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Synthesis and Toxicity Study of Silver Oxide Nanoparticles on Human Gingival Fibroblasts

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ABSTRACT

Background and Objective: Silver oxide nanoparticles have very high activity against a wide range of microbes and parasites and also have lower toxicity to human cells compared to other biocides and heavy metals. The present study was conducted to determine the cytotoxic effects of silver oxide nanoparticles on gingival fibroblasts.

Methods: This experimental in vitro study was conducted on 15 samples in 4 groups at concentrations of 10, 20, 30 and 40 µg/ml within 2, 24, 48 and 72 hours. Dynamic light scattering (DLS) and scanning electron microscopy (SEM) were used to determine the properties of silver oxide nanoparticles, and the MTT technique was used to determine the toxicity of silver oxide nanoparticles.

Findings: According to the results, after 2 hours of proximity to silver oxide nanoparticles, the mean difference was statistically significant between the control group (0.19±0.01) and the 40 µg/ml group (0.17±0.01), and also between the 10 µg/ml group (0.19±0.01) and the 40 µg/ml group (0.17±0.01) ($p<0.001$). After 48 hours of proximity, the mean difference in OD between the control group and concentrations of 40 µg/ml (0.25±0.01) and 30 µg/ml (0.25±0.01) was statistically significant ($p<0.001$). After 72 hours, the mean difference in OD between the control group and concentrations of 40 µg/ml (0.30±0.01) and 30 µg/ml (0.31±0.01) was statistically significant ($p<0.001$).

Conclusion: The results of the study showed that the highest viability was related to the concentration of 10 µg/ml and after 2 hours. Therefore, this concentration of silver oxide nanoparticles has acceptable biocompatibility.

Keywords: *Silver Oxide Nanoparticles, Gingival Fibroblasts, Cytotoxicity*.

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Introduction

Nanomaterials usually refer to tiny solid particles with diameters of 1-100 nm. Nanomaterials are promising in antibacterial therapies due to their enhanced and unique physical and chemical properties, which include very small size, larger surface-to-volume ratio, and enhanced chemical reactivity. Nanoparticles exist in nature and are widely used in our daily lives (1, 2). With the advancement of technology, silver nanoparticles have been introduced as effective antimicrobial components in prosthetic materials, adhesives, and implants, to arrest dental caries, to prevent biofilm formation, and to induce bone formation. Consequently, it is reasonable to predict that AgNPs (nanoparticles) will play an important role in oral health care in the near future (3).

Nanoparticles are small enough to pass through the smallest capillaries in the body and biological membranes and affect the physiology of every cell type in the body (4, 5). Recently, there have been reports of their toxicity to liver, spleen, kidney, and reproductive tissues (6), and it has even been shown that silver nanoparticles with a diameter of less than 80 nm cross the brain barrier (7-9). According to reports, neurological and respiratory damage, circulatory problems, and some other toxic effects of nanoparticles are the most important concerns in the use of nanoparticles (10-12). Cell culture techniques are very useful for evaluating the biocompatibility of dental materials. These techniques are cheaper, more reproducible, and more reliable. One of the cells that has been of interest in cytotoxicity studies is the fibroblast, which is the main cell in the synthesis and formation of collagen fibers and mesenchymal cells that can differentiate into cementoblasts and osteoblasts (13-15).

Satyavani et al. synthesized nanoparticles from the leaves of Suaeda monoica (S. monoica) using 1 mM silver nitrate and evaluated the effect of the synthesized silver nanoparticles on human epidermoid larynx carcinoma cell line using MTT colorimetric technique. The effect of silver nanoparticles on human epidermoid larynx carcinoma cell line showed dose-dependent toxicity to the tested cell and the viability of Hep-2 cells was reduced to 50% (IC50) at a concentration of 500 nM (16).

In recent years, some scientists have concluded that the application of nanoparticles in drug delivery systems can enhance tumor suppression and reduce drug side effects by using silver nanoparticles. In addition, the combination of traditional drugs with nanotechnology provides an opportunity for the development of new antimicrobial drugs (17).

