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Concentration- and Time-Dependent Cytotoxicity of Silver Nanoparticles on Normal Human Skin Fibroblast Cell Line

Mansoureh Paknejadi [1], Mansour Bayat [1], Mona Salimi [2,*] and Vadood Razavilar [1]

[1] Department of Pathobiology, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2] Physiology and Pharmacology Department, Pasteur Institute of Iran, Tehran, Iran

[*] *Corresponding author*: Physiology and Pharmacology Department, Pasteur Institute of Iran, Tehran, Iran. Tel: +98-2164112264, Fax: +98-2164112834, Email: salimimona@pasteur.ac.ir

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Abstract

Background: A wide use of Silver Nanoparticles (AgNPs) in consumer products, industry, pharmaceuticals, and medical sciences, including dermatology, has always caused concerns related to its adverse effects on the environment and human safety. Nano-sized particles can be uptaken by the body through various routes, especially the skin, which is the widest organ and the first contact surface for different nanomaterials and other foreign agents.
Objectives: The aim of this investigation was to assess the viability of a vulnerable normal skin cell line to cytotoxicity of AgNPs with the same effective size and concentrations used for various microbial agents, such as *Candida* species.
Methods: Synthesized AgNPs were characterized using UV-visible absorption spectroscopy, X-ray diffraction, Dynamic Light Scattering (DLS), and Transmission Electron Microscopy (TEM). Different concentrations of the aqueous colloidal AgNPs (2.17 to 69.5 μg/mL) were tested on normal Human Skin Fibroblast (HSF) cell line using MTT assay after 24 and 48 hours of exposure.
Results: The analysis demonstrated that the synthesized AgNPs were mainly spherical with an average size of 6.03 nm. The MTT findings showed a significant concentration- and time-dependent manner in the reduction of cell viability. The calculated IC_{50} values were 30.64 and 14.98 μg/mL for 24 and 48 hours of incubation, respectively.
Conclusions: The cytotoxic effect of AgNPs was higher for Human Skin Fibroblast cells after 48 hours of exposure compared with 24 hours. According to the observed results, small nano-sized silver particles could potentially have cytotoxic effects on normal cells, especially at high concentrations and prolonged exposure times.

Keywords: Cytotoxicity, Dermatology, Human Skin Fibroblast cell line, MTT assay, Silver nanoparticles

1. Background

Silver metal is considered a valuable element and has significantly been consumed for thousands of years in human civilization to manufacture antiques. Among the various applications of silver, its antimicrobial property seems to be more important for hygienic and medical purposes. In this regard, disinfectants containing silver were used to control and treat infections in ancient Greece, Rome, Egypt, Macedonia, and even during World War (1-4). In 2000, the United States of America was the initiator of the National Nanotechnology Initiative Program and later on, many countries constituted similar programs. Gradually, with fast progress in the field of nanotechnology, silver metal was converted to nanoparticles containing a diameter ranging from 1 to 100 nm in one structural dimension (3-6). These engineered Ag nanoparticles were introduced as one of the most plentiful NPs, which were manufactured and applied all over the world (7, 8). The engineered NPs possess unique physicochemical characteristics including small size, large surface area, specific shape and structure, charge, optical properties, quantum mechanics, conductivity, magnetic, catalytic, and chemical properties, such as reactivity and thermal activity (2, 4, 7). These features led to extensive applications of AgNPs in various fields, including the industry, agriculture, and medicine (2, 4, 6-11).

According to different studies, AgNPs are gradually being entered in the surroundings as a result of various production processes, consumer products, and disposal of wastes (9, 10). Therefore, a hazardous material would be available to terrestrial and aquatic organisms like humans, fish, plants, and insects (3, 9). Following the release of AgNPs, some significant changes occur in nanoparticles that lead to environmental and biological toxicity. The toxicity can be related to Ag_2O generation, silver ions release, interaction with other surface compounds, and AgNPs aggregation/agglomeration (2, 4, 8, 12-14). These changes may be re-

lated to unique physicochemical properties of NPs, tissue type, chemical compounds of media and cells, exposure time, mode of entrance and uptake, precipitation rate, biocompatibility, biological activity, and intracellular interaction (4, 15, 16).

