In vitro Cytotoxicity of Silver Nanoparticles on Human Periodontal Fibroblasts

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Silver nanoparticles (NNPs) are extensively used for all kinds of antimicrobial applications in medical research. Their efficacy has been demonstrated against Streptococcus mutans, which is associated with dental caries. However, their cytotoxic effects on human periodontal tissue are not completely understood. ***Objective:*** *The aim of this study was to evaluate the possible toxic cellular effects of different concentrations and sizes of silver nanoparticles, less than 10 nm, 15–20 nm, and 80–100 nm, respectively, on human periodontal fibroblasts.* ***Study design:*** *Primary culture cells isolated from human periodontal tissue were exposed to 0–1,000 μM silver nanoparticles of each size for 24-, 72-, and 168-hour periods. Cytotoxicity was evaluated with a nonradioactive, soluble MTS/PMS assay.* ***Results:*** *The results demonstrated that silver nanoparticles of less than 20 nm increased cytotoxicity in human periodontal fibroblasts in a dose- and time-dependent manner.* ***Conclusion:*** *The 80–100-nm-sized nanoparticles did not modify the viability of human primary culture cells.*

Keywords: *nanoparticles, silver, cytotoxicity, fibroblast*
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INTRODUCTION

Silver salts have been widely used medically as bactericides in water purification systems, antiseptic solutions such as prosthetic heart lining, pulp capping, and catheters.[1,2] In dentistry, silver has been alloyed with other metals as amalgam[3] as well as in commercial products whose active ingredient, silver diamine fluoride, has been proven to prevent caries, increase enamel resistance, inhibit plaque formation, and reduce dental hypersensitivity.[4,5]

Recently, several studies have investigated the bactericidal and bacteriostatic properties of silver nanomolecule solutions on various species of microorganisms and viruses.[6] In previous research we showed that *Streptococus mutans*, the principal organism associated with tooth decay, is targeted by silver nanoparticles (NNPs).[7] Silver ion toxic effects are rare in humans, but chronic exposure increases the formation of skin (argyria) and eye (ocular argirosis) deposits of this metal.[8-10] In the oral mucosa, amalgam dental restorations cause the development of a blue-black coloration in the surrounding tissue. These toxic effects in humans are reversible once exposure is stopped. The metal is eliminated primarily by hepatic metabolism and renal filtration.[11]

In 1984, the International Organization for Standardization (ISO) established its norms for the development of dental material biocompatibility tests *in vivo* and *in vitro*.[12] However, due to the recent introduction of nanomaterials in various preparations, the potential toxicity have not been fully evaluated for human use. The development of animal cell cultures *in vitro* has successfully established a reproducible experimental biology model for biomedical research. The primary cell cultures derived from these methods have been shown to retain the genotypic, morphologic, metabolic, and functional characteristics of the tissue from which they were isolated as well as a high degree of differentiation.[13-17]

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The objective of this study was to evaluate the cytotoxic effect of NNPs on an *in vitro* model of primary fibroblast cultures isolated from human periodontal tissue.

MATERIALS AND METHODS

Silver nanoparticles (NNPs)

The nanoparticle manufacturing procedure included consecutive colloidal solution reactions with salt oversaturation, forming a precipitate by homogeneous and heterogeneous nucleation. Subsequently, the nanoparticles were grown by diffusion. The concentration gradient, temperature changes, agitation, and surfactants controlled the nanoparticle size. The procedure produces monodisperse, non-agglomerated particles of uniform size.

