Original Article

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The cytotoxicity of silver nanoparticles on human gingival fibroblast cells: An *in vitro* study

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Objective: The aim of this study was to evaluate the toxic effects of silver nanoparticles (AgNPs) on human gingival fibroblast (HGF) cells at different concentrations and treatment durations.

Methods and Material: AgNPs were synthesized via the chemical reduction of silver nitrate with sodium borohydride in a chitosan solution at the Pharmacology Department, Faculty of Dentistry, Mahidol University, Thailand. HGF cells were exposed to 0.5, 1.5, 2.5, 5, or 10 µg/mL AgNPs for 2 min and 24 h. The positive control was 0.12% Chlorhexidine, while the negative control was growth medium. Cell viability was determined using a Methylthiazol tetrazolium bromide assay, and images were obtained using a light microscope. The uptake of 0.5 µg/mL AgNPs by HGF cells after a 24-h incubation was observed using transmission electron microscopy (TEM).

Results: A 2-min exposure to 0.5–10 µg/mL AgNPs did not induce cytotoxicity. In contrast, a 24-h exposure led to a significant decrease in cell viability, however, it remained above 70% compared with the control. Exposure to AgNPs for 24 h resulted in a lower density of HGF cells and more degenerative cells when the concentration of AgNPs increased observed with the microscope. TEM analysis revealed the absorption, internalization, and dissemination of 0.5 µg/mL AgNPs in HGF cells after a 24-h incubation.

Conclusions: The cytotoxicity of AgNPs was concentration- and exposure time-dependant, as evidenced by the intracellular accumulation observed using TEM after a 24-h exposure. Concentrations below 10 µg/mL can be considered non-cytotoxic during short-term oral cavity exposure. Based on our results, the use of AgNPs in dentistry is likely to be safe for oral application.

Keywords: cytotoxicity, human gingival fibroblast cell, silver nanoparticles

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Introduction

The use of silver nanoparticles (AgNPs) has increased in dentistry due to their unique properties. Studies have consistently demonstrated their profound inhibitory effect on a range of bacteria and fungi, such as *Streptococcus mutans*, *Candida albicans*, and *Enterococcus faecalis*. This effect plays a crucial role in reducing secondary caries, fungal infection, endodontic failure, and dental implant failure [1, 2]. Therefore, AgNPs, ranging from 1.5–5 nm [3], are recognized for their ability to combat caries-causing bacteria and prevent dental caries [4-6].

The manufacturing process and superior benefits compared with typical therapy make AgNPs an intriguing agent. Synthesizing AgNPs is simple and cost-effective, with various techniques proposed, including laser ablation, gamma irradiation, and electron irradiation. The chemical reduction method, which uses both organic and inorganic reducing agents, is the most commonly used approach for AgNP synthesis [7]. Santos *et al.* successfully synthesized AgNPs with

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a size below 5 nm using the chemical reduction method, employing chitosan as a stabilizer and sodium borohydride ($NaBH_4$) as the reducing agent. These AgNPs have been reported to interact with a broad range of microorganisms. Furthermore, nanosilver fluoride has outperformed silver diamine fluoride as an anti-caries agent, improving aesthetics without staining, while effectively killing bacteria. Despite being an interesting novel therapy, the safety issues during the clinical use of AgNPs remain a concern [8, 9].

Evaluating AgNPs' cytotoxicity is crucial prior to their use. Their specific toxicological mechanisms are still being researched. Studies have shown that AgNPs can cross the cell membrane, accumulate in mitochondria, and contribute to oxidative stress, inflammation, and apoptosis[10]. Higher concentrations of AgNPs generally result in increased toxicity, influenced by factors such as cell type, particle size, and duration of exposure. Therefore, the optimal concentration range for clinical use varies depending on the target microorganism and cell type [9].

Human gingival fibroblasts (HGF) are vital for maintaining the health and integrity of gingival tissue in the oral cavity. Evaluating the cytotoxicity of dental materials on these cells offers valuable insights into the potential effects of the materials on gingival tissue and oral health. Previous studies have reported varying cytotoxic concentrations of AgNPs, ranging from 2.5—10 µg/mL after a 24-h exposure [3, 11]. However, concentrations below 1.5 µg/mL are more likely to demonstrate biocompatibility. Although assessing the cytotoxicity of AgNPs on gingival fibroblasts helps to identify potential adverse effects during their interaction with gingival tissue, research on their impact remains limited. This underscores the need for a better understanding of their safety to facilitate the clinical use of AgNPs in dentistry.