Furthermore, AgNPs can be easily uptaken through the respiratory system, gastrointestinal tract, and skin. These particles can move freely in the blood circulation or lymph stream and subsequently enter various organs, such as the spleen, bone marrow, liver, brain, heart, and kidneys through endocytosis and macropinocytosis. Sometimes, AgNPs enter the reproductive organ and penetrate in the blood-testis barrier. In addition, they may cross the blood-brain barrier, then enter in the cerebellum and the olfactory bulbs (2, 7-10, 15, 16). Clinical cytotoxic studies reported that nanoparticles can be absorbed in the bloodstream and organs after placing the dressing coated with AgNPs on dermal wounds (8, 17). For instance, antimicrobial drugs of Flamazine and Acticoat had high cytotoxicity yet PolyMem silver, and a novel burn wound hydrogel dressing showed lower adverse effects, respectively (13, 18). The critical mechanisms defined for AgNPs toxicity in the cells include mitochondrial and lysosomal damage, cell membrane degradation, Reactive Oxygen Species (ROS)-oxidation stress, and genotoxicity (2, 4, 8, 12-14).

Considering the above-mentioned report and knowing that AgNPs are a real hazard to normal cells, the data about the toxicity of this material is still insufficient. In addition, based on the literature review, there are minimal studies related to the cytotoxicity of silver nanoparticles at the size used in this research on normal human skin fibroblast cells. Thus, small size of AgNPs was produced, which can be suitable for antimicrobial applications, and investigate the cytotoxicity of several concentrations of colloidal silver nanoparticles on normal HSF cells.

2. Objectives

The purpose of this study was to evaluate the viability of a vulnerable normal skin cell line to cytotoxicity of AgNPs with the same size and concentrations effective in yeasts, causing cutaneous and mucosal candidiasis.

3. Methods

3.1. Materials

To prepare the silver colloidal nanoparticles, silver nitrate ($AgNO_3$), sodium citrate ($Na_3C_6H_5O_7$), and polyvinylpyrrolidone (PVP) were acquired from Merck (Darmstadt, Germany). Dulbecco's Modified Eagles Medium (DMEM), Fetal Bovine Serum (FBS), penicillin-streptomycin were obtained from Gibco (Life Technologies Ltd, Paisley, UK), and normal human skin fibroblast cell line (HSF-PI-16) was supplied by the cell bank of Pasteur Institute, Iran (NCBI). MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and other chemicals were purchased from Merk company.

3.2. Synthesis of Silver Nanoparticles

Clear colloidal aqueous suspension of AgNPs was synthesized according to Monteiro et al.'s method (19-21). Briefly, AgNPs were prepared via reduction of an aqueous solution of silver nitrate (5.0×10^{-3} mol/L) by sodium citrate (0.3 mol/L) at 90°C. The AgNPs were formed when the colloidal suspension turned yellow. This solution was stabilized by adding 102 g/L of PVP after cooling at room temperature. The colloidal stock of AgNPs was sterilized using a 0.22-μm sterile syringe filter (Merck KGaA, Darmstadt, Germany). In order to prevent endotoxin interference in the AgNPs toxicity, all the synthesis steps were performed in a clean laboratory environment.

In order to estimate the initial concentration, the Varian Atomic Absorption Spectrometer (SpectrAA-200, Canada) was used. To determine the hydrodynamic diameter of particles, 1 mL of AgNPs suspension was diluted in water and the solution was measured by Dynamic Light Scattering (DLS, Malvern Instrument Ltd, Malvern, UK). Next, the synthesized AgNPs colloidal solution was centrifuged and the supernatant was eliminated. The pellet NPs was washed using purified water three times, then, the pellet was transferred to a freeze dryer. The crystalline metallic pattern of dried NPs powder was evaluated by X-Ray diffraction (Philips X'pert PRO SUPER X-ray diffractometer). Finally, this lyophilized powder was re-dissolved in pure water using sonication; Then, the formation and stability of AgNPs were determined by Ultraviolet absorbance spectroscopy (Carry 100 Bio, UV-Vis spectrophotometer, Varian, Australia) and their shape and size were measured by Transmission Electron Microscopy (TEM, LEO906, 100KV, Zeiss, Germany) (22).