First, a silver solution was prepared with sodium tetrahydroborate as a reducing agent. The silver/reducing agent ratio was greater than 1. A stabilizer agent was added to control the nanoparticle size and uniformity. Three different-sized particles were produced in distilled water solutions at a concentration of 10^{-2} moles/l: less than 10 nm NNPs (pH 11—modified at pH 7), 15–20 nm NNPs (pH 10—modified at pH 7), 80–100 nm NNPs (pH 7). The pH modifications were made after synthesis of the nanoparticles by the addition of nitric acid.[18-20] Silver nanoparticle size was controlled by electron transmission microscopy and dynamic light scattering (DLS) analysis.[21]

Human periodontal fibroblast primary cultures

Fibroblast cell primary cultures were isolated from human periodontal tissue explants of third molars extracted for orthodontic reasons from healthy donors. After extraction, the teeth were rinsed twice in modified buffered Hank´s saline solution (130 mM NaCl, 2.9 mM KCl, 0.98 mM Na_2HPO_4, 15 mM HEPES, 2.3 mM $NaHCO_3$, 5 mM D-glucose, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B), and then were placed in Petri dishes containing Dulbecco´s modified Eagle medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. Periodontal tissue was carefully removed from the coronal section and 1 mm^2 explants were incubated with 2 mg/mL collagenase F, 2.5 mg/mL papain, and 0.125% trypsin in a buffered saline solution (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 8 mM $MgCl_2$, 20 mM HEPES, pH 7.4, 10 mM D-glucose, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.4 mg/mL DTT) for 2 hours at 37°C, 5% CO_2, and 95% humidity. After tissue disaggregation, the cell suspension was centrifuged at 400 × g, for 7 minutes, and the cell pellet was grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 15 mM HEPES, 20 mM D-glucose at pH 7.4, 100 U/mL penicillin, and 100 µg/mL streptomycin in 75 cm^2 flasks. Confluent fibroblast monolayers were trypsinized (0.125%/0.5 mM trypsin-EDTA solution), and the cells were harvested and suspended in fresh medium (10^6 cells/mL) to be subcultured in either 75 cm^2 polystyrene flasks or 96-well culture microplates for experiments.

Cytotoxicity assay

The cytotoxic effect of silver nanoparticles and the cell viability of human periodontal fibroblast monolayers were evaluated by MTS/PMS assay, which measures soluble formazan formation. Five × 10^4 cells/0.32 cm^2/well were grown in microplates and incubated with 0–1,000 µM of 3 different NNPs sizes: less than 10 nm, 15–20 nm, and 80–100 nm, for 24 hours, 72 hours, and 7 days at standard conditions. Then, 20 mL of MTS/PMS (1:20) mix were added to each well and incubated for 1 hour at 37°C in the dark. The reduction in cell viability was determined based on the absorbance of the MTS/PMS formazan at 490 nm for each sample in a microplate reader (BioRad Laboratories, Hercules, CA). Measurements were performed in triplicate for each point, and the experiments were repeated at least 3 times. Results were expressed as percentage of cell viability relative to cells treated with vehicle (control), and the mean and standard deviation of each point were calculated.

Statistical analysis

Three identical experiments were performed in triplicate, then were analyzed with ANOVA followed by an LSD post hoc analysis using the statistical program SPSS v. 18. (SPSS, IBM, Chicago, IL, USA). Differences with $P < .05$ were considered statistically significant.

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham F12 medium, and certified fetal bovine serum were obtained from GIBCO-BRL (Life Technologies BRL, Gaithersburg, MD, USA). MTS/PMS CellTiter 96® AQueous Non-Radiactive Cell Proliferation Assay kit were purchased from Promega Co (Madison, WI, USA); other chemicals and materials were obtained from Sigma-Aldrich (St Louis, MO, USA).

RESULTS

Time and concentration dependence of less than 10-nm-sized, NNPs-induced cytotoxicity of human periodontal fibroblasts

To evaluate the cytotoxic effect of <10-nm-sized NNPs exposure time on cell viability, monolayers of human periodontal ligament fibroblasts were incubated in the presence of either 1–1,000 µM NNPs or the control for 24 hours, 72 hours, and 7 days. As shown in Figure 1, cell viability at 24 hours was significantly decreased in the <10-nm-sized NNPs-treated fibroblasts with 250, 500, and 1,000 µM (66.6 ± 18, 23.1 ± 12.38, 7.4 ± 0.6, respectively, $P < .05$). Similar results were observed at 72 hours (36.3 ± 12.4 % with 500 µM, and 25.8 ± 11.3 % with 1,000 µM vs 100% with control, $P < .05$) and 7 days (12.4 ± 5.9% with 500 µM, and 11.3 ± 4.5% with 1,000 µM, $P < .05$). However, after 7 days of NNPs exposure, a significant increase in fibroblast cell viability was observed at concentrations of 50, 100, and 250 µM (116.1 ± 9.4%, 119.7 ± 7.2%, 108.1 ± 5.5%, respectively, vs control, $P < .05$).

