In vitro effect of Nanosilver toxicity on fibroblast and mesenchymal stem cell lines

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Abstract

Nanotechnology presents countless opportunities to develop new and improved consumer products for the benefit of the society. A most prominent nanoproduct is nanosilver. Nanosilver particles are generally smaller than 100 nm and contain 20–15,000 silver atoms. Despite the wide application of nanomaterials, there is a serious lack of information concerning their impact on human health. In the previous study we reported the cytotoxic of nanosilver on osteoblast G292 cancer cell line and the amount of IC_{50} determined as 3.42 µg/ml (Moaddab et al., Iran. Nano Lett., Vol. 1, No. 1, January 2011, pp. 11-16). The purpose of the present study is to assess the biological assay of nanosilver on two normal cell lines of fibroblast (HF2), and mesenchymal stem cells. The effect of nanosilver on these cells is evaluated by light microscopy, and by cell proliferation and standard cytotoxicity assays. The results demonstrate a concentration-dependent toxicity for the cells tested, and IC_{50} was determined as 6.33, and 6.68 µg/ml in mesenchymal stem cell, and fibroblast HF2, respectively. There is no significant difference between the 24 h and 48 h of cells exposure to nanosilver. The results show that Nano-Ag possesses low toxicity to normal cells and can display potential application in cancer chemoprevention and chemotherapy.

Keywords: Nanosilver, toxicity test, mesenchymal stem cell, fibroblast (HF2)

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Introduction

Metallic silver is subjected to new engineering technologies with resultant extraordinarily novel morphologies and characteristics. Instead of being made "big", metallic silver is engineered into ultrafine particles whose size is measured in nanometres (nm). When these particles have at least one dimension, which is less than 100 nm, they are named nanoparticles (Oberdorster et al., 2005; Warheit et al., 2007). Nanoparticles are now used to target synthetic peptides, proteins, oligonucleotides, and plasmids to specific cell type while protecting these macromolecules from enzymatic degradation (Chavany et al., 1994; Janes et al., 2001). In addition, nanoparticles have been proposed for the treatment of many diseases that need constant drug concentration in the blood or drug targeting to specific cells or organs (Moghimi et al., 2001; Panyam and Labhasetwar, 2003; Soltani et al., 2011). In this respect, nanoencapsulated therapeutic agents such as antineoplastic drugs have been used with the aim of selectively targeting antitumor agents and obtaining higher drug concentration at the tumor site (Chawla and Amiji, 2002; Sahoo et al., 2004). This achievement appears to be important because many antineoplastic agents have several adverse side effects. Nanoparticles can be used to treat diseases that require a sustained presence of the drug at several anatomical sites (Panyam and Labhasetwar, 2003; Li et al., 2003). Use of nanosilver is becoming more and more widespread in medicine and related applications. This has led to an increasing number of medical applications of silver nanoparticles. Some of the products which are already available in the market include wound dressings, contraceptive devices, surgical instruments and bone prostheses (Cheng et al., 2004; Chen et al., 2006; Cohen et al., 2007; Lee et al., 2007; Zhang et al., 2007).

The present study is aimed to target osteoblast G292, fibroblast HF2, and mesenchymal stem cell lines to determine the cytotoxic threshold of nanosilver in these cancerous and non cancerous cell lines. Moreover, in a time-course study, we found that the monodispersed Nano-Ag could keep stable at least for 12 months in the soluble state (data not shown). This stabilization of nanosilver is an important issue for its medical applications.

Materials and methods

Nanoparticle production

The method of the present invention is to provide a simple preparation method for silver nano particles with well-controlled sizes in a surfactant solution. The nano silver colloid is prepared by the following steps: (1) dissolving silver nitrate crystal in distilled water, (2) adding surfactant, LABS (Linear alkyl benzene sulfonate) to the solution and (3) adding reducing agent to the solution. The method is performed based on undisclosed US-patent and the produced nanosilver is nominated as Nanocid® (Rahman-Nya, 2009).

Cell culture

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The cell lines were obtained from the Cell Bank of Pasteur Institute of Iran. Fibroblast cells were seeded in flasks containing RPMI medium. The medium had contained 5-10% fetal bovine serum (FBS). Stem cells were cultured in DMEM containing low glucose. The cells were incubated at 37°C in a 5% CO2 atmosphere. After 24-48 h the attached cells were trypsinized for 3-5 min and centrifuged (1,400 rpm, 5 min). The live cells were counted and distributed in 96 well ELISA plate with 100 µl of culture medium containing 10^4 cells in each well. The plate was incubated 24-48 h at 37°C in a 5% CO2 atmosphere to allow the cells to attach to the bottom of the well (Mosmann, 1983).