3.3. Cell Culture and Cytotoxicity Assay

The HSF cell line was cultured as a monolayer in a 25-cm^2 flask (Nunc, Denmark) containing complete DMEM enriched with 10% FBS, a mixture of 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were preserved in an incubator containing 5% CO_2 and 95% humidity at 37°C. Then, their morphology was investigated, and photographed under an inverted microscope (Figure 1A). After ultrasonic dispersion and a vigorous vortex of the AgNPs colloidal stock, serial dilutions were provided ranging from 69.5 to 2.17 μg/mL (ppm), and their cytotoxic effects were immediately tested on standard HSF cell line using MTT assay. Briefly, the cells were placed in 96-well microplates (Nunc, Denmark) and maintained at 37°C and 5%

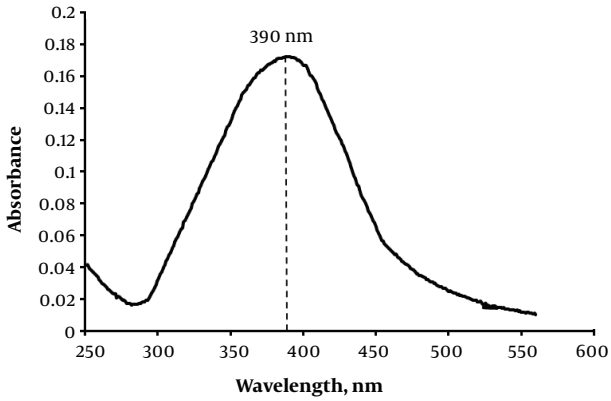

Figure 1. UV-visible spectroscopy spectrum of AgNPs

CO_2 in a humidified atmosphere. Then, the cells were incubated at different concentrations for 24 and 48 hours. Untreated cells served as the control. The HSF cells were exposed to MTT (0.5 mg/mL in phosphate buffered saline) for four hours at 37°C to reduce tetrazolium to insoluble formazan. Subsequently, the medium was eliminated and crystal formazan dye was solubilized in DMSO. The absorbance measurement was calculated at 545 nm, and then the cell viability percentage was compared with untreated control cells. The IC_{50} values of AgNPs that showed 50% inhibition of cell growth were calculated following 24 and 48 hours of exposure times. Three independent experiment, performed with at least three repeats, were applied for these measurements (23-25).

3.4. Statistical Analysis

All data were represented as the mean ± standard deviation for at least three replicates. The obtained results were analyzed using GraphPad Prism software version 6.0 for Windows, (GraphPad Software, La Jolla, California, USA), and one- and two-way Analysis of Variance (ANOVA). The value of $P < 0.05$ was considered statistically significant.

4. Results

4.1. Characterization of Silver Nanoparticles

The findings of SpectrAA indicated that the concentration of AgNPs colloidal stock was 556 μg/mL (ppm). This was necessary for preparing serial dilutions of AgNPs suspension. The UV-Vis spectrum of AgNPs presented a strong curve at 390 nm, which emphasized the character of nanocrystalline particles (Figure 1).

As shown in Figure 2, X-ray diffraction spectra were reported on a Philips X'pert PRO SUPER X-ray diffractometer using graphite monochromatized CuKα radiation (λ = 1.541874 Å). The five diffraction spectra (111), (200), (220), (311), and (222) were observed, which were consistent with the standard pattern.

The results of DLS estimated that the hydrodynamic diameter range of AgNPs was approximately 3.765 to 18.92 nm. The size distribution histogram is shown in Figure 3.