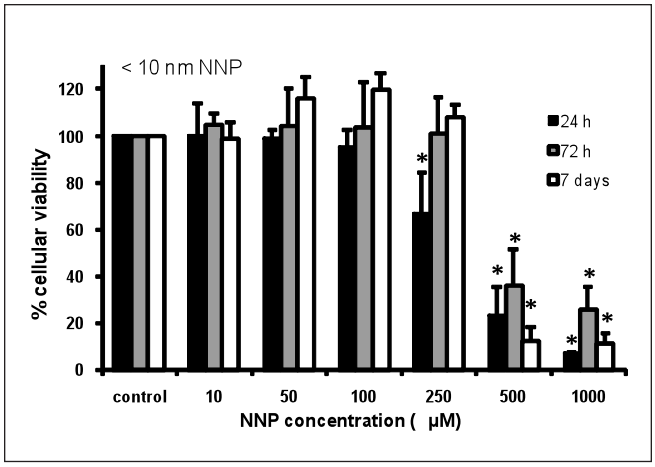

Figure 1. Time and concentration effect of less than 10-nm-sized silver nanoparticles (NNP) on cell viability of human periodontal ligament fibroblasts. Primary cultures of human periodontal ligament fibroblasts were exposed to 0–1,000 μM of <10-nm-sized NNP for 24 hours to 7 days. Experiments were performed using the MTS/PMS assay with absorbance measurements at 490 nm as described in Methods. The results are expressed as a percentage of control (vehicle) and represent the means ± standard deviation of 3 independent experiments, with each point determined in triplicate.
*P < .05, NNP vs control.

Time and concentration dependence of 15–20-nm-sized, NNPs-induced cytotoxicity of human periodontal fibroblasts

When human periodontal ligament fibroblasts were exposed to 15–20-nm-sized silver nanoparticles for 24 hours to 7 days, treatment with NNPs at concentrations of 10 to 1,000 μM resulted in a time- and concentration-dependent cytotoxic effect (Figure 2). Reduction in cell viability was evident in NNPs-treated cells at 24 hours (86.7 ± 6.7%, 72.5 ± 13.3%, 40.1 ± 11.73%, and 28.7 ± 3.97%, with 100, 250, 500 and 1,000 μM, respectively, vs control, P <.05); 72 hours (40.3 ± 14.4%, 32.3 ± 17.5%, and 25.5 ± 5.4%, with 250, 500, and 1,000 μM, respectively, vs control, P < .05), and 7 days (7.75 ± 3.2%, 7.3 ± 0.8%, and 7.2 ± 1.3%, with 250, 500, and 1,000 μM, respectively, vs control, P < .05).