Cell treatment with nanosilver

Different concentrations of nanosilver were added to wells in duplicates. The amounts of 1, 3, 5, 10, 15, 25, 50, 100 µg/ml nanosilver were inoculated into as-grown ($1x10^4$ cells/well) and the cell population was then determined by optical microscopy at 24 and 48 hours.

MTT assay

Cell viability was evaluated by the MTT colorimetric technique (Mosmann, 1983). Briefly, 100 µl of the yellowish yellow tetrazolium MTT solution (Sigma) (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) without phenol red, (5 mg/ml in PBS) was added to each well. The plates were incubated for 3-4 h at 37 °C, for reduction of MTT by metabolically active cells, partially by dehydrogenase enzymes activity, to generate reducing equivalents such as NADH and NADPH. By adding MTT crystals the resulting intracellular purple formazan was solubilized and quantified by spectrophotometry and then the supernatants were removed. For solubilization of the MTT crystals, 100 µl of isopropanol or DMSO was added to each well. The plates were placed on a shaker for 15 min to complete solubilization of crystals and then the optical density of each well was determined. The quantity of produced formazan measured in the absorbance of 545 nm, is directly proportional to the number of living cells in culture. Each experiment was done in duplicate. The relative cell viability (%) related to control wells containing cell culture medium without nanoparticles as a vehicle was calculated by [A]test/ [A]control×100. [A]test is the absorbance of the test sample and [A]control is the absorbance of the control sample (Mosmann, 1983).

Statistical Analysis

All experiments were done in duplicate, and the results were presented as mean ± standard deviation. The experimental data were analyzed using SPSS. Statistical significance was accepted at a level of $p < 0.05$. To calculate IC50 values, concentration–response curves were graphed using Excel. The IC50 of the curves was calculated by non-linear regression analysis and interpolation according to the method of Alexander et al. (1999).

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Results

Characterization of Nanosilver

The morphology and microstructure of Nano-Ag was examined by the transmission electron microscopy (TEM) method to synthesize size-controlled Nano-Ag. The images revealed that the uniform spherical nanoparticles were obtained. The size distribution and average diameter of Nano-Ag in aqueous solution were further characterized by LLS. The result was described elsewhere (Moaddab et al., 2011). Nano-Ag in water would easily aggregate with particle size distributed between 5–45 nm with an average diameter of 10 nm.

Cytotoxic effects of Nano-Ag on two normal human cell lines

The *in vitro* cytotoxic effects of Nano-Ag were screened against two normal human cell lines by means of MTT assay. As shown in Fig. 1, Nano-Ag showed lower cytotoxicity toward normal cells (M-Stem cell and human fibroblasts (HF2), with an IC50 value of 6.38 and 6.68 µg/ml, respectively (Fig. 2A, B).

