



## An In Vitro Assessment of Antimicrobial and Cytotoxic Effects of Nanosilver

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### ABSTRACT

**Background:** The antimicrobial activity of silver nanoparticles has been investigated in medical fields in recent years, but there are few studies regarding its effect on oral microorganisms. The aim of the present study was to evaluate the in vitro antimicrobial and toxicity properties of nanosilver against two dental plaque microorganisms and Human Gingival Fibroblast (HGF) cell line.

**Methods:** Antibacterial effects of nanosilver colloidal solution were determined by minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) using microdilution method. Standard strains of *Streptococcus sanguis* and *Actinomyces viscosus* were used. For toxicity assessment, MTT and LDH tests were performed under controlled conditions. Different concentrations of nanosilver were prepared and their toxic effects on HGF were determined after 24, 48 and 72 hours.

**Results:** The MIC of nanosilver solution for *S. sanguis* and *A. viscosus* were 16 and 4 µg/ml, respectively. The MBC of nanosilver was 64 µg/ml for *S. sanguis* and 16 µg/ml for *A. viscosus*. MTT results showed that after 24 hours the concentrations of ≥ 0.5 µg/ml of nanosilver solution affected cell viability when compared with control group. After 48 and 72 hours only the concentration of ≥ 5 µg/ml showed significant effect on cultured cell viability. LDH release test demonstrated toxic effect only after 48, 72 hours by 20 and 50 µg/ml of nanosilver.

**Conclusion:** The results demonstrated that beside its antibacterial activity against *S. sanguis* and *A. viscosus*, nanosilver mediated a concentration and time dependent cytotoxicity on HGF.

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## Introduction

Colonization of microorganisms in mouth begins immediately after birth. More complex microbial flora forms in mouth after tooth eruption. Accumulation of specific microorganisms on dental and adjacent gingival surfaces as dental plaque results in decay and periodontal diseases and subsequently tooth loss (1). Many people cannot effectively remove microbial plaque from their teeth surfaces by mechanical methods like tooth brushing, therefore applying chemicals such as mouthwashes, is an effective way to help microbial plaque control (2).

Antimicrobial effects of silver have been known for long time. The term nanosilver refers to 5-50 nm sized-particles of silver (3). The results of many studies indicate that, common antibiotics usually have lethal and inhibitory effects only on bacteria, but nanosilver has lethal effects on a wide range of fungi, protozoa and even viruses (4-8). Nanoparticles exhibit different properties in comparison to conventional sizes due to their greater surface areas, surface energies and increased surface reactivity (9). Possible mechanisms by which nanosilver shows its antimicrobial effects include attachment to cell membranes, changing its permeability, passing through it and generating reactive oxygen species intercellularly (10-12). This means that nano-particles are able to access biologic sites which are not accessible for larger particles (13). For this reason and also because of increasing interests in the application of nanomaterials, the possible toxicity of these materials on different aspects must be examined. A number of studies have been directed to assess the toxic effects of nano-

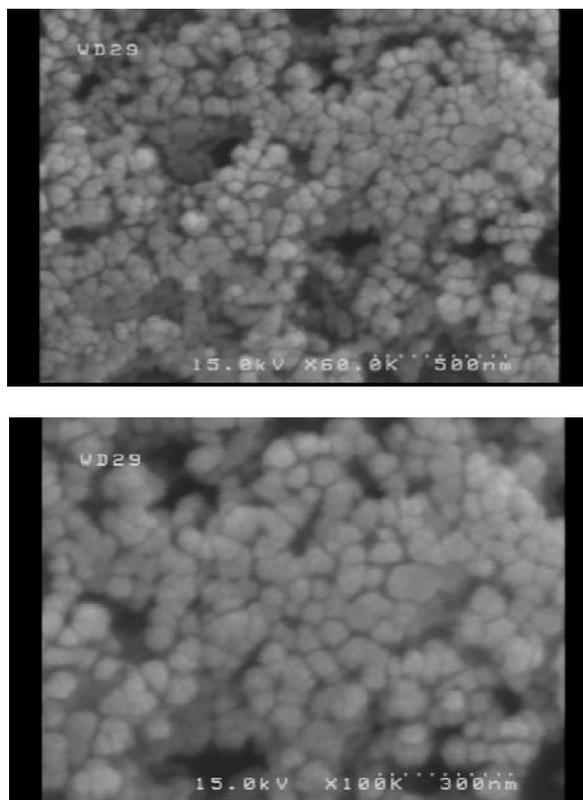
silver on different cells (3, 14-18). Also, few studies have been conducted upon the impacts of nanosilver on the microorganisms responsible for the formation of dental microbial plaque (19). *Streptococcus sanguis* and *Actinomyces viscosus* are two microorganisms that play a role as early colonizers in dental plaque formation (1, 20). Nanosilver should exhibit good cell compatibility as well as antimicrobial properties in order to be applicable in the formulation of a mouthwash. If this material is used after oral surgeries for mouth disinfection, it will affect many different cells beside microorganisms. Fibroblasts are the preponderant cellular element of connective tissue and play a major role in synthesis, maintenance and repair of connective tissues of extracellular matrix components (1, 21). The purpose of the present study was to evaluate antimicrobial properties of nanosilver on the above mentioned microorganisms and the toxicity of silver nanoparticles on cultured human gingival fibroblasts.

