Cytotoxicity of Silver Nanoparticles on Human Gingival Epithelial Cells: An *In Vitro* Study

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Abstract

Objective: Nanosilver has numerous applications in medicine due to its potent antibacterial activity. However, data regarding the bio-safety of its effective concentrations is scarce. This study aims to assess the toxicity of silver nanoparticles on human gingival epithelial cells under *in-vitro* conditions.

Methods: This *in vitro* study evaluated the toxic effects of filtered and unfiltered nanosilver solution on human gingival epithelial cells obtained from the Pasteur Institute of Iran using the methylthiazol tetrazolium bromide (MTT) assay (mitochondrial function)and membrane leakage of lactate dehydrogenase (LDH) at 24, 48 and 72h time points. The concentrations of silver nanoparticle solution used were 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20 and 50µg/ml.

Results: The MTT assay showed that nanosilver solution at high concentrations (20 and 50µg/ml) significantly decreased the viability of cells at all time points. The lower non-fatal concentrations at 24 and 48h were capable of causing cell death or significantly inhibit cell growth at 72h. The LDH assay demonstrated that death of epithelial cells only occurs at high concentrations of nanosilver (20 and 50µg) and no significant toxicity was seen at lower concentrations.

Conclusion: Based on the results, silver nanoparticles have toxic effects on human gingival epithelial cells and this effect is time and dose-dependent.

Key words: Cytotoxicity, Epithelial cells, Nanosilver

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Introduction:

At present, nanotechnology is among the hot topics in scientific communities worldwide making a revolution in research and manufacturing of different products (1). Nano-materials are defined as substances measuring 1-100 nm. Nano-technology deals with nano-materials and production of nano-substances (2, 3). Nano-materials have different physical and chemical properties compared to those of the same bulk material (4-6). Silver nanoparticles have been produced by nanotechnology. Silver nanoparticles smaller than 100nm include 15 to 20 thousand silver atoms. The antimicrobial effects of silver have long been recognized. Manufacturing silver in nanocrystalline structure has significantly enhanced its biological and antimicrobial properties (7). Silver nanoparticles provide greater surface area than bulk silver. This issue may be responsible for the greater antimicrobial effect of nanosilver (8-12). Nanosilver particles bind to proteins containing sulfur in the bacterial membrane, penetrate into the bacteria and by changing the morphology and membrane permeability and affecting the

cellular respiration cycle and mitosis lead to cell death (12,13). Studies have shown that in contrast to conventional antibiotics that only have bactericidal and bacteriostatic properties, nanosilver exerts its antimicrobial effect on a wide spectrum of microorganisms namely bacteria, fungi, protozoa and even viruses (13-17). Despite the extensive use of nano-products, limited studies have investigated their biological and toxic effects on human cells (18-23). Thus, further studies in this respect seem necessary. This study aimed to assess the toxicity of silver nanoparticles on human gingival epithelial cells under *in vitro* conditions.

Methods:

Nanosilver with medium 10nm particles and primary concentration of 0.1mg/ml was purchased (Plasmachem, Berlin, Germany). After initial evaluation and ensuring its applicability in cell culture medium (in terms of causing contamination), various concentrations of the solution were prepared in the cell culture medium and added to the cells. Volume of each well was 200 µl and the final concentration of nanosilver in wells was 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05 and 0.02 µg/ml.

As the primary source for obtaining the cells, one KB epithelial cell flask was purchased from the cell bank of Pasteur Institute of Iran. The characteristics of this cell line have been described in the brochure available at cell bank website as follows:

These cells were first taken from the mouth of a Ghafghazi male. Their culture medium is EMEM (EBSS)+ 1% NEAA+ 10% FBS that became compatible with RPMI 1640 + 10% FBS in the Pasteur Institute of Iran.