In this study, we synthesized self-prepared AgNPs in our laboratory with the aim of formulating them for various dental treatments and oral hygiene prevention. Therefore, the objective of this study was to investigate the toxic effects of the self-prepared AgNPs on HGF using a colorimetric assay at different concentrations and treatment durations. We also observed the interaction between the cells and AgNPs using light and transmission electron microscopy.

Material and Methods

AgNP synthesis and preparation

The methodology employed in this study was adapted from Santos *et al.* [8]. Chitosan was selected as the stabilizer, while sodium borohydride ($NaBH_4$) was utilized as the reducing agent. AgNP synthesis was performed at the Pharmacology Department, Faculty of Dentistry, Mahidol University, Thailand. To prepare the colloidal silver, a solution of 1 g chitosan in 200 mL 2% (v/v) acetic acid was subjected to overnight stirring and subsequent filtration. Subsequently, the prepared chitosan solution was combined with silver nitrate (0.012 mol/L) before adding sodium borohydride ($NaBH_4$). During the reduction process, the color of the solution transitioned from colorless to light yellow and ultimately to reddish brown as the silver ions (Ag+) were reduced. The AgNPs were stored at -80°C until further use and subsequently diluted in growth medium at 0.5, 1.5, 2.5, 5, and 10 µg/mL.

AgNP cytotoxicity

Human gingival fibroblasts, CRL-2014 cells, obtained from the American Type Culture Collection (ATCC-HBT-55) were cultured in Dulbecco's Modified Eagle's Medium (ATCC® 30-2002), a high glucose medium (4.5 g/L) supplemented with sodium pyruvate (0.11 g/L),

10% fetal bovine serum, and 1% penicillin/streptomycin. The cells were enzymatically detached using trypsin-EDTA and seeded at 10,000 cells per well in a 96-well plate for 24 h before being exposed to 0.5, 1.5, 2.5, 5, or 10 µg/mL AgNPs for 2 min and 24 h. The positive control was 0.12% chlorhexidine, while the negative control was growth medium. Each group was assayed in triplicate wells in each experiment. The cytotoxicity of the gingival fibroblast cells was assessed using the Methylthiazol tetrazolium bromide (MTT) assay. Images were obtained using a light microscope (Olympus, CKX53SF), and the absorbance at 570 nm was measured using a Microplate Spectrophotometer Hybrid Multi-Mode Microplate Reader (Synergy H1 MFD). The results were calculated as a percentage relative to the control values of the unexposed cells using the following equation:

% cell viability = (experimental absorbance [abs] 570 nm of exposed cells/ abs 570 nm of unexposed cells) × 100.

Transmission electron microscopy (TEM) analysis of intracellular AgNPs

The interaction and uptake of 0.5 µg/mL AgNPs by gingival fibroblast cells after a 24-h incubation were investigated using transmission electron microscopy (TEM). The cells were washed three times with phosphate-buffered saline (PBS), digested, and centrifuged. The resulting cell pellets were rapidly fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer for 2 h before fixing with 2% osmium tetroxide for 45 min. After washing, the cell pellets were dehydrated in a series of graded alcohol concentrations and transferred to an embedding medium containing ERL 4221, Diglycidyl ether of polypropylene glycol (DER 736), Nonenyl succinic anhydride (NSA), and Dimethylaminoethanol (DMAE). Ultrathin sections were prepared with an Ultramicrotome (RMC Products, USA) equipped with a diamond blade (DiATOME, Switzerland). The ultrathin sections were stained with uranyl acetate and lead citrate prior to observation using a transmission electron microscope (Jeol, JEM 1400, Japan, Voltage 100kV).

Statistical analysis

The data are presented as the mean ± standard error of three independent experiments. Statistical analysis of the mean difference among multiple groups was determined using one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison tests. The Chlorhexidine group was not included in the statistical analysis. For comparisons between both treatment durations at each concentration, two-tailed Student's t-tests were performed. A *p*-value less than 0.05 was considered significant.

Results

AgNP cytotoxicity

The objective of the experiment was to assess the viability of gingival fibroblast cells when exposed to 0.5, 1.5, 2.5, 5, or 10 µg/ ml AgNPs using the MTT assay. The negative control consisted of growth medium, while the positive control was 0.12% chlorhexidine. The viability of the positive control cells was 3.66% after 2 min and 3.92% after 24 h. Figure 1 demonstrates that a 2-min exposure to AgNPs did not affect cell viability. However, after 24 h, HGF viability significantly decreased at all concentrations, remaining above 70% compared with the negative control group. The microscopic images revealed spindle-shaped HGFs attached to the cell culture flask in the absence of AgNPs.