The TEM images indicated that synthesized AgNPs had a spherical shape with an average size of 6.03 nm (Figure 4).

4.2. Cytotoxic Effects of AgNPs on HSF Cell Line

After exposing HSF cells to different concentrations of AgNPs, the cytotoxic effects were evaluated by the MTT assay (Figure 5).

As shown in Figure 6, the synthesized AgNPs at concentrations ranging from 8.68 to 69.5 μg/mL and from 2.17 to 69.5 μg/mL decreased the viability percentage on HSF cells in a concentration-dependent manner at 24 hours and 48 hours, respectively. The results also indicated time-dependent viability towards HSF cells. In this regard, 2.17 and 4.34 μg/mL of AgNPs could not alter cell viability at 24 hours, whereas the viability of the cells was affected by these two concentrations at 48 hours. In fact, all the used concentrations showed a significant effect on HSF cell viability after 48 hours of exposure (Figure 6B). In order words, the current findings indicated that HSF cells were more sensitive to small size NPs at 48 hours compared with 24 hours, with IC_{50} values of 14.98 and 30.64 μg/mL, respectively. Additionally, as shown in Figure 7, a significant difference was evident in the cytotoxicity of all AgNPs concentrations between 24 and 48 hours of incubation.

5. Discussion

Regarding the abundant applications of AgNPs-containing compounds, their exposure to human and other organisms in the environment is dramatically developing. Recently, the use of AgNPs in hygienic and cosmetic products and pharmaceuticals has revolutionized the dermatology and other domains of medical science (3, 6, 8). Due to antimicrobial properties, it was reported that appropriate concentrations of AgNPs with small sizes can be used to fight against the growth of undesirable microorganisms. This application is considered as the main purpose in the majority of scientific researches. Thus, the current research prepared a small size of AgNPs and selected concentrations, at which they can inhibit yeast growth caused by cutaneous and mucosal candidiasis (19-21).

Since selecting a cytotoxicity assay is necessary for obtaining an accurate result, the current study used MTT assay as a sensitive method compared to the other methods,