NCBI Code	Designation	Species	Tissue	Morphology
C152	KB	Human	Mouth	Epithelial-like

In this study, cells from a single-layered flask were used. In order to familiarize the obtained cells after transferring them from the primary medium of the cell bank to a fresh medium, cell flask was stored in an incubator at 37˚C for 24h in an environment containing 5% CO_2 and 95% water vapor. This medium was evacuated again and warm RPMI medium was added for 15-20 min at 37˚C in an incubator. For irrigation, 5cc of the medium and for replacement (refreshment), 5.4 cc of the medium along with 5.0 cc FBS serum (a combination of 90% RPMI and 10% FBS) were conventionally used. Then, cell passage was performed. To ensure compatibility and health of cells before their transfer to the 96-well plate, we tried to perform at least 2 successful passages for each cell line. Then, cells were transferred to a 96-well plate for testing and counting.

After completion of cell culture in plate, 18h time was allowed and after ensuring cell growth, different concentrations of drug were added. Considering the risk of contamination of the culture medium, in the first step, the drug was sterilized using a filter. Considering the risk of reduction of particles following filtering, the experiment was repeated twice with the filtered and unfiltered drugs.

After preparation, the drug was added to the cell plates. The medium in each well was gently and carefully removed by a sampler. Given the concentration of drug, serum-containing medium was added to each row and then a specific amount of drug was added to each row to obtain the desired concentrations of drug (50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05 and 0.02µg/ml). After adding the drug, the plate was placed on a shaker with moderate agitation for less than one minute and then placed in an incubator.

In this study, MTT and LDH assays were used to

assess thecytotoxicity of nanoparticles. MTT assay evaluates the viability of cells. In other words, it evaluates viable cells and its reduction indicates cell death (24, 25). In the LDH assay, the amount of LDH released from the dead cells is measured. This value corresponds to the cell death rate (26). After conversion of absorbance obtained by MTT and LDH assays to the percentage of toxicity using the ELISA reader, the concentration of drug causing the death of 50% of cells was calculated.

The results of various groups were statistically analyzed using Student's t-test and post-hoc tests. $p<0.05$ was considered statistically significant.

Results:

A. Cytotoxicity assessment using MTT assay:

As observed in Diagram 1, the viability of cells significantly decreased at high concentrations of nanosilver at 24, 48 and 72 hours. Student t-test demonstrated that this reduction was statistically significant and was greater at 72h. An important point is that lower doses that do not cause cell death can lead to cell death or at least growth inhibition at longer periods (particularly 72h) compared to the control group.

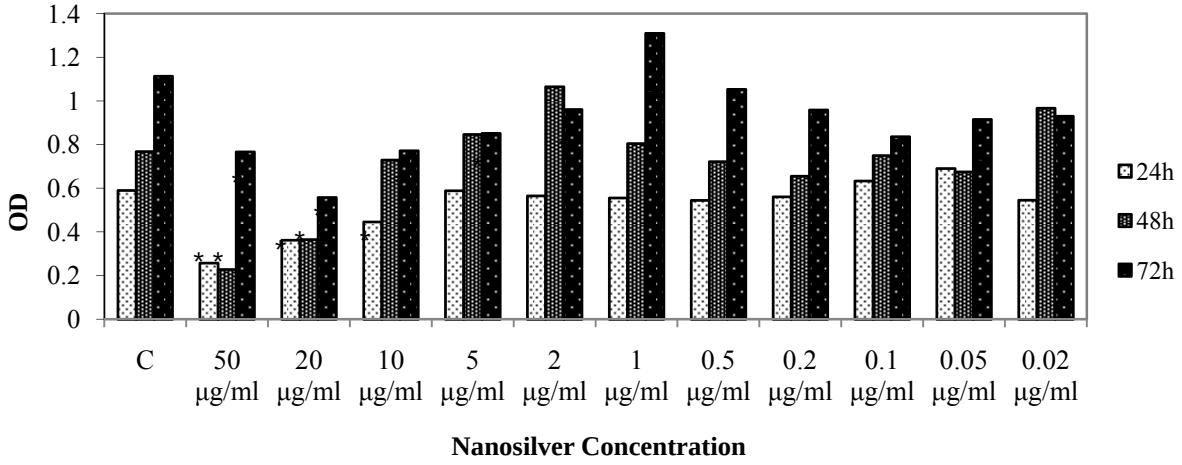

Diagram 1- Optical density indicating the effect of nanosilver on epithelial cells at 24, 48 and 72h