Tunç investigated the anticancer, antimicrobial and proapoptotic activities of silver nanoparticles (AgNPs) and carboplatin-loaded silver nanoparticles (AgNPs-Car). DLS, EDX-STEM and FTIR techniques were used to identify and analyze the synthesized nanoparticles. The antiproliferative and proapoptotic effects of these nanoparticles were also evaluated using XTT and Annexin V tests. Furthermore, the MIC (minimum inhibitory concentration) test was used to measure the antimicrobial activity. The results showed that the anticancer activity of AgNPs-Car was very high in MCF-7 (human breast adenocarcinoma), A549 (human lung carcinoma) and C6 (brain glioma) cells. The highest selective cytotoxic activity was observed in C6 cells. In addition, it was shown that AgNPs-Car and AgNPs cause DNA degradation and ultimately increase apoptosis in cells. In evaluating the antimicrobial activity of AgNPs and AgNPs-Car on gram-positive and gram-negative pathogenic microorganisms and yeast fungi, the results showed that AgNPs-Car had the most successful results compared to other nanomaterials. Overall, AgNPs-Car performed very successfully in targeting C6 glioma cells by facilitating drug entry into the cell. Moreover, the anticancer activity of these nanoparticles was high in MCF-7 and A549 cells, while their toxicity was relatively low. Due to the characteristics and positive effects of silver nanoparticles, these materials are preferred for the design of drug delivery systems (18).

Due to the significance of using silver oxide, as well as its inexpensive and practical use in disease prevention, as well as the feasibility of the study and limited number of studies in this field, this study was conducted to determine the cytotoxic effects of silver oxide nanoparticles on gingival fibroblasts at different concentrations at different times.

Methods

This experimental in vitro study was conducted after approval by the Ethics Committee of Qazvin University of Medical Sciences with code IR.QUMS.REC.1400.415. According to statistical studies, the number of samples was estimated to be 15. Ag2O nanoparticles were prepared at concentrations of 10, 20, 30, and 40 ppm. Culture medium without FBS was used to obtain the required concentration of nanoparticles because if alcohol or water is used as a solvent, there is a possibility of cytotoxic effects of these materials and they may affect the test result, therefore RPMI was used as the safest material (16).

At least three wells of 4 plates (for different times of 2, 24, 48 and 72 hours) were considered for each concentration of the tested substances and 3 wells were also considered for the control group. 10,000 fibroblast cells were placed in each well of a 96-well cell culture plate and after 24 hours of incubation (5% CO2 pressure, 98% humidity, 37 °C temperature), the supernatant was removed. Then, Ag2O nanoparticles at concentrations of 10, 20, 30 and 40 ppm were applied to the cells in the wells of the 96-well plate, while complete culture medium was poured as a 100% non-toxic material in the 3 control wells.

Fibroblast cell culture: Human gingival fibroblast (HGF) cells purchased from the Pasteur Institute of Iran were used to carry out this research. First, the frozen cells were thawed and cultured in special flasks with culture medium (GIBCO RPMI) and placed in an incubator at a temperature of 37°C, atmospheric pressure of 1 ATM, 100% humidity, and 5% carbon dioxide concentration. Before each passage stage, cell viability was assessed by microscopy. Cell passage was performed in order to multiply the cells and reach the required number. To perform cell passage, first the culture medium was removed from the flask and then the flask was washed with warm physiological serum and its content was discarded. Then, cells were separated from the bottom of the plate with EDTA and trypsin and new culture medium was added. Half of the contents of the flask was transferred to another flask and then these media were placed in the incubator for 1 week. After several passage steps and ensuring cell viability, 10,000 cells were placed in each well of a 96-well plate. Then, the volume of cells was adjusted to 100 µl with complete culture medium, RPMI, to nourish the cells, along with 100 units/ml penicillin and 100 micrograms/ml streptomycin to prevent bacterial growth, and the plates were placed in an incubator for approximately 20 hours. During this time, the coating process, i.e., the adhesion of cells to the bottom of the plate, was performed, and the viability of all wells was evaluated again. The chemical reduction method was used to synthesize nanoparticles in this study.