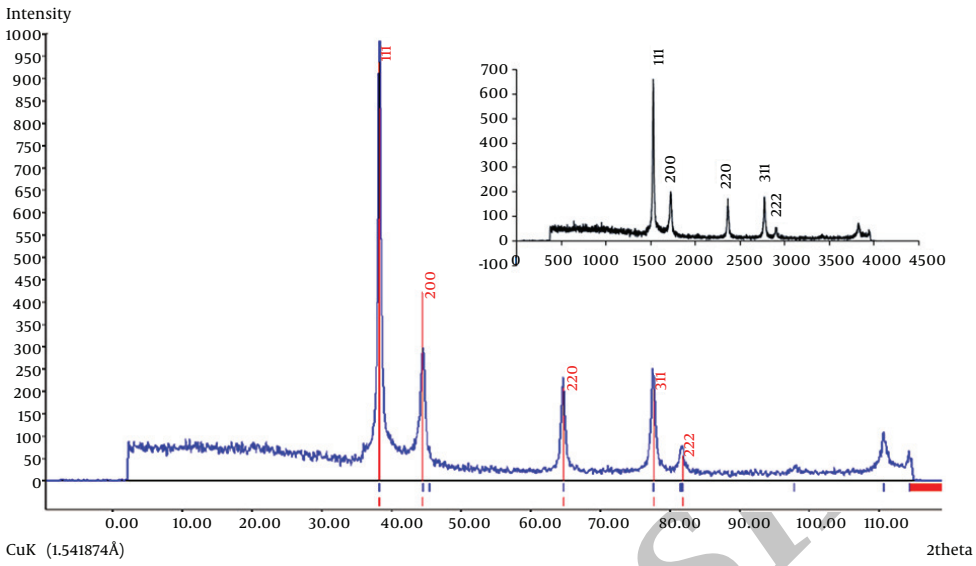

Figure 2. XRD pattern of synthesized silver nanoparticles

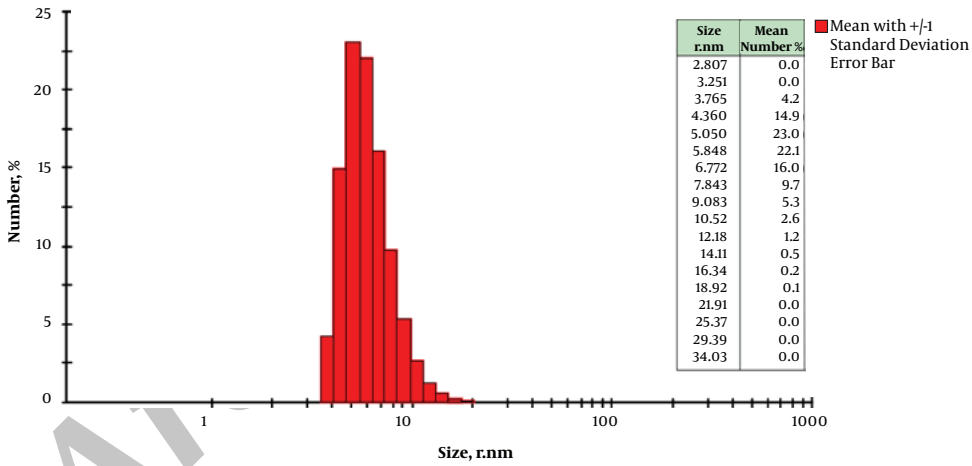

Figure 3. Size distribution histogram of AgNPs by DLS analysis

including Commusie Blue, Neutral Red, and Alamar Blue in order to assess the prepared AgNPs toxicity on the standard cell line (1, 2, 5, 8, 9, 16).

Nowadays, people are using different commercial cosmetics, topical drugs, and other products, whose safety standards are not generally high. For this reason, dermal contact is considered one of the most ordinary ways of exposure to nanoparticles (26-28). Connected with this point, the Royal Society proposed that NPs can be used in cosmetics if their effects and absorption capability are evaluated on the skin by the European Commission's safety committee (27). Therefore, the use of a sensitive standard human skin cell line instead of cancerous cells would be more effective to screen the cytotoxicity of NPs *in vitro*. It is worth mentioning that cancerous cells are much more likely to be sensitive to AgNPs than normal cells, yet they are only used to evaluate the effectiveness of NPs for cancer chemoprevention and chemotherapy (2, 5, 8, 26-31).

In the current research, a non-cancerous and also sensitive human skin fibroblast cell line was selected to evaluate the cell viability percentage after exposure to AgNPs. In accordance with the current study, results obtained by