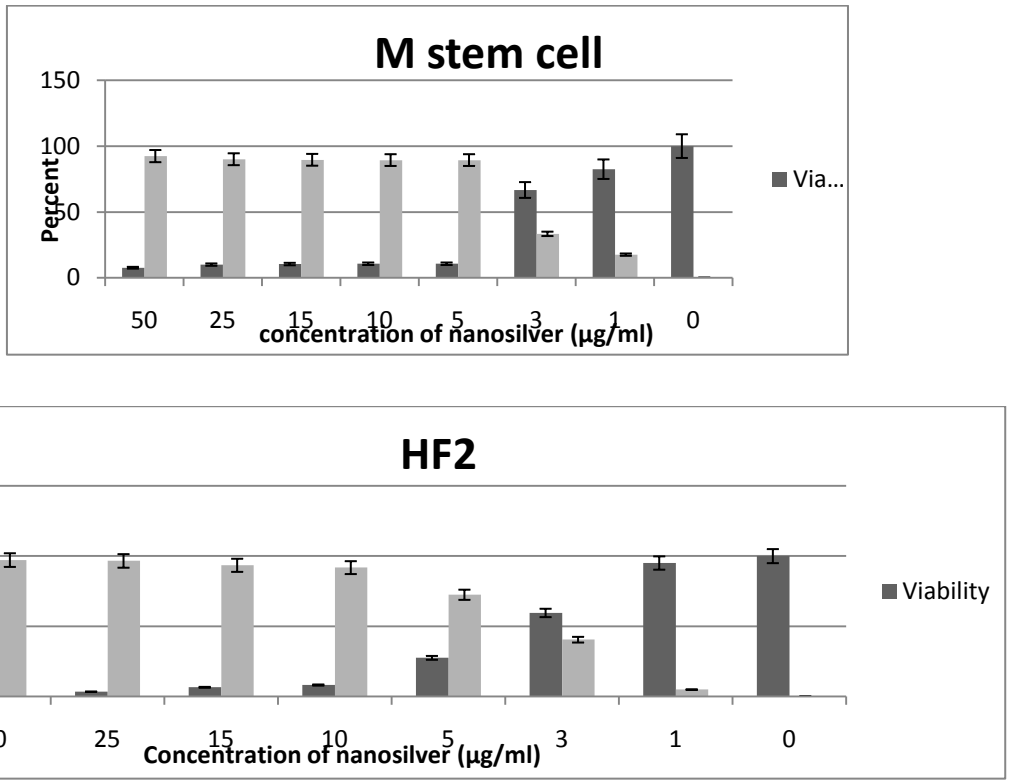


Figure 1: The percent of viability measured by MTT assay on mesenchymal stem cell and human fibroblast (HF2) cells after treatment with nanosilver (1.0 µg/mL to 100 µg/ml) for 24 h. The data are expressed as mean ± standard deviation (SD) of three independent experiments. An OD value of control cells (unexposed cells) was taken as 100% viability (0% cytotoxicity).

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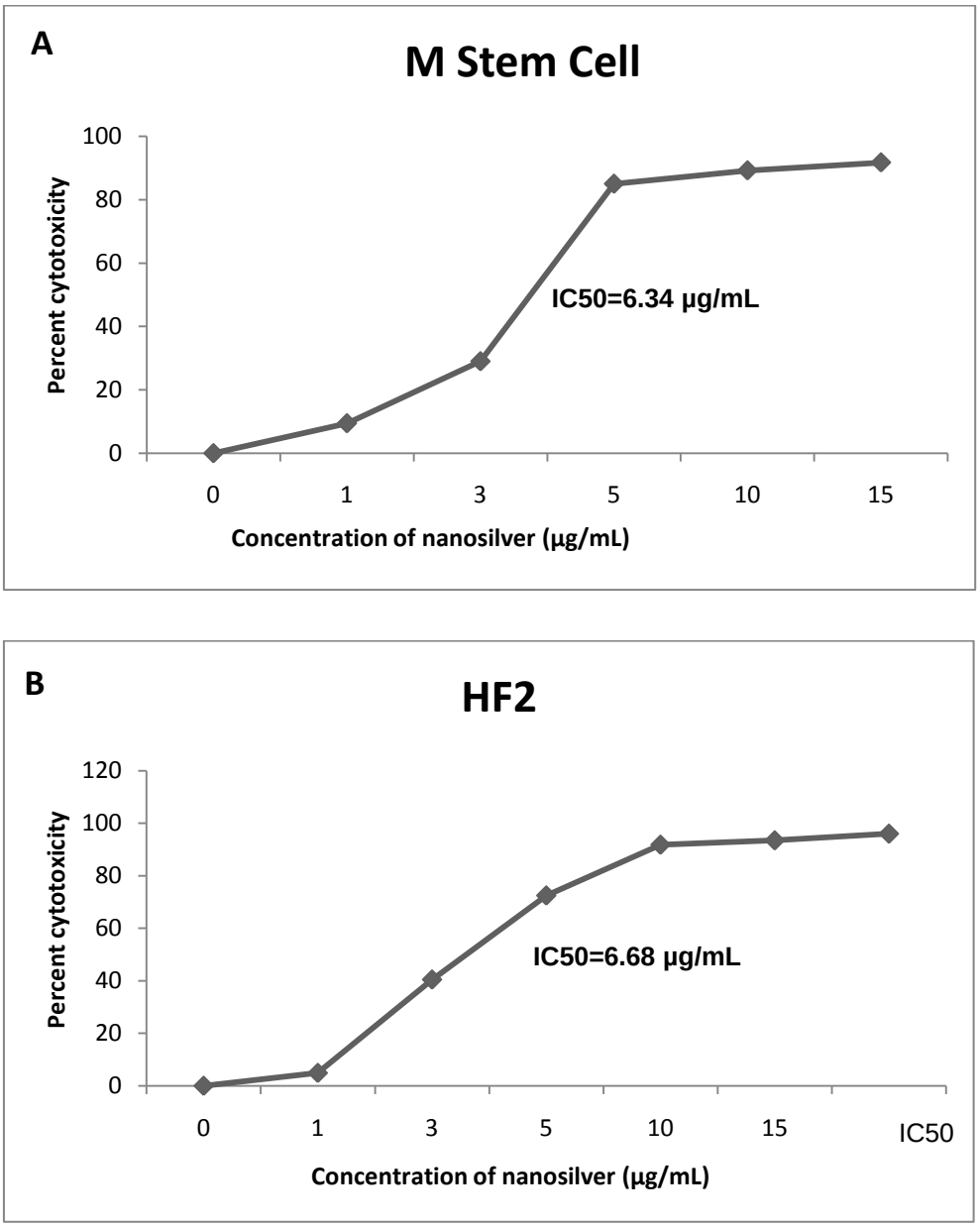