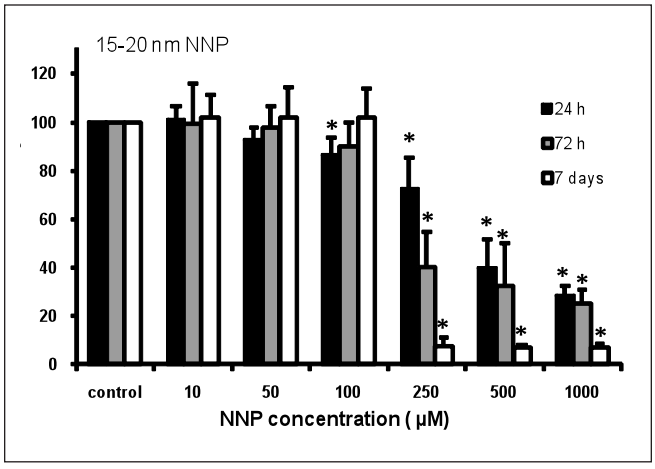

Figure 2. Time and concentration effect of 15–20-nm-sized silver nanoparticles (NNP) on cell viability of human periodontal ligament fibroblasts. Primary cultures of human periodontal ligament fibroblasts were exposed to 0–1,000 μM of 15–20-nm-sized NNP for 24 hours to 7 days. Experiments were performed using the MTS/PMS assay with absorbance measurements at 490 nm as described in Methods. The results are expressed as a percentage of control (vehicle) and represent the means ± standard deviation of 3 independent experiments, with each point determined in triplicate.
*P < .05, NNP vs control.

Time and concentration dependence of 80–100-nm-sized, NNPs-induced cytotoxicity of human periodontal fibroblasts

Finally, there was a no significant decrease in cell viability of human periodontal ligament monolayers incubated with 80- to 100-nm-sized NNPs with all the concentrations and times studied, as shown in Figure 3.

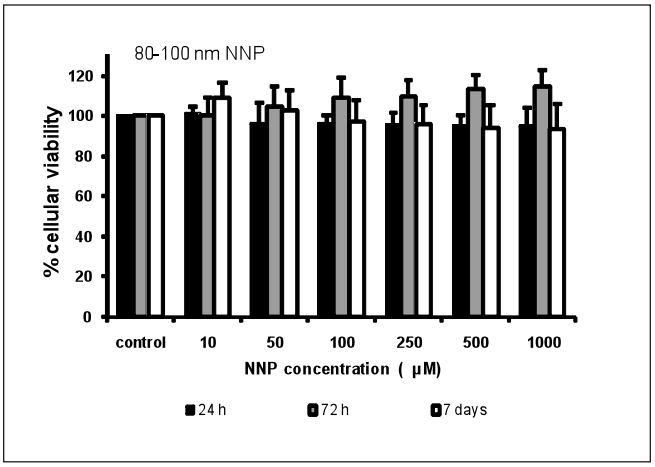

Figure 3. Time and concentration effect of 80–100-nm-sized silver nanoparticles (NNP) on cell viability of human periodontal ligament fibroblasts. Primary cultures of human periodontal ligament fibroblasts were exposed to 0–1,000 μM of 15–20-nm-sized NNP for 24 hours to 7 days. Experiments were performed using the MTS/PMS assay with absorbance measurements at 490 nm as described in Methods. The results are expressed as a percentage of control (vehicle) and represent the means ± standard deviation of 3 independent experiments, with each point determined in triplicate.
*P < .05, NNP vs control.

DISCUSSION

Primary cultures offer the advantage of having the same characteristics as the original tissue; cytotoxicity studies should be performed in vitro using the same cells that will be exposed to the compound to be analyzed. Other researchers prefer to use established cell lines, which are morphologically and physiologically more homogeneous because they have been replanted repeatedly to modify the original tissue karyotype. Also, they are mostly isolated from animal cells such as cell line L929 (mouse fibroblast), BRL 3A (mouse liver cells), neoplastic tissue cells (HeLa, human epithelial cells of cervical cancer), or human tissue of a different type such as human epithelial skin fibroblast NCTC 2544.[22-25] Another advantage of primary cultures is that, in evaluating samples from different patients, it is possible to have cellular response heterogeneity in each organism to the presence of a toxic agent, material, or drug due to patients' biological variability.

