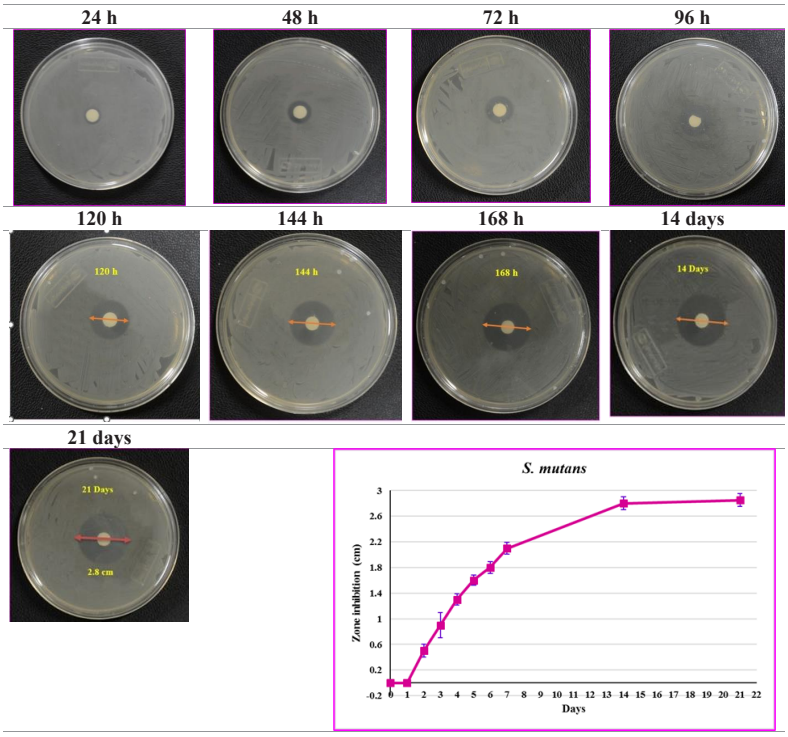

Fig. 7. The changes in the diameters of the inhibition zone on the S. mutans medium plate after different periods

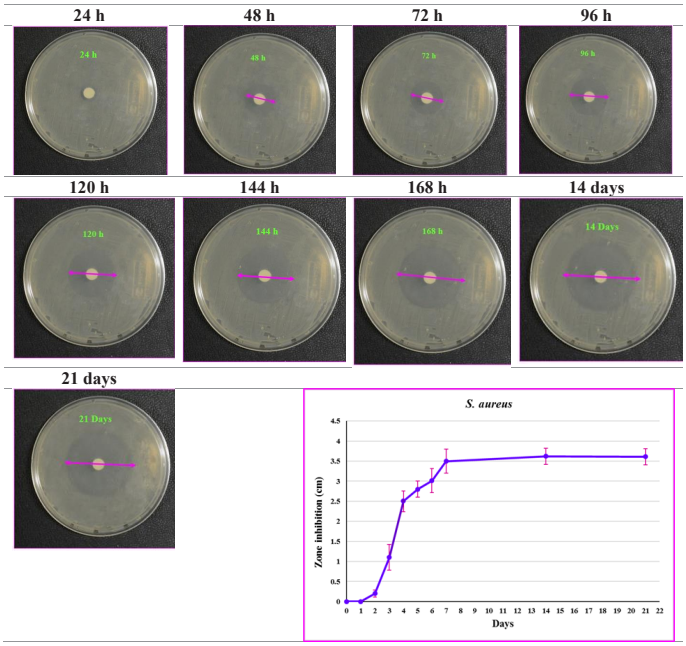

Fig. 8. The changes in the diameters of the inhibition zone on the S. aureus medium plate after different periods

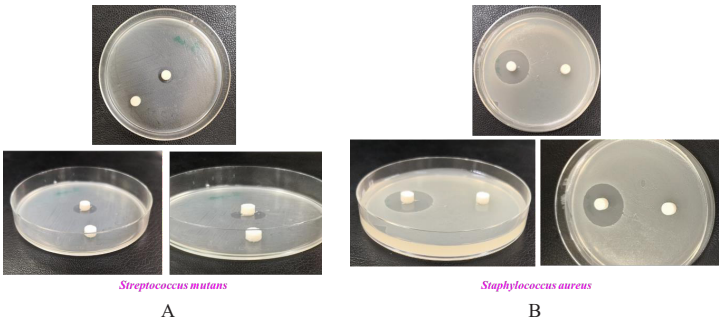

Fig. 9. The evaluation of antibacterial activities of Ag/ZnO NPs loaded composite scaffolds using inhibition zone on the medium plate of *S. mutans* and *S. aureus*

the scaffolds with Ag/ZnO NPs, which was 2.8 cm for *S. mutans*, while it was significantly higher for *S. aureus* at 3.5cm.

Cytotoxicity evaluation

The evaluation of cytotoxicity tests conducted on specimens after 24 and 48 h using three different NPs concentrations showed that Ag/ZnO NPs loaded PLC/CS composites had no cytotoxic effects on the fibroblast cells after more than 1 day, and all three concentrations of NPs resulted in more than 70% of cell viability. After 24 h, the cell viability of the scaffolds without NPs was slightly higher than those with NPs, and the concentration of 25 µg/mL showed the highest cell viability compared with 50 µg/mL and 100 µg/mL (Fig. 10).