Figure 2: IC50 measurement of two cell lines; Cytotoxicity measured by MTT assay on (A) M-Stem, and (B) HF2 cells with (1.0–50µg/ml in 24 h. The data are expressed as mean ± standard deviation (SD) of three independent experiments. An OD value of control cells (Unexposed cells) was taken as 100% viability (0% cytotoxicity). A statistically significant difference compared to control ($p < 0.05$).

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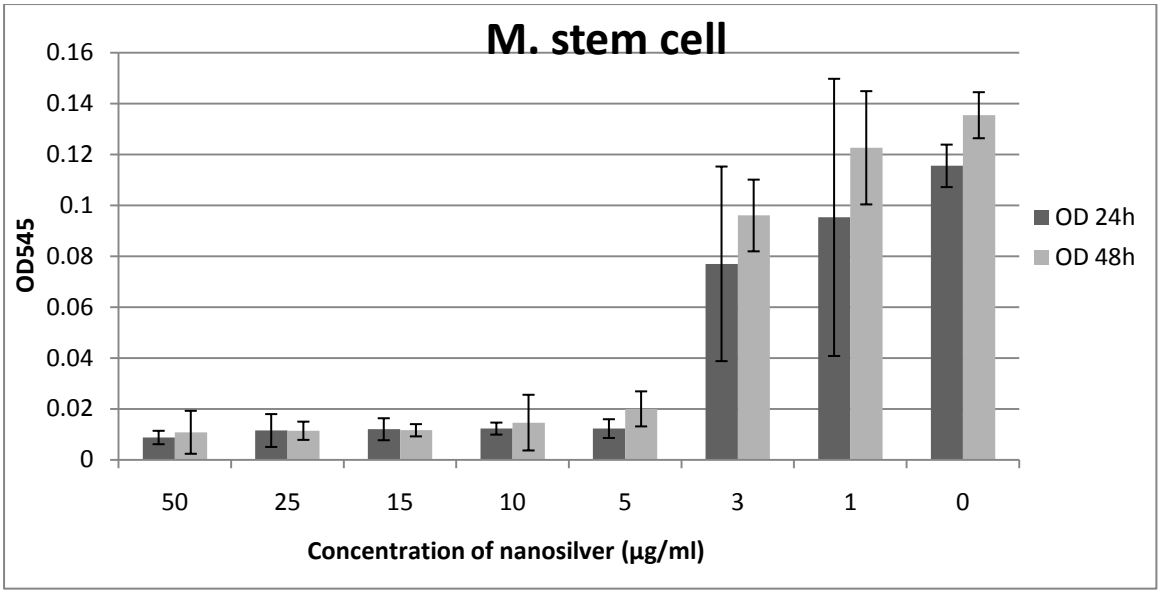


Figure 3: The percent of viability measured by MTT assay on mesenchymal stem cells. Treatment with nanosilver (1.0 µg/ml to 50 µg/ml) was determined after 24 and 48h. The data are expressed as mean ± standard deviation (SD) of three independent experiments. An OD value of control cells (unexposed cells) was taken as 100% viability (0% cytotoxicity).