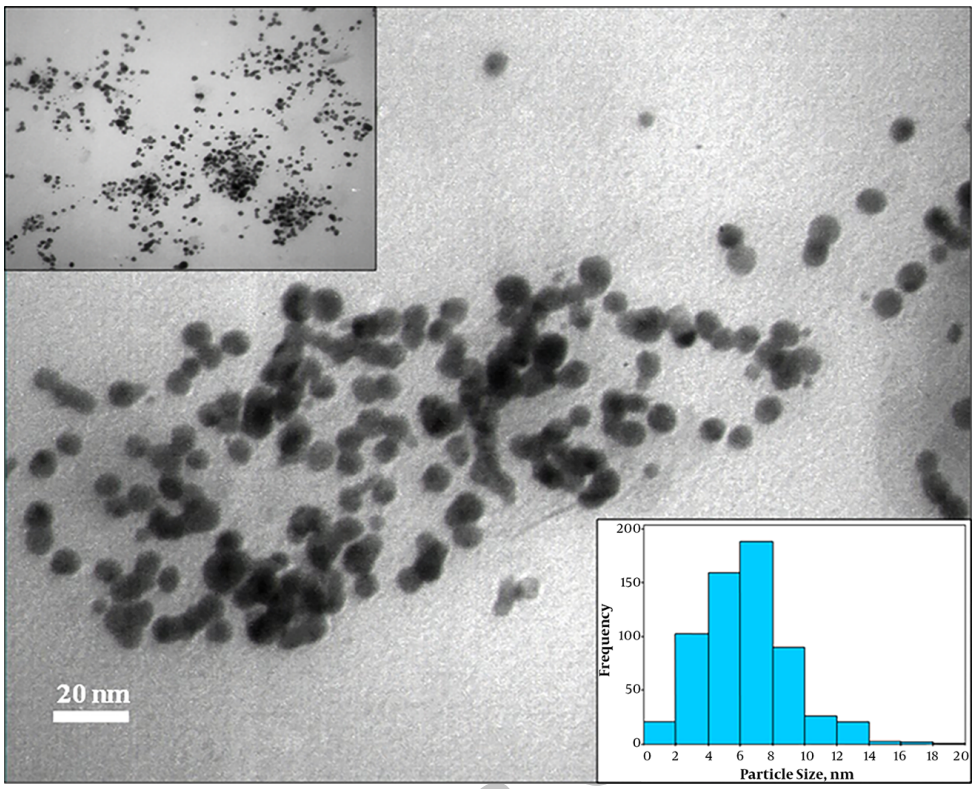

Figure 4. TEM images and histogram of silver nanoparticles size distribution

Chun et al. showed that Normal Human Epidermal Fibroblast (NHEF) cell line was more sensitive to AgNPs or Ag^+ ions than keratinocyte cells (NHEK) (31). Peng et al. also declared that mouse L-929 fibroblast cell line had a higher resistance to AgNPs than human fibroblast (16). In fact, researchers found that significant differences in the sensitivity of cells could be related to cellular characteristics, including penetrability of the plasma membrane and proliferation, as well as their natural antioxidant levels (1, 27).

Prolonged exposure to Ag^+ ions or AgNPs is a serious concern for human health. Argyrosis or argyria, a permanent bluish-gray discoloration of eyes and skin, is one of the most well-known symptoms of prolonged exposure to silver (1, 10, 29). Due to the importance of exposure time, the current study compared the cytotoxic effect of various concentrations of AgNPs at 24 and 48 hours. The findings of the MTT assay showed that in addition to time-dependent cytotoxicity, a statistically significant concentration-dependent manner was observed at 24 and 48 hours. However, a dramatic reduction of cell viability was found following 48 hours of exposure, even at lower concentrations. Importantly, a significant difference was evident in the cytotoxicity of all the concentrations between 24 and 48 hours of exposure time.

The current findings are consistent with Shahbazzadeh et al. study, who worked on HSF and HMS cell lines. They reported a significant concentration-dependent cytotoxicity for AgNPs; however, no significant difference was observed between cytotoxicity at 24 and 48 hours in all used concentrations of AgNPs (5). In this regard, Peng et al., AshaRani et al. and Dziedzic et al. also assessed the AgNPs cytotoxicity on normal and cancerous cell lines (EBFs, L-929, IMR-90, U251 and SCC-25) and their findings were in agreement with the current report, demonstrating a time and concentration dependency in the cytotoxicity of AgNPs (16, 29, 32).

Many researchers believe that in order to achieve more effective antimicrobial properties, they have to use AgNPs below 10 nm (9, 19-21, 33). Our results showed that PVP-coated AgNPs of 6.03 nm had a high cytotoxicity for the human normal cell line, especially at higher concentrations. Other studies performed by Liu et al. confirmed the current findings and displayed that all four tested cell lines (SGG-7901, HepG2, A-549, MCF-7) were more sensitive to the cytotoxicity of 5 nm AgNPs than 20 and 50 nm (34). In this regard, Ivask et al. evaluated the cytotoxicity of five