## Materials and Methods

### Materials

Nanosilver colloidal solution in water (Plasma Chem Company, Berlin, Germany) with the concentration of 0.1 mg/ml (w/v) and average particle size of 10nm was used for this experiment.

The size of silver nanoparticles were evaluated using field emission scanning electron microscopy (Hitachi S4160), which showed particle diameters within nano-size range (*Figure 1*).



**Figure 1.** Field Emission Scanning Electron Microscopy images

#### Antibacterial tests

The antimicrobial activity of nanosilver was tested against standard strains of *S. sanguis* (PTCC 1449) and *A. viscosus* (PTCC 1202). To activate the lyophilized bacteria, 1ml Mueller Hinton broth was aseptically added to it and mixed well. After that, a portion of bacterial suspension was transferred to a blood agar culture medium. *S. sanguis* grew after 24 hours incubation at 37°C but, *A. viscosus* grew after 48 hours in an incubator containing 5% CO<sub>2</sub> at 37°C. The strain grown on the blood agar was immediately transferred to a medium containing phosphate buffer solution of sodium-phosphate [pH = 7.4] (PBS: 0.13 m NaCl, 10 mM), 5% bovine serum albumin or 5% fetal calf serum and 10% dimethyl sulfoxide [C<sub>2</sub>H<sub>6</sub>SO] and stored in a

freezer at -70°C for short periods until the time of antimicrobial tests (22).

#### Broth microdilution method to determine antimicrobial properties of nanosilver

MIC of nanosilver solution against *S. sanguis* and *A. viscosus* was determined by broth microdilution method using Mueller Hinton broth (23). The cell suspension was prepared by suspending growth from agar plate in 2 ml of sterile Mueller Hinton broth in a way to reach an optical density equivalent to 0.5 McFarland. Suspension was further diluted to obtain an inoculum of 10<sup>6</sup> CFU/ml (22).

Twelve sterile test tubes (16×100 ml) were filled with one ml Mueller Hinton broth medium except 1<sup>th</sup> and 12<sup>th</sup> tubes. Tube No 11 was used as a positive control which has no nanosilver solution and only contained the microorganisms. Tube No 12 was negative control which has neither nanosilver solution nor microorganisms (it only contained the Mueller Hinton broth medium). The nanosilver suspension was diluted in a series of two-fold dilutions in these test tubes in a way, that they contained 0.0625 to 32 g/ml nanosilver.

In the next phase, 1 ml of prepared bacterial suspension containing 10<sup>6</sup> CFU/ml was added to each tube. Therefore, there were 5×10<sup>5</sup> CFU/ml bacteria in each test tube which was in line with the broth micro-dilution method standard. Then, the concentration of nanosilver was calculated in the final volume (2 ml), which varied from 16 g/ml in the first tube to 0.0312 g/ml in the tenth one. The tubes incubated with agitation at 37°C for 24 hours. After incubation, the lowest concentration of nanosilver showing no growth which was determined by no opacity in its

tube, was considered as the MIC of nanosilver against microbial strain (23).