Discussion

Nanotechnology, which deals with devices typically less than 100 nanometer in size, is expected to make a significant contribution to the fields of computer storage, semiconductors, biotechnology, manufacturing and energy. Nanomaterials can enter human tissues through several ports via the lungs after inhalation, through the digestive system and possibly through the skin (Jani et al., 1990; Warheit et al., 2007). Systemic distribution of nanoparticles has been demonstrated after inhalation and oral uptake (Jani et al., 1990; Oberdorster et al., 2002), and nanoparticles have been found to cross the blood–brain barrier, reaching the olfactory bulb and the cerebellum (Oberdorster et al., 2004). Chen and colleagues also reported that nanoparticles can penetrate the blood-testis barrier (Chen et al., 2003). Although organ- or cell-specific drug delivery through nanoparticles is a promising area of medicine, and nanoparticles might be used some day as sensors for intracellular mechanisms, few toxicology studies are available. Many of the artificially manufactured nanoparticles are made of nonbiodegradable pollutants, such as carbon black and metals, and the long-term behavior of such substances is unknown. Nanosilver is a liquid product that is being used as a potent antibacterial. In the previous study we showed that nanosilver can induce toxicity on osteoblastoma G292 cancerous cell line with a concentration-dependent toxicity for the cell tested (Moaddab et al., 2011).

In the present study the effect of nanosilver on cell viability was estimated using MTT assay. The mesenchymal stem

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cell and fibroblast HF2 were used as normal human cell lines. The cytotoxicity result showed that the IC_{50} of nanosilver in these cell lines were 6.33 and 6.68 µg/ml, respectively. It means that nanosilver is twice less toxic to normal cells compared with cancerous cell such as osteoblast G292 with IC_{50} of 3.42 µg/ml (Moaddab et al., 2011). The result obtained in this study is in accordance with the studies done by other researchers. Morphology of the C18–4 spermatogonial stem cells after incubation with silver nanoparticles (SNP) (Ag—15 nm) at concentrations higher than 5 µg/ml became necrotic and detached from the culture dishes and dramatic changes were induced by SNP at concentrations of 10 µg/ml and above. In addition to necrotic areas, some cells retained an intact plasma membrane, indicating that apoptosis had occurred (Braydich-Stolle et al., 2005). Meanwhile, silver carbonate, generally considered to be non-hazardous, had no significant cytotoxic effect on mitochondrial functions and cell viability up to concentrations of 100 µg/ml. The EC50 of silver carbonate was calculated at 408 µg/ml. On the other hand, silver added to the cells as a nanoparticulate (diameter of 15 nm) reduced mitochondrial functions and cell viability drastically. The toxic effect of SNP started between 5 and 10 µg/ml, with an EC50 calculated at 8.75 µg/ml. Higher concentrations of silver nanoparticles could not be tested because of particle clumping and precipitation above 10 µg/ml (Braydich-Stolle et al., 2005). Also a slight increase in LDH leakage was observed with silver nanoparticles, indicating that these particles interfere with cell metabolism rather than disrupting the plasma membrane. Thus, they might promote cell apoptosis rather than necrosis. The EC50 of SNP was evaluated at 2.5 µg/ml (Braydich-Stolle et al., 2005). Upon exposure to silver nanoparticles for 24 h, morphology of primary fibroblast cells remained unaltered up to 25 µg/ml, although with a minor decrease in confluence. In this in vitro study, the IC50 value for primary fibroblasts, which were revealed by XTT assay, was 61 µg/ml, and Caspase-3 activity assay indicated that the SNP concentrations required for the onset of apoptosis were found to be 3.12 µg/ml compared to the necrotic concentration of 100 µg/ml. These observations were confirmed by confocal laser scanning microscopy (CLSM) studies by exposure of cells to ½ IC50 SNP (resulting in apoptosis) and 2× IC50 cells (resulting in necrosis) (Arora et al., 2009). The mechanisms by which SNP induces this toxicity in cancer cells are not well known. Our results show that normal cells possess much higher SNP tolerance than the cancerous cells, maybe due to more oxidative stress in cancer cells compared to normal cell lines which facilitate the possible human applications of nanosilver in next steps. As shown in Fig. 3, there is no significant difference between the 24 h and 48 h of mesenchymal stem cell exposure to nanosilver.

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In summary, our studies show that silver nanoparticles exert similar toxicity in the fibroblast and mesenchymal stem cells which is nearly two times more than the amount needed for cancerous cells such as osteoblst G292 which was reported in the previous work (Moaddab et al., 2011). These results suggested that Nano-Ag possesses low toxicity to normal cells and can display potential application in cancer chemoprevention and chemotherapy.

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