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The Comparison of Silver and Hydroxyapatite Nanoparticles Biocompatibility on L929 Fibroblast Cells: An *In vitro* Study

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Abstract

Background and aims: Nano particles are tiny materials (<1000 nm in size) that have specific physicochemical properties different to bulk materials of the same composition and such properties make them very attractive for commercial and medical development. In contrast to many efforts aimed at exploiting desirable properties of nano particles for medicine, there are limited attempts to evaluate potentially undesirable effects of these particles. Therefore, there is a pressing need for careful consideration of benefits and side effects of the use of nano particles in medicine. The purpose of this research was to compare the nano silver and hydroxyapatite nanoparticles' biocompatibility on L929 fibroblast cells.

Materials and methods: To evaluate the biocompatibility of nano silver and hydroxyapatite nanoparticles (nHA), L929 fibroblast cells were cultured on a 96-well plate. Cells were exposed to nHA and nano silver at the following concentrations: 5, 10, 20, 30, 40, 50 ppm after 24, 48 and 72 hrs. Later, for measuring the biocompatibility of materials, MTT method was utilized.ANOVA test was used for statistical analysis.

Results: None of the nHA experimented concentrations were toxic, but vitality of cells exposing to nano silver particles were the least after 24 hours and increased after 48 hours and ANOVA analyze indicated that there was significant difference between groups ($p<0.05$). Also the result showed nano silver particles at concentrations more than 20 ppm within 24 and 48 hours are toxic to fibroblast cells and it was significant ($p<0.02$).

Conclusion: NHA were more biocompatible rather than nano silver particles on L929 fibroblast cells.

Keywords: Hydroxyapatite nano particles; Nano silver; L929 fibroblast cells; Cytotoxicity; Photo absorption

Introduction

Nanotechnology is the understanding and control of matter at dimensions of roughly 1-100 nanometers, where unique phenomena enable novel applications [1,2]. In the last decade, engineered nano particles have become an important class of new materials with several properties that make them very attractive for researchers [3,4]. Nano silver and hydroxyapatite nano particles are the most used of these compounds [5,6]. Antimicrobial effects of silver, has been known for long time but after it has been developed as nano-particles [7-9], the contact surfaces have been increased and its antimicrobial effects have been improved more than 99% and because of its substantial antibacterial, anti viral and anti fungal effects, its uses has been strongly approved in medicine [10,11]. Also, nHA has been introduced for augmentation procedures in osseous defects [8-11]. The nHA particles has already been used for treatment of human periodontal bony defects [12,13] and various types of metaphyseal fractures such as the calcaneus and tibia in orthopedic surgery [14], as well as tooth perforations [15], jaw cysts [16], and peri implantitis lesions [17,18], also nHA particles are currently being investigated to be used as delivery vehicles in various medical applications, including the delivery of growth factors antibiotics [19], anticancer drugs [20]. Despite of wide spread use of nanosilver and nHA there is no enough studies about its side effects on human [21,22]. Degradation products of nano materials are potentially toxic [23,24]. Thus, it is essential to assess biocompatibility of these materials before their usage in clinical applications. The purpose of this research was to compare the nano silver and hydroxyapatite nanoparticles' biocompatibility on L929 fibroblast cells by using the MTT assay.

Materials and Methods

Preparation and sterilization of nHA and nano silver particles

In this study, nano sized, rod- like hydroxyapatite particles (Figure 1) and spherical nano silver particles (Figure 2) provided from NANOSHEL corporation (Batch No#290090621 and 280080521 respectively) and were precisely sterilized by UV for 24 hrs.

Preparation and culture of L929 fibroblast cells

Murine L929 fibroblast cells were prepared from Iran-Pastoor institute, after defreezing the cells, they were stored in special flasks. Microscopic image of L929 fibroblast cells were cultured in flasks was shown in figure 3. We have used DMEM (Grand Island, NY) medium,

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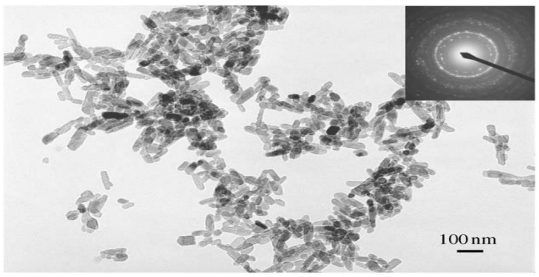

Figure 1: Transmission electron micrograph of nHA particles.