#### *Determination of MBC*

MBC was determined by removing 100 microliters of inoculum from each tube that did not show any turbidity and cultivating on Mueller Hinton agar. All the plates were incubated at a temperature of 37°C for 24 hours. The number of bacterial colonies was counted and compared with the number of living cells per volume of original inoculum. Since the anti-bacterial drugs do not always kill bacterial population completely, the lowest concentration of the antimicrobial substance which reduces the living bacteria to less than 0.1% of original inoculum will be considered as the minimal bactericidal concentration (23). For each test 5 replicates were undertaken in order to ensure the accuracy of the test.

#### *In vitro toxicity tests*

The toxicity of nanosilver was tested on Human Gingival Fibroblast cell line (HG Fcells) (National cell bank of Pasteur Institute, Tehran, Iran) was tested. The cells were cultured in RPMI-1640 (Gibco) containing 10% Fetal Bovine serum (FBS) and maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for 24 hours, then seeded onto 96-well culture plates at 1×10<sup>4</sup> cells per well. After 18 hours (overnight adherence at 37°C) cells were treated with different concentrations of Nanosilver (0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 µg/ml). To obtain a nanosilver solution without microbial contamination, it was passed through 0.2 µm pore filters. The experiment was per-

formed with both filtered and non-filtered solutions.

Each concentration of nanosilver and also the control solution media which had no nanosilver were added to 5 wells of a 96-well plate. The solvent of original nanosilver solution was ultrapure water; therefore, for evaluating the effect of solvent on cell culture, ultrapure water was added to one row of wells. Two different cytotoxicity testing were performed for cell viability evaluation, MTT and LDH. In MTT assay, the viability of HGF was assessed using MTT [3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide]. After incubation with various nanosilver concentrations, cells were treated with 0.5 mg/ml MTT solution, for 4 hours at 37°C. After that, they were rinsed with PBS, and 500 µl of 0.04 M HCl in isopropanol was added to each well. These solutions were transferred to 96 well ELISA plate. The number of viable cells was determined by uptake of MTT. The absorbance which is relative to cell vitality was measured at 570 nm (ASYS HiTech Expert plate reader).

In LDH assay, 50 µl of 96-well plate cell culture supernatant was removed and added to LDH detection kit (Roche, Applied sciences) in a 96-well plate. Then NAD<sup>+</sup>, Diphosphoresis (Cytochrom b5 reductase) and Iodine tetrazolium chloride (INT) solution were added to wells. After 30 minutes, absorbance and cell lysis of supernatant was measured at 492 nm (ASYS HiTech Expert plate reader). In this assay, in addition to negative or low controls (LC) which had no nanosilver, there were positive or high controls (HD) that contained 2% Triton-X100 with high level of toxicity.

### Statistical Analysis

Variations between groups were compared using a one way ANOVA and Student T-test. *p*-Values less than 0.05 were statistically considered significant.

## Results

### In vitro antimicrobial properties

The results indicated that the MIC of nanosilver against *S. sanguis* and *A. viscosus* was 16 and 4  $\mu\text{g/ml}$ , respectively (Table 1). The MBC of nanosilver was 64 for *S. sanguis* and 16  $\mu\text{g/ml}$  for *A. viscosus* (Table 1).