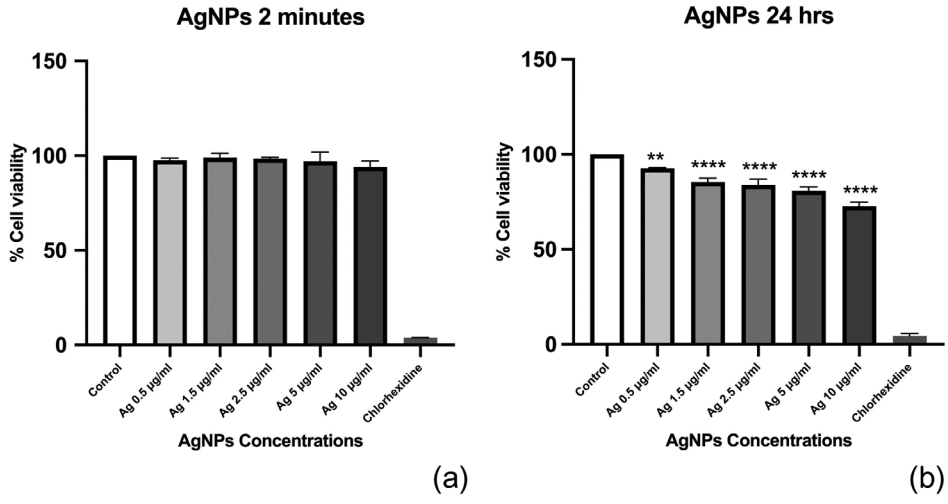

Figure 1 The cell viability percentage after exposure to different AgNP concentrations. The results indicate a high percentage of cell viability when human gingival fibroblasts were exposed to AgNPs for 2 min (a). However, as the concentration of AgNPs increased, cell viability decreased after 24 h (b). Treatment with 0.12% chlorhexidine resulted in reduced cell viability both after a 2-min and 24-h exposure. Statistical comparisons were performed without the chlorhexidine group, and significant differences are denoted as follows: (**) *p*-value <0.01, (***) *p*-value <0.001, (****) *p*-value <0.0001.

However, with increasing concentrations of AgNPs, the cell morphology degenerated and detachment from the culture flask was observed. We also found a lower density of HGF cells and more degenerative cells when the concentration of AgNPs 2.5 µg/ml after a 24-h exposure, as observed using the light microscope (Figure 2).

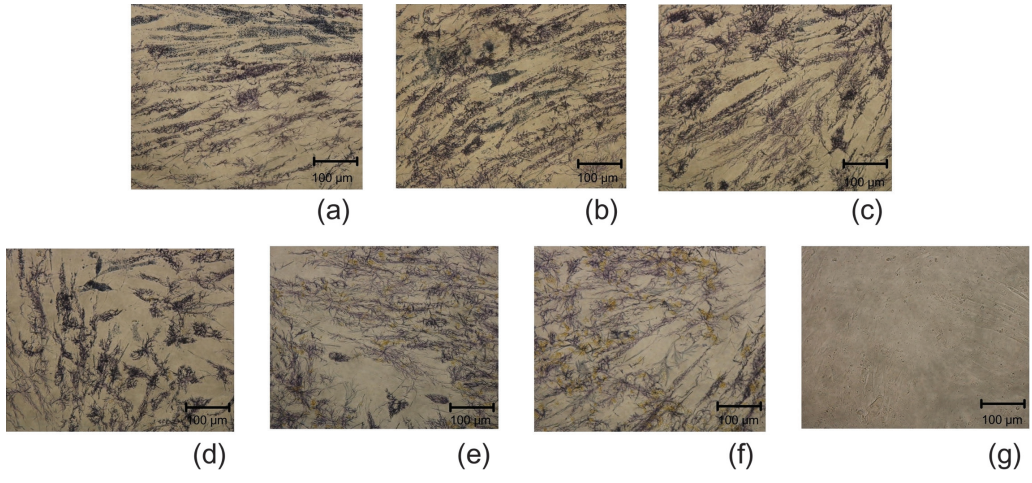

Figure 2 Gingival fibroblast cells-MTT were exposed to different concentrations of AgNPs for 24 h (magnification 40X). The images depict: (a) Untreated cells (Negative controls); (b) 0.5 µg/ml AgNPs; (c) 1.5 µg/ml AgNPs; (d) 2.5 µg/ml AgNPs; (e) 5 µg/ml AgNPs; (f) 10 µg/ml AgNPs; (g) Chlorhexidine (positive control). A lower density of HGF cells and more degenerative cells were observed after a 24-hour exposure when the concentration of AgNPs ≥ 2.5 µg/ml.