Dynamic light scattering (DLS) and FE-SEM microscopy were used to study the size of silver nanoparticles. FE-SEM microscopy imaging was also used to examine the morphology of the fabricated silver nanoparticles (Figure 1).

Cell viability was measured using the MTT test at 2, 24, 48 and 72 hours after exposure to nanoparticles. After the desired time, the cell culture media was removed from the incubator and one-tenth of the cell supernatant was added to each well of Dimethylthiazol-2 yl 2-5 diphenyl tetrazolium bromide (MTT) solution and the plate was incubated for 4 hours. Then, the plate was removed and isopropanol acid was added to the wells by extracting the supernatant to dissolve the purple crystals formed in the surviving cells and a uniform liquid was formed. This colored liquid was transferred to the wells of the ELISA

plate and its absorbance was read using an ELISA READER at a wavelength of 570 nm with a 620 reference filter. The results were evaluated in terms of optical density (OD), so that the higher the number read by the ELISA-reader, the more mitochondrial enzymes were able to perform the MTT reduction process.

Data analysis was performed using SPSS version 25. One-way ANOVA was used to compare the viability of fibroblast cells at different concentrations and times, and two-way ANOVA was used to examine the simultaneous effect of concentration and time. Pairwise comparisons were performed using Tukey's post hoc test, and $p<0.05$ was considered significant.

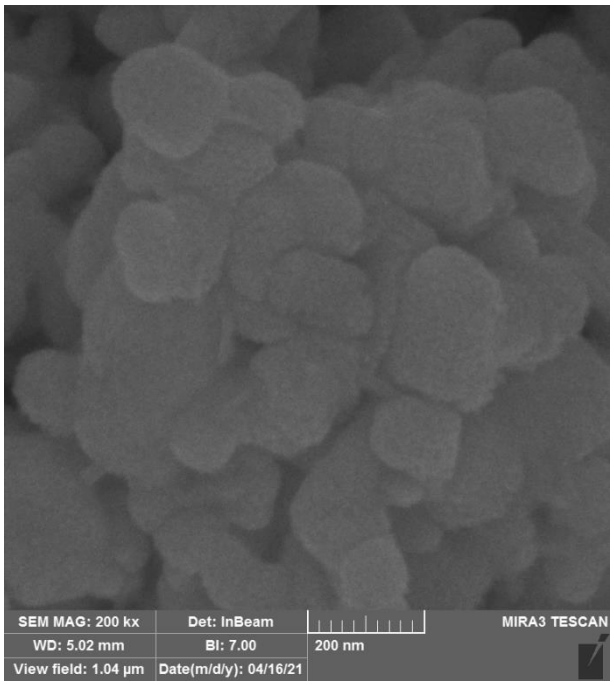

Figure 1. Field emission scanning electron microscopy (FESEM) image of synthesized Ag2O nanoparticles at 200 kX magnification

Results

After 2 hours, a significant difference was observed between the different groups of silver oxide nanoparticles in terms of mean Optical Density (OD) ($p=0.014$). The highest survival and viability of fibroblast cells was at a concentration of 10 (0.19±0.01), with 98.85% of the cells remaining alive. In a pairwise comparison between different concentrations, only the mean difference between the control group and the concentration of 40 µg/ml (0.17±0.01), as well as concentration of 10 µg/ml and 40 µg/ml was statistically significant (Table 1).

After 24 hours, the highest survival and viability of fibroblast cells was at a concentration of 10 (0.23±0.01), with 96.74% of the cells remaining alive. Overall, no significant difference was observed between different groups of silver oxide nanoparticles in terms of mean OD ($p=0.427$) (Table 2).