**Table 1.** MIC and MBC of nanosilver against *S. sanguis* and *A. viscosus*

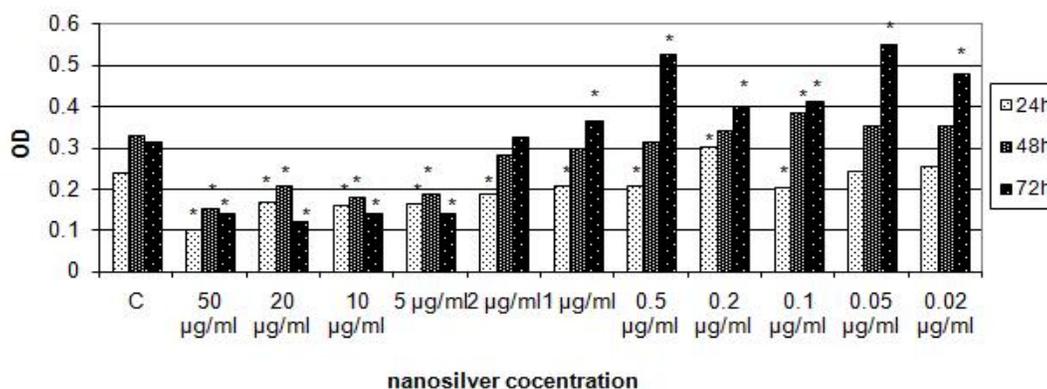
Bacteria	MIC	MBC
<i>S. sanguis</i>	16 $\mu\text{g/ml}$	64 $\mu\text{g/ml}$
<i>A. viscosus</i>	4 $\mu\text{g/ml}$	16 $\mu\text{g/ml}$

### In vitro toxicity assessment

HGF were incubated with different concentrations of nanosilver solutions ranging from 0.02  $\mu\text{g/ml}$  to 50  $\mu\text{g/ml}$ . Cell viability was assessed after 24, 48 and 72 hours of incubation.

### MTT results

The values of OD have shown in Figure 2. These data showed that after 24 hours the concentrations of 0.5  $\mu\text{g/ml}$  and higher of non-filtered nanosilver solution had statistically significant effect on cells vitality when compared to control group. After 48 and 72 hours, only the concentration of 5  $\mu\text{g/ml}$  and higher showed significant effect on cultured cells. A significant increase in OD occurred after 72 hours in low concentration ( $\leq 1$   $\mu\text{g/ml}$ ) which indicated these doses of nanosilver had a stimulating effect on fibroblast cells and increased their vital activity.



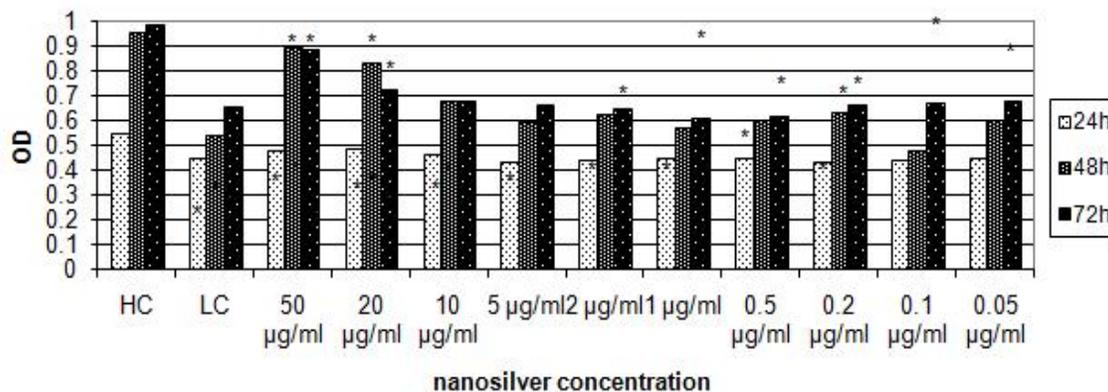
**Figure 2.** Optical Density (OD) by MTT test for different concentration of non-filtered nanosilver solution after 24, 48 and 72 hours.

\*represents significant difference compare to control group.

### LDH results

In LDH vitality assay, non-filtered nanosilver solutions were used. During 24 hours, there was no statistically significant LDH release by different concentrations of nano-

silver solution (Figure 3). After 48 and 72 hours, there was a significant increase in LDH release by 20 and 50  $\mu\text{g/ml}$  concentrations (Figure 3).



**Figure 3.** Optical Density (OD) by LDH test for different concentration of non-filtered nanosilver solution after 24, 48 and 72 hours.

\*represents significant toxicity compare to control group.

## Discussion

In this research the antimicrobial effects of silver nanoparticles against two dental plaque microorganisms, i.e. *S. sanguis* and *A. viscosus*, were evaluated by determining MBC and MIC. The applied in vitro method was broth micro dilution which is used as a standard method in laboratories and is more accurate and reliable compared with other methods such as disk diffusion also its results can be interpreted easier (2, 23).