The literature on NNP toxic effects on different biological systems, organs, and tissues is scarce. An in vitro study conducted with rat liver cell cultures, cell line BRL 3A, 15

to 100 nm NNP, and 0–50 mg/mL concentrations for 24 hours, showed a decrease in incubated cell viability compared with 5 mg/mL NNP, and a very evident cytotoxic effect at 25 and 50 g/mL concentrations with NNP of 100 nm. This same work also showed that the NNP altered the mitochondrial membrane potential, reduced glutathione concentration, and increased the reactive oxygen species (ROS) concentration, suggesting that the cytotoxic effect mediated by the 15- and 100-mn NNP may also be mediated by oxidative stress.[26] Similar findings have been reported recently,[27,28] with an in vitro model of alveolar macrophages cultured in the presence of 15, 30, and 55 nm NNP for 24 hours. The cell viability decreased in a concentration-dependent manner in the presence of the 15- and 30-nm NNP, and it was observed that the concentration of ROS and proinflammatory cytokines increased such as tumor-alpha (TNF-a), IL-1, and the macrophage inhibitory protein-2 (MIP-2), suggesting the involvement of oxidative stress mechanisms in the development of cytotoxicity.

Previous studies in our laboratory have demonstrated a concentration-dependent, in vitro bactericidal effect of 60 nm-diameter silver nitrate nanoparticles on *S mutans*,[7] one of the main microorganisms associated with the development of caries. Based on these findings and with the aim of establishing the potential usefulness of silver nitrate nanoparticle solutions as bactericidal agents in the prevention of caries in the human oral cavity, this study evaluated 3 silver nitrate nanoparticle solutions ≤10, 15–20, and 80–100 nanometers in diameter at different concentrations and exposure times in the development of cytotoxicity in healthy human periodontal tissue fibroblast cell primary cultures. The cytotoxic effect produced by the silver nanoparticles in human periodontal tissue fibroblasts was evaluated in relation to the decrease in cell viability.

The results demonstrated that fibroblast cell viability of cells isolated from human periodontal tissue decreased depending on the size of silver nitrate nanoparticles ≤10, 15–20, and 80–100nm, for the same concentration and incubation time. However, silver nanoparticles ≤ 100 nm did not show decreased periodontal tissue fibroblast cell viability with any of the concentrations and incubation times tested, and contrary to what was observed with the ≤10 nm nanoparticles, we observed with the ≤ 20 nm nanoparticles a partial increase in cell viability. When the effect of the ≤10 nm-diameter silver nitrate nanoparticles was analyzed, we noted that fibroblast viability significantly decreased from a concentration of 250 μM (27 mg/mL) at 24 and 72 hours and from 500 mm (54 g/mL) after 7 days of incubation. The fibroblast viability in the presence of 15–20 nm NNP decreased significantly from 100 μM (17 mg/mL) at 24 hours to 250 μM at 72 hours to 7 days. The effect of NNP concentration, independently of the biomaterial diameter and the incubation time of cell viability, showed significant cytotoxic effect starting at 250 μM. In addition, when we increased the 3 different incubation times in the development of cytotoxicity, independently of the concentration and diameter of the silver nanoparticles, there were no statistically significant differences.

Our results suggest that smaller diameter (10 to 20 nm) silver nitrate nanoparticles in direct contact with oral tissue could produce a moderate cytotoxic effect, depending on the concentrations and exposure times. However, the bactericidal and bacteriostatic properties of this diameter NPP on *S mutans* or other microorganisms have not been evaluated. Previous results from our laboratory[7] showed that the bacteriostatic and bactericidal concentrations of a solution of 40–60 nm diameter silver nitrate nanoparticles for *S mutans* correspond to 4.25 mg/mL and 6.25 mg/mL respectively, so that the bactericidal and bacteriostatic concentration calculated for NNP is 2 to 3 times lower than the concentrations that produced a significant cytotoxic effect in human periodontal tissue fibroblast primary cultures. The differences in our cytotoxicity results in human periodontal tissue fibroblasts compared with those reported by other authors could be attributed to (1) the cell type analyzed or (2) the pH of the solutions of the NNP (not specified in the other studies) given that generally the size of the nanoparticle is determined by this variable, and in our study, the pH of the solutions was adjusted from 10–11 to 7 so as to not alter the final conditions of the cultured cells that grow in a pH range of 7.0–7.4.

CONCLUSION

The silver nanoparticles 80–100 nm in diameter produced no apparent cytotoxic effect on fibroblasts at the human periodontal tissue concentrations or incubation times studied; however, there was an increase in cell viability.

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