B. Cytotoxicity assessment using LDHassay:

MTT assay revealed that unfiltered nanosilver does not cause any contamination in the culture medium. On the other hand, filtering the drug may decrease the number of nanosilver particles. Thus, for assessment of cytotoxicity using LDH assay, unfiltered nanosilver was used. According to the manufacturer's instructions provided in the LDH kit, the optical density (OD) results can be converted to the percentage of cytotoxicity in the respective cells using the formula. Thus, according to the instructions, two control groups were used: one control group without the nanosilver (culture medium only, negative control, without cell death) and another control group containing 2% Triton X100 to achieve the highest toxicity and LDH production by the respective cells. The control group with only the culture medium was named the low control (LC) and the one with high cytotoxicity as high control (HC) group.

The amount of LDH due to the effect of nanosilver on KB epithelial cells was measured at 24, 48 and 72h and the P-value relative to the LC group was calculated by Student's t-test (Diagram 2).

As observed in Diagram 2 according to the LDH assay, cell death only occurred at high concentrations of silver nanoparticles and no significant toxicity was seen at low concentrations.

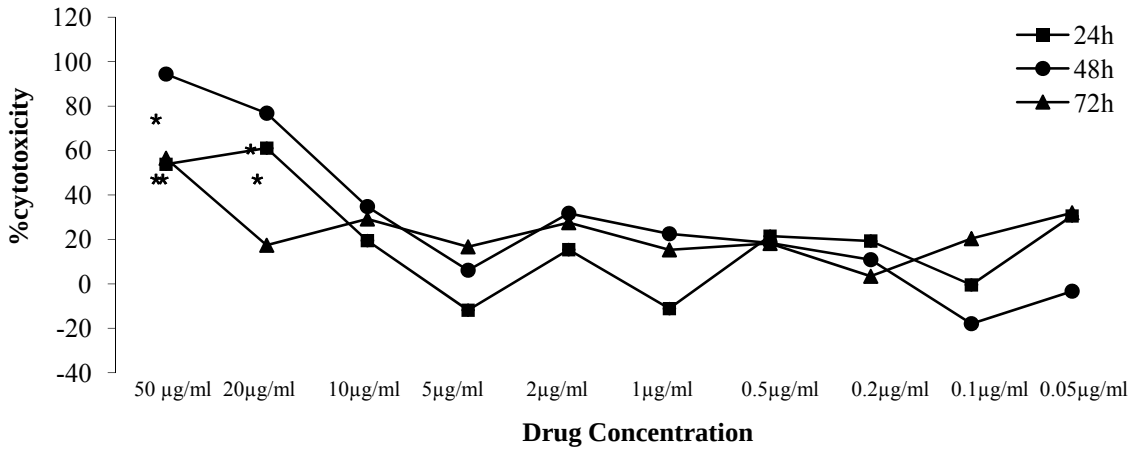

Diagram 2- The percentage of cytotoxicity due to the effect of nanosilver on epithelial cells at 24, 48 and 72h

The IC50 results (the concentration causing 50% mortality) are shown in Table 1. As observed, according to the MTT assay cells better manifest the damages due to nanosilver in short time. However, higher doses of nanosilver are required to see serious damages in long term. This finding was also confirmed by LDH assay.

Table 1- IC50 of the effect of nanosilver particles on gingival epithelial cells in µg/ml

	IC50 in MTT Test	IC50 in LDH Test
24 hours	39.51	38.01
48 hours	33.75	20.67
72 hours	62.85	47.47

Using repeated measures ANOVA, the effect of time and various doses of drugs as well as the effect of filtered and unfiltered nanosilver were evaluated.

Discussion:

In the recent years, nanotechnology has been suggested for use in preventive dentistry. This technology has been used for the manufacture of nanosilver toothbrush and toothpastes. Nanosilver has excellent antibacterial and antiviral properties (27). Since nanosilver particles are used because of their optimal antimicrobial properties when in contact with the oral environment (first contact with epithelial cells), evaluation of their toxicity is necessary. The present study was the first to assess the cytotoxicity of nanosilver solution on human gingival epithelial cells (obtained from a cell bank).