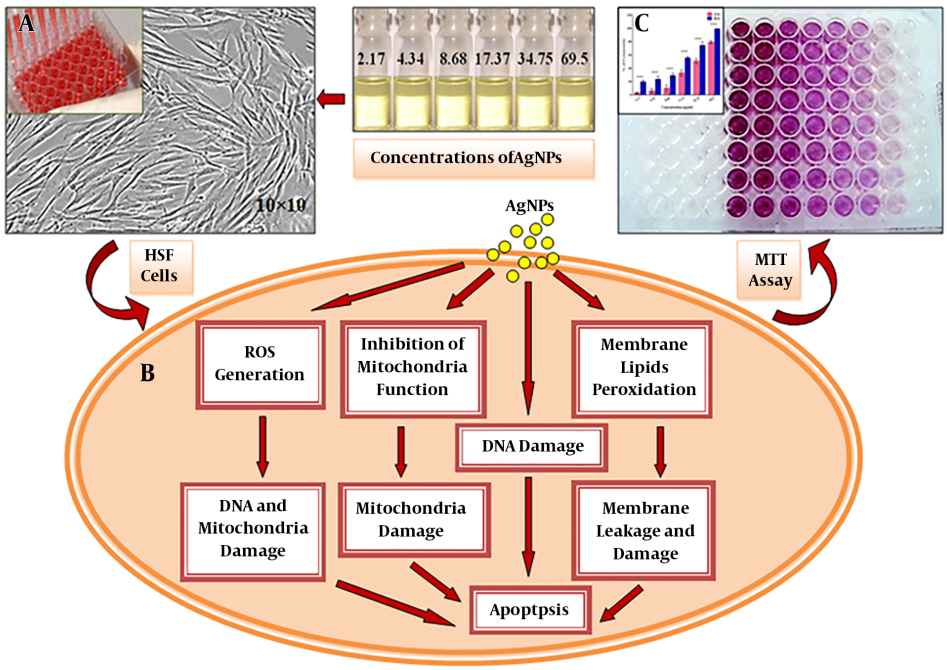

Figure 5. A, Observation of HSF-PI-16 cells under Nikon inverted microscope (10 × 10). B, Adverse effects of AgNPs on normal cells. C, Evaluation of AgNPs cytotoxic effect on normal HSF-PI-16 cell line using MTT assay

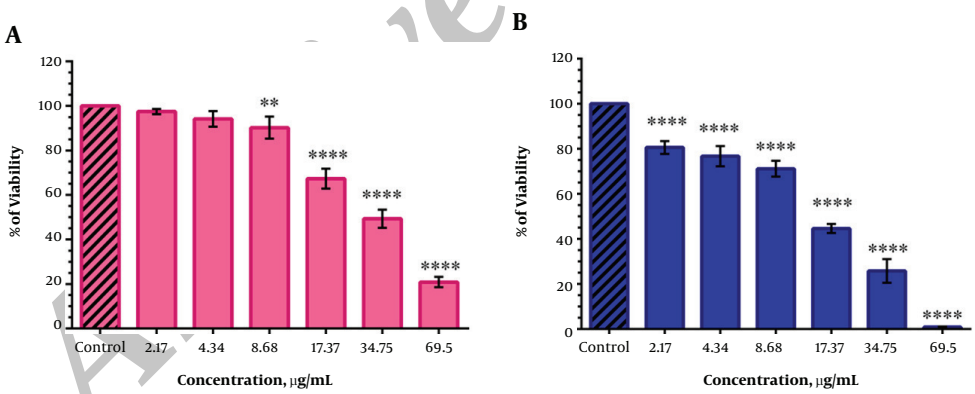

Figure 6. Assessment of HSF cell viability using MTT assay after exposure to different concentrations of AgNPs at 24 hours (A) and 48 hours (B). The values are described as the mean ± standard deviation (SD) of three independent examinations. All data were compared with the control (ANOVA test, ** P < 0.01, **** P < 0.0001).