The highest viability rate of fibroblast cells was observed in 48 hours of their proximity to silver oxide nanoparticles at concentrations of 10 (0.27±0.01) and 20 (0.27±0.01), with 92.62% of cells remaining alive. In a pairwise comparison between different concentrations, the difference in mean OD between the control group and concentrations of 40 (0.25±0.01) and 30 µg/ml (0.25±0.01) was statistically significant ($p<0.001$)

(Table 3). After 72 hours, the highest viability of fibroblast cells was at concentration of 10 (0.34±0.01), with 93.55% of cells surviving. In a pairwise comparison between different concentrations, the difference in mean OD between the control group and concentrations of 40 (0.30±0.01) and 30 (0.31±0.01) was statistically significant. Also, the concentration of 40 was significantly different from the concentrations of 10 and 20 µg/ml (0.33±0.01) ($p<0.0001$) (Table 4).

Table 1. Survival and viability of fibroblast cells after 2 hours

Silver oxide nanoparticles concentration (µg/ml)	Number	Fibroblast survival and viability (%)	Mean±SD	p-value
Control	5	100	0.19±0.01[a]	0.014
10	5	98.85	0.19±0.01[a]	0.014
20	5	95.22	0.18±0.02[ab]	0.014
30	5	92.73	0.18±0.01[ab]	0.014
40	5	90.23	0.17±0.01[bc]	0.014

Similar lowercase letters indicate lack of statistically significant difference.

Table 2. Survival and viability of fibroblast cells after 24 hours

Silver oxide nanoparticles concentration (µg/ml)	Number	Fibroblast survival and viability (%)	Mean±SD	p-value
Control	5	100	0.24±0.03	0.427
10	5	96.74	0.23±0.01	0.427
20	5	95.48	0.23±0.02	0.427
30	5	91.22	0.21±0.01	0.427
40	5	90.55	0.22±0.02	0.427

Table 3. Viability of fibroblast cells after 48 hours

Silver oxide nanoparticles concentration (µg/ml)	Number	Viability of fibroblasts (%)	Mean±SD	p-value
Control	5	100	0.29±0.01[a]	0.001
10	5	92.62	0.27±0.01[ab]	0.001
20	5	92.62	0.27±0.01[ab]	0.001
30	5	86.82	0.25±0.01[b]	0.001
40	5	84.23	0.25±0.01[b]	0.001

Similar lowercase letters indicate lack of statistically significant difference.

Table 4. Survival and viability of fibroblast cells after 72 hours

Silver oxide nanoparticles concentration (µg/ml)	Number	Fibroblast survival and viability (%)	Mean±SD	p-value
Control	5	100	0.36±0.02[a]	0.0001
10	5	93.55	0.34±0.01[ab]	0.0001
20	5	92.60	0.33±0.02[ab]	0.0001
30	5	85.38	0.31±0.01[bc]	0.0001
40	5	83.60	0.30±0.01[cd]	0.0001

By controlling the effect of time, a significant difference was observed in terms of survival and viability of fibroblast cells between different concentrations ($p<0.001$) (Chart 1). Also, by adjusting for the effect of concentration, the changes in time were independently significant. The mean survival and viability increased over time at different concentrations. The highest mean survival and viability was at 10 µg/ml at all times.