The results of MTT assay showed that concentrations higher than 10 µg/ml of the respective substance at 24 and 48h had toxic effects on epithelial cells; however, lower concentrations did not have significant effect on the viability of cells. At 72h, in almost all concentrations, a slight inhibition of growth (or viability) was observed compared to the control group. This reduction was small (10-15%) but statistically significant and may indicate the regulatory effect of nanosilver on cell growth; which requires longer time to manifest.

The result of LDH assay which is indicative of cytotoxicity revealed that nanosilver only at 20 and 50 µg/ml concentrations had a toxic effect on epithelial cells at all understudy time points.

The results of IC50 test (concentration that causes 50% mortality) showed that damage due to exposure to silver nanoparticles is better manifested in short time periods and higher doses of nanosilver are required to cause significant damage at longer periods.

Park *et al.*, in 2007 evaluated the effect of zinc, aluminum, nickel and mercury nanoparticles

with a mean diameter of 150 nm on human epithelial cell line (A549). They exposed human pulmonary epithelial cells to different concentrations of these nanoparticles for 24h and assessed morphologic changes using two-color flow cytometry. Their results demonstrated that these nanoparticles cause various degrees of cytotoxicity; which is dose-dependent (18). They evaluated the effect of various concentrations of nanoparticles on pulmonary epithelial cells after 24h; whereas, we evaluated the effect of various concentrations of nanosilver particles on gingival epithelial cells that are exposed to different oral hygiene products containing nanosilver. Our study results are probably more documented.

Alt, *et al*. (2004) evaluated the antimicrobial effect and cytotoxicity of a bone cement containing 1% nanoparticulate silver and showed its optimal antimicrobial properties and no toxicity against mouse fibroblasts and human osteoblasts (19). These results are in accord with our findings; however, our study evaluated the effect of various concentrations of nanosilver on human gingival epithelial cells; which is different from their study.

Hsin, *et al*. in 2008 by using MTT assay demonstrated that 50µg/ml and higher concentrations of this product had toxic effects on fibroblast and endothelial smooth muscle cells of rat at 24h (20). Such finding is in contrast to our results. This difference may be attributed to the fact that they evaluated fibroblast and endothelial smooth muscle cells of rat.

Ahamed in 2011 investigated the toxic effects of Nickel nanoparticles at 0, 1, 2, 5, 10 and 25 µg/ml concentrations on pulmonary epithelial cells at 24 and 48h using MTT, LDH, GSH, ROS and LPO as factors indicative of toxicity. He found that Nickel nanoparticles had toxic effects on pulmonary epithelial cells (28). However, his study is different from ours since he evaluated the effect of Nickel nanoparticles.

Hussain, *et al*. in 2005 assessed the toxic effects of silver, aluminum, molybdenum, iron oxide and titanium dioxide nanoparticles on mouse hepatocytes using MTT, LDH, GSH, ROS and MMP factors. The results showed that silver nanoparticles at all concentrations had high toxicity on mouse hepatocytes; which was significantly higher than that of other elements (23). These results are not in agreement with our findings. In our study, toxic effects were evaluated on human gingival epithelial cells at 24, 48 and 72h. Different reactions of these cells from those of rat hepatocytes and also the time of evaluation might be responsible for the different results.

Kawata, *et al*. in 2009 evaluated the toxicity of silver nanoparticles on human hepatocytes and found that nanosilver does not have toxicity at low concentrations but it has toxic effects on human hepatocytes at high concentrations (29). These results are in concord with our findings.

The results of the present study demonstrated that the toxic effect of silver nanoparticles on human gingival epithelial cells is time and dose-dependent. This finding has also been reported in some previous studies on fibroblasts (20, 22). Different toxic concentrations reported by previous studies may be due to the different type of fibroblasts used, different preparation method of silver nanoparticles (24) or size of the particles (26).

Conclusion:

Study results revealed that silver nanoparticles have toxic effects on human gingival epithelial cells and this effect is time and dose-dependent.

Conflict of Interest: "None Declared"

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