AgNPs diameters (10, 20, 40, 60 and 80 nm) on five different cells (mammalian cells, bacteria, and so on.) *in vitro*. The findings indicated that nanoparticles of 10 nm possessed greater cytotoxicity than 20 to 80 nm, although it had better antimicrobial property (33). Gliga et al. and Zapor proved a specific size-dependent cytotoxicity. They found that AgNP at the 10 nm diameter was more toxic to standard cell lines (BEAS-2B, RAW264.7, 15P-1, CHO-9) than particles of 40, 75, and 100 nm (7, 9). Thus, silver nanoparticles of < 10 nm are as much beneficial for destructing cancerous cells and microorganisms, as they are detrimental to normal cells (9, 13, 33). Adverse effects of nanoparticles below 10 nm can be related to a broader surface area for interaction of surface Ag° atoms with O_2 (Ag_2O) and also to other physicochemical characteristics (12). On the other hand, geno- and cell-toxicity, including mitochondrial respiratory chain disruption, increase ROS-dependent oxidative stress, DNA damage, and cell cycle arrest (G_1, S, G_2/M),

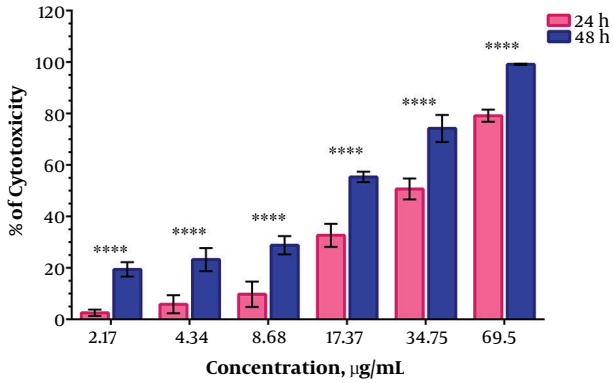

Figure 7. Comparison of cytotoxicity of AgNPs at different concentrations towards HSF cell after 24 and 48 hours of exposure. The values are described as the mean ± standard deviation (SD) of three independent examinations. (ANOVA test, **** $P < 0.0001$).

and apoptosis can be intensified as a result of secondary cytotoxicity even at low concentrations of small NPs (9, 12, 16, 29, 35). This occurs because of better solubility of small AgNPs and releasing more toxic Ag^+ by Ag_2O interaction with various compounds of intracellular or cell culture media, such as FBS, proteins, sulfur, and chloride (1, 14, 36, 37). In addition, secondary cytotoxicity can be correlated with more precipitation of small AgNPs inside the cells, especially in sticky cells at the bottom of Petri dishes and also increase in the size of NPs by agglomeration/aggregation (1, 9, 14).

Based on these findings, the current researchers suppose that high cytotoxicity observed in this study could be strengthened by secondary cytotoxicity. Considering the different factors involved in the effects of AgNPs, controversial challenges exist on their toxicity towards normal cells (3). Thus, it is recommended to apply a safe standard method in order to make NPs, a suitable cytotoxicity assay coincident with the aim of the study, and an appropriate culture media without interfering materials along with a proper cell line (11, 13, 14, 29).

5.1. Conclusions

In the present research, the impact of various concentrations of small nano-sized silver was evaluated on normal human skin fibroblast cell line after 24 and 48 hours of incubation. Based on the results of the MTT assay, the cell viability decreased in a time- and concentration-dependent manner. Also, the calculated IC_{50} values showed that cytotoxicity following 48 hours of exposure was much higher than that of 24 hours. Based on the potential cytotoxicity of small AgNPs on normal cells, it will definitely need future researches to produce safer NPs with protective concentrations for application in medicine, hygienic and industrial products.

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Footnotes

Authors' Contribution: Study concept, design, acquisition of data and drafting of the manuscript: Masoureh Paknejadi; analysis and interpretation of data: Masoureh Paknejadi and Mona Salimi; critical revision of the manuscript for important intellectual content: Mona Salimi; statistical analysis: Masoureh Paknejadi and Mona Salimi; study supervision: Mona Salimi, Masoureh Paknejadi, Mansour Bayat and Vadood Razavilar.

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