TEM analysis of intracellular AgNPs

Gingival fibroblasts treated with 0.5 µg/ml AgNPs for 24 h showed the presence of AgNPs in the cells, mainly scattered in the cytoplasm (Figure 3). Both individual nanoparticles and agglomerated nanoparticles were observed. No specific intracellular organelle was found to accumulate the AgNPs.

Discussion

The use of AgNPs in dental treatment has gained attention due to their antimicrobial properties. However, before their clinical application, addressing concerns regarding their toxicity to human cells is crucial. We developed AgNPs that show promise as an anti-caries agent without causing tooth discoloration [12]. To ensure the safety of our self-prepared AgNPs, the aim of this study was to investigate their cytotoxicity in a dose and time-dependent manner. Various concentrations of AgNPs ranging from 0.5-10 µg/ mL were tested, and the cell viability remained above 70% after a 2-min and 24-h exposure. The toxicity increased with higher doses and longer exposure times, and TEM revealed intracellular accumulation after 24 h of exposure to AgNPs.

The viability of human gingival fibroblast cells was assessed after exposure to AgNPs at concentrations ranging from 0.5-10 µg/mL for 2 min and 24 h. The results did not show a significant change in cell viability after a 2-min exposure. Although after 24 h, there was a significant decrease in cell viability, it remained above 70% compared with untreated cells, indicating non-cytotoxicity according to ISO 10993-5 recommendations. Notably, a lower density of HGF cells and more degenerative cells after a 24-h exposure were observed. Consistent with a previous study [3], significant cytotoxicity was observed for concentrations above 2.5 µg/mL. The maximum concentration used in this study was 10 µg/mL,

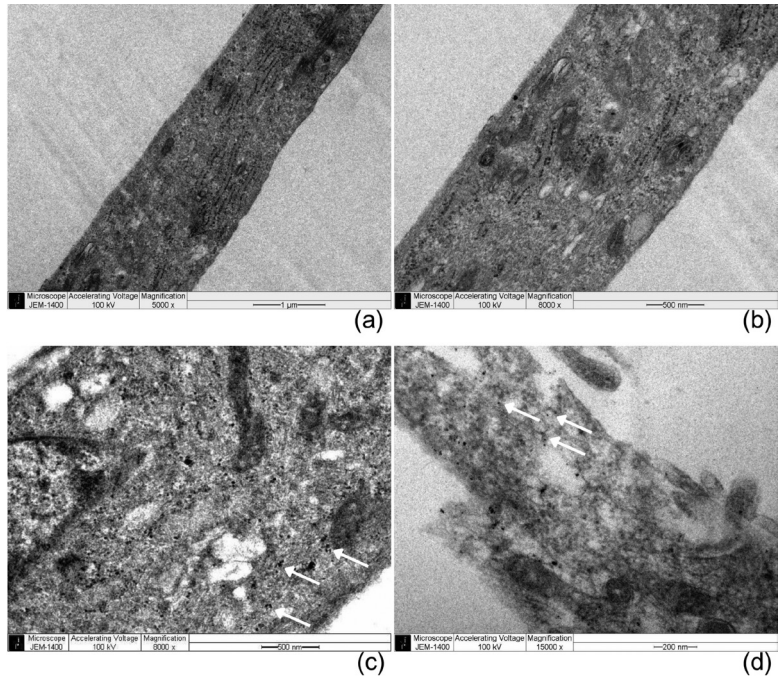

Figure 3 Observation of AgNP accumulation in gingival fibroblast cells using a transmission electron microscope. Images of the untreated cells (controls) are shown in (a) and (b), while images of the treated cells are displayed in (c) and (d). The microscopic images revealed that after exposing the cells to 0.5 µg/mL AgNPs for 24 h, the AgNPs were internalized and dispersed in the cells as indicated by the presence of AgNPs (white arrows).

because higher concentrations (>10 µg/mL) had detrimental effects on human gingival epithelial cells, resulting in decreased viability [11]. At 50 µg/mL, cell viability was reported as 50%, and at 200 µg/mL, most fibroblasts died within 24 h [6]. Our study focused on a 2-min treatment time, which corresponds to the duration of applying an AgNP solution to carious teeth [8, 13, 14] and the recommended brushing time with toothpaste according to the American Dental Association guidelines. The observed cell compatibility during this short exposure indicates that our laboratory-synthesized AgNPs are safe for further dental application.