The MIC of silver nanoparticles against *S. sanguis* and *A. viscosus* was 16 and 4 g/ml and the MBC was 64 and 16 g/ml, respectively. It seems that *A. viscosus* is more sensitive to this substance compared with *S. sanguis*.

It should be mentioned that although nanosilver is effective against those bacteria in vitro, these concentrations may not have the same impacts in clinical conditions. In the mouth environment, microbial populations are different from free-living microorganisms and they exist within the biofilms which act as a barrier (1). The biofilm plays an important role in protecting microorganisms from

the effect of mouthwashes and other local antimicrobials. Saliva also plays an effective role in diluting the substance and changing the pH. Other factors, which affect the results, are blood and different potential of oxidation and reduction in various part of the mouth (1). Also, in the test tubes antimicrobials are in contact with the microorganisms longer than when these are used as mouthwashes. In our study, silver nanoparticles were applied in pure, not in the formulation of a mouthwash which contains many other substances. The presence of other chemical substances may affect its antimicrobial property (24).

In this study, we also evaluated the cytotoxicity effect of different concentrations of nanosilver solution on human gingival fibroblast cell line (HGF cells) using MTT and LDH viability assays. In order to prevent microbial contamination, nanosilver solution was passed through filters with 0.2 µ pores. The process of filtering might decrease the concentration of solution because particles could be trapped in the filter structure. For this reason MTT assay was performed with both filtered solutions and non-filtered solu-

tions. At the end of this part of examination, no microbial contamination was observed in non-filtered solutions, so only non-filtered solution was used for LDH release test.

The result of MTT test showed that after 24 hours, nanosilver solutions with the concentrations of  $\geq 0.5$   $\mu\text{g/ml}$  had a transient toxic effect on fibroblast cells but after 48 and 72 hours  $\geq 5$   $\mu\text{g/ml}$  concentrations had such effect. On the other hand, toxicity decreased with the culturing days. Another study has shown similar results (25). This result may suggest that at relatively low concentrations some kind of biocompatibility between the nanosilver and the cell could be achieved with the culturing time. Another interesting observation was the increase in OD at low concentrations after 72 hours, which represents that nanosilver particles may augment the activity of cells or induce their proliferation or both.

LDH test results showed that none of the nanosilver concentrations had toxic effect on cells after 24 hours, but after 48 and 72 hours the concentrations of  $\geq 20$   $\mu\text{g/ml}$  showed toxicity. So, results showed a time and concentration-dependent manner of reduction in cell viability which was consistent with other studies (14, 15, 18). According to the results of toxicity tests, MIC of nanosilver against *S. sanguis* and *A. viscosus* and its MBC against *A. viscosus* seem to be within the safe doses of nanosilver.

The results of two cytotoxicity tests were different. An explanation of this difference is that MTT mostly represents cell vitality but LDH assay shows cell toxicity. On the other hand,  $\geq 5$   $\mu\text{g/ml}$  concentrations of nanosilver decrease the optical density which means a reduction in cell activity and viability but not

necessarily cell death. In comparison between the results of the two tests, more emphasize can be put on LDH results.

Alt *et al* (2003), in their study on a bone cement containing 1% nanosilver, showed that this material had no toxic effect on mouse fibroblast and human osteoblasts (3). In another study on mouse fibroblasts (L929) using MTT test, nanosilver particles at concentrations more than 20 ppm demonstrated toxic effects (25). Hsin *et al* (2008), showed that nanosilver was toxic on muscle cells at concentrations of  $\geq 50$   $\mu\text{g/ml}$  using MTT assay (18).

The variation of the result reported by different studies can be interpreted in two ways. First, the types of cells used for experiment were different. Second, the difference between nanosilver preparation (12, 26) and particle sizes (12).

In conclusion, the result of our study demonstrated that although nanosilver mediated a concentration and time dependent cytotoxicity on HGF, it has a good antibacterial activity against *S. sanguis* and *A. viscosus*. It is suggested that the antimicrobial properties of this material be assessed against periopathogens and cariogenic bacteria. Also, its cytotoxic effect on other cells should be examined.

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### Conflict of Interest

None declared conflicts of interest.

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