After 48 h, the scaffolds with NPs showed higher cell viability than those without NPs. The

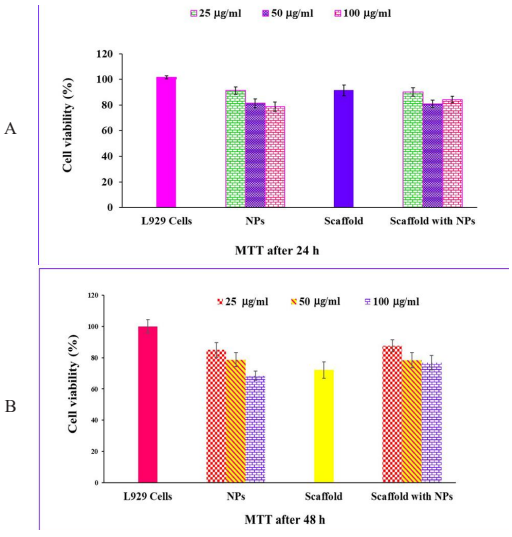

Fig. 10. The results of MTT after 24 h (A) and 48 h (B)

25 µg/mL concentration resulted in the highest percentage of cell viability with a negligible error rate in L929 fibroblasts. The lowest cell viability was seen in the scaffolds without nanoparticles (Fig. 10).

DISCUSSION

In the present study, two types of PCL/ Chitosan composite scaffolds were made: 1 without Ag/ZnO NPs, and 2. with Ag/ZnO NPs. Due to PCL's mechanical properties and biocompatibility, this polymer is generally used as a composite with other polymers to control the degradation rate and improve cell adhesion [12, 31]. Chitosan is a biocompatible substance with moderate antimicrobial activity that can be used as a drug carrier due to its biodegradability [14]. In this study, these two polymers were used to synthesize a scaffold that can be loaded NPs for dental applications. The effects of Ag and ZnO nanoparticles have been studied separately in previous studies. Yoshida found that the Ag NPs have antibacterial properties against *S. mutans*, the most important bacteria in the tooth decay micro-flora [11]. Jones suggested the application of ZnO NPs for their antibacterial activities because they can inhibit the growth of *S. aureus* [32].

Recently, the evaluation of the antibacterial and cytotoxicity effects of coupled nanoparticles attracts the researchers' attention. It has been found that the effect of ZnO NPs can be enhanced when coupled with Ag [33-35]. A study by Kasraei showed that composite resins containing Ag nanoparticles and ZnO have antibacterial activity against *S. mutans* and *Lactobacillus* [36]. The results of our study revealed that Ag/ZnO NPs can increase the antibacterial properties of composite polymers. Applying Ag/ZnO NPs on both *S. mutans* and *S. aureus* medium plates led to the creation of a clear ring of inhibition zones which can show the antibacterial activities of the NPs against these 2 important pathogenic oral microflorae (Fig. 9). These activities were considerable against both of the microorganisms during 3 weeks. However, it was more significant against *S. aureus* rather than *S. mutans.* The diameter of the inhibition zone of *S. aureus* was significantly higher than that for *S.* mutans during all days of the examination period. Especially, in a short period (a week) the disparity between these diameters was more considerable (3.5 cm for *S. aureus* and 2.2 for *S. mutans*) (Fig. 7 and Fig. 8).

Moreover, the results of the present study showed that the antibacterial property of the scaffold made of PCL and chitosan only depends on the presence of Ag/ZnO NPs. The scaffolds without NPs were not able to create any inhibition zone on the bacterial medium plates, therefore, PCL/CS scaffolds do not have antibacterial activity (Fig. 9).

The antibacterial activity of Ag/ZnO NPs can be correlated to the concentration of NPs. In this study, the NPs construction had increased over time and the highest concentration was seen on the 21st day (Fig. 5). As the highest diameter of the inhibition zone on both bacterial medium plates was seen on the 21st day, they can also be correlated.

The cytotoxicity effects of NPs are known related to different parameters. In a study conducted by Sufyani, the size of NPs is considered to be related to its cytotoxicity. In this study, chemically synthesized Ag NPs with the smallest size had a less cytotoxic effect on GF01 human gingival fibroblast cells [19]. This result suggests that the size of NPs might affect the effect of NPs on L929 fibroblasts. Avci found that in laboratory conditions, MTT assay shows better cell viability in PCL specimens with encapsulated Ag NPs than Ag NPs and pure PCL nanofibers [37]. It is suggested that the toxicity of ZnO NPs can depend on the production of reactive oxygen species (ROS) in mitochondria and the induction of apoptosis [18].

In this study, we found no cytotoxic effects of Ag/ZnO NPs, and applying all concentrations of NPs resulted in more than 70% cell viability. MTT assay showed that time can be one of the other parameters affecting NPs' cytotoxicity. After 24 h, the cell viability of scaffolds without NPs was slightly more than those with NPs. In comparison, after 48 h, the cell viability had increased in the groups of the scaffolds with NPs and significantly more than those without NPs. So, cell viability can increase over time. In the scaffolds with NPs group, the highest cell viability was seen at 25 µg/mL concentration of NPs after 24 and 48 h. The lowest cell viability was seen at 100 µg/mL concentration. The cytotoxicity effect was evaluated on the fibroblast cells, one of the important cells in the skin, therefore, Ag/ZnO NPs can be used on the skin, as well.

CONCLUSION

The results of this study showed that Ag/ZnO

NPs loaded polycaprolactone/chitosan composites have controlled slow-released properties. These NPs have antibacterial effects on oral microfilms and can inhibit the growth of *S. mutans* and *S. aureus*. Moreover, they are safe with no cytotoxicity effects on the fibroblast cells and can be used in the oral cavity and skin. We suggest that AgZnO/PCL/CS scaffold analysis is conducted in further animal and clinical trial studies.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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