AgNPs have been found to be internalized by cells, leading to various effects depending on the particle size and cell type. Previous studies have demonstrated that a concentration of 15 µg/ml AgNPs was absorbed by B16 mouse melanoma cells, dispersing into the cytoplasm and nucleus and localizing within membrane-bound structures, such as endocytic vesicles, particularly early endosomes, and lysosomes [15]. In HGF, AgNPs penetrated the cell membrane and predominantly accumulated inside the mitochondria, forming agglomerates after a 24 h incubation [3]. Nanoparticles smaller than 40 nm also passed through the nuclear pore complexes and entered the nucleus [16, 17]. In our study, using a minimum concentration of 0.5 µg/ml of the self-prepared AgNPs, we observed particle absorption, internalization, and dissemination into the gingival fibroblast cell cytoplasm after a 24-h incubation, through passive diffusion [15], with no specific intracellular organelle accumulation observed under TEM. Intracellular accumulation can yield antimicrobial effects, known as “Zombie effects”, which extend the killing effects of AgNPs after treatment [18]. Long-term degenerative effects may also occur in human cells, posing a potential threat to clinical use. However, our study was unable to assess the prolonged toxic effects of AgNPs. Furthermore, our dental applications target the tooth surface, where there are no living cells or a limited exposure time of two minutes.

The cytotoxicity of AgNPs can be attributed to their interaction with cell membrane proteins and activation of signaling pathways, leading to the production of reactive oxygen species (ROS) and damage to proteins and nucleic acids, resulting in inhibited cell growth and increased apoptosis [19-21]. Generally, AgNPs exhibit high toxicity at concentrations ranging from 5—10 µg/mL and sizes of 10—100 nm, causing disruption of mitochondrial function [22, 23]. The primary toxic effects of AgNPs are mediated through the mitochondrial pathway by reducing glutathione levels and inducing lipid peroxidation. AgNPs and silver ions generate ROS and induce oxidative stress at various levels within cells, including molecules, organelles, and the cell as a whole. AgNPs also increase DNA damage, cell apoptosis, and necrosis by causing significant oxidative damage to cell membranes and organelles, such as lysosomes, mitochondria, and the nucleus [9,19, 24-26]. Cellular responses to AgNPs are influenced by factors, such as dose, exposure duration, size, shape, surface chemistry, and cell type [24].

This preliminary in vitro study lays the foundation for future dental application research. The results suggest that concentrations ranging from 0.5—10 µg/mL AgNPs are safe for human gingival fibroblast cells during short oral exposures. Additionally, within this concentration range, our self-prepared AgNPs inhibited *S. mutans* biofilm formation, as previously reported [12]. However, it's important to note that other factors, such as interactions with saliva, the presence of various oral tissues, and the dynamic oral environment, were not within the scope of our investigation. Furthermore, when considering higher concentrations (>10 µg/mL) of AgNP products, dental applications should prioritize the protection of oral tissues and direct application to carious teeth to mitigate the potential toxicity of AgNPs while optimizing their efficacy.

Conclusion

Our study indicates that the self-prepared AgNPs have relatively low harmful effects on gingival fibroblasts during short-term exposure. However, prolonged exposure exceeding 24 h results in the accumulation of AgNPs in the cells, potentially leading to long-term adverse effects. Thus, future studies should use long-term animal models to assess the cytotoxicity and genotoxicity of these compounds, ensuring their safety.

Author contributions

Thanapha Putthanuparp contributed to conducting the entire methodology of the study, analysis, and manuscript writing. Nisarat Ruangsawasdi contributed to the conception and design of the study, verifying the data interpretation and revising the manuscript. Praphasri Rirattanapong contributed to supervision and study design.

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Supplementary data

Table 1 The cell viability percentage after exposure to various AgNP concentrations.

		% Cell Viability (mean)					
AgNP (m)	Control	AgNP 0.5	AgNP 1.5	AgNP 2.5	AgNP 5	AgNP 10	*p*-value
2 Minutes	100	97.59	98.9	98.4	97.08	94.07	0.1754 (ns)
24 Hr	100	92.68	85.41	83.94	80.79	72.71	<0.0001 (****)
p-value		0.0017 (**)	0.0019 (**)	0.0013 (**)	0.0059 (**)	0.0007 (***)	

Significance level: (ns) non significant, (**) *p*-value <0.01, (***) *p*-value <0.001, (****) *p*-value <0.0001

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