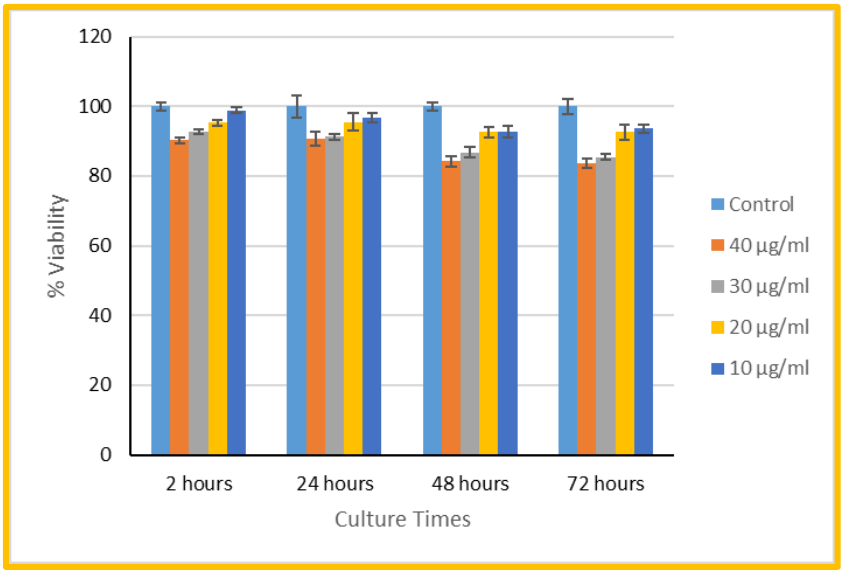

Chart 1. Mean percentage of survival and viability of fibroblast cells at concentrations of 10, 20, 30, and 40 µg/mL after 2, 24, 48, and 72 hours

Discussion

The results of the study showed that the highest survival and viability of fibroblast cells occurred after 2 hours of exposure to silver oxide nanoparticles at a concentration of 10, with 98.85% of the cells maintaining their viability.

The results of the study by Mohsenzadeh et al. on peripheral blood mononuclear cells showed that there was no toxicity to the cells at concentrations less than 10 ppm (19). As a general result, the lowest toxicity was observed at shorter times and concentrations less than 10, which is consistent with the results of the present study.

In a study by Yen et al., the cytotoxic effect of small-sized nanoparticles on macrophages was more pronounced than that of medium-sized or large-sized nanoparticles for both silver and gold nanoparticles, and AuNPs had a greater effect than AgNPs of the same size. The findings also ensured the biosafety of silver nanoparticles for antimicrobial applications at a dose of less than 10 ppm, which is consistent with the results of the present study (20).

According to a study by Xinping et al., the results showed that silver nanoparticles (NPs) can significantly increase their antibacterial activity with increasing concentration because their toxicity increases with increasing concentration, which is consistent with the present study (21).

In a study conducted by Shahoon et al., the cytotoxicity of rod-shaped hydroxyapatite nanoparticles on fibroblast cells was investigated at different concentrations and at times of 2, 24, 48 and 72 hours, and the results showed that with increasing time and concentration, the mean vital activity of the cells decreased (22), which is consistent with our study.

According to a study by Movagharnia et al., the lowest toxicity and lethality were related to the concentration of 125.3 PPM and the highest to the concentration of 100 PPM. As a result, the toxicity increases with increasing concentration (23), which is consistent with the present study.

Liu et al. investigated the toxic effects of silver nanoparticles at concentrations of 0.005–2.5 μg/mL on normal 293HEK cells compared to the HeLa cancer cell line and concluded that 0.4 μg/mL is the minimum IC50 value for AgNPs among cell lines, which is considered normal (24). Therefore, consistent with the present study, increasing the concentration of nanoparticles increases their toxicity.

According to these results, the synthesized nanoparticles have different effects. Since silver nanoparticles have a higher surface-area-to-volume ratio than larger silver particles, they are more chemically active and are converted to its ionized form to a greater extent. Silver nanoparticles can bind to different tissues and have toxic and destructive effects due to the production of oxygen free radicals and cause cell death. The strength of our study was in conducting detailed laboratory studies and using up-to-date devices, as well as carrying out all control steps, which makes the results more reliable. The weakness of this study was the excessive cost of the equipment and materials, which forced us to use a minimum sample size.

Given that the oral environment contains different layers of connective, epithelial, and immune cells, further research will clarify the exact mechanism of the effect of nanoparticles on different tissues. Considering that the present study is a laboratory study and the test was performed on a cultured cell layer, the generalization of the results of this study to clinical conditions should be done cautiously.

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