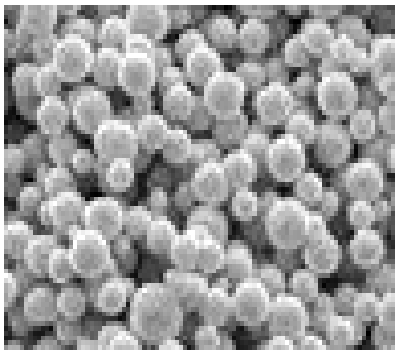

Figure 2: Transmission electron micrograph of nano silver particles.

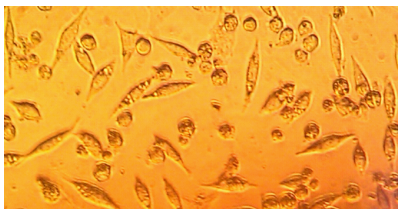

Figure 3: Microscopic image of L929 fibroblast cells cultured in flasks.

in order to cultivating the cells. We also added 100 IU/ml Penicillin (Sigma, USA) and 100 IU/ml Streptomycin (Sigma, USA) to sterilize the medium. To enrich the cultivating medium, 10% FBS (GIBCO, USA) were added. The cell suspension was distributed in each well in triplicate on a 96-well culture plate and cultured at 37°C in humidified air containing 5% CO_2.

Exposure L929 fibroblast cells to nHA and nano silver particles

10,000 fibroblast cells were exposed to nHA and nano silver particles at the following concentrations: 5, 10, 20, 30, 40, 50 ppm. For measuring the biocompatibility of these materials, MTT method was utilized after 24, 48, 72 hrs.

Cell viability assay

The viability of L929 fibroblast cells was assessed using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay. This Method outlines a simple assay to determine the viability/number of colored product (in a mitochondria-dependent reaction) to which the cell membrane is impermeable [25]. Sample solutions were removed after incubation with the various nHA preparations and MTT was added at the concentration of 0.5 mg/ml in medium for 4 h at 37°C. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehdrogenase enzymes. Cells were rinsed with PBS and 500 ml of extracting solution (0.04 M HCl in isopropanol) was added to each well so the water insoluble formazan can be solubilized. Plates were incubated for 15 min at room temperature to dissolve the dye and 200 ml of dye solution was transferred to 96 well plates. Absorbance was measured at 570 nm (ASYS HiTech Expert plate reader) and cell viability was expressed as percent relative to the control.

Results

Results of this study showed that although the mean of L929 fibroblast cells' vitality exposing to nHA was decreased by increasing concentration and time elongation (Table 1) but ANOVA analyze indicated that there was no significant difference between groups ($p>0.05$).

Cytotoxicity percentages of L929 fibroblast cells and were shown in figure 4 for each concentration in all time durations. Noted that nHA at 50 ppm concentration after 72 hrs had the maximum percentage of cell's mortality (19.4%).

Vitality of L929 fibroblast cells exposing to nano silver particles were the least after 24 hours, but the vitality of cells increased after 48 hours (Table 2) and ANOVA analyze indicated that there was significant difference between groups ($p<0.05$). Also the result showed concentrations over 20 ppm within 24, 48 hours are toxic to fibroblast cells and it was significant ($p<0.02$). However within 72 hours there are no significant findings in cells vitality ($p>0.05$).

Cytotoxicity percentages of L929 fibroblast cells were shown in figure 5 for each concentration in all time durations Noted that nano

time / Concentrations of nHA	24 hours	48 hours	72 hours
5	0.299 ± 0.005	0.295 ± 0.009	0.259 ± 0.007
10	0.306 ± 0.01	0.300 ± 0.005	0.267 ± 0.006
20	0.309 ± 0.002	0.297 ± 0.003	0.262 ± 0.002
30	0.298 ± 0.009	0.299 ± 0.009	0.256 ± 0.002
40	0.304 ± 0.004	0.295 ± 0.007	0.238 ± 0.002
50	0.308 ± 0.009	0.295 ± 0.005	0.236 ± 0.004

Table 1: Mean and standard deviations of vitality of L929 fibroblast cells after exposure to different concentrations of nHA at 24, 48 and 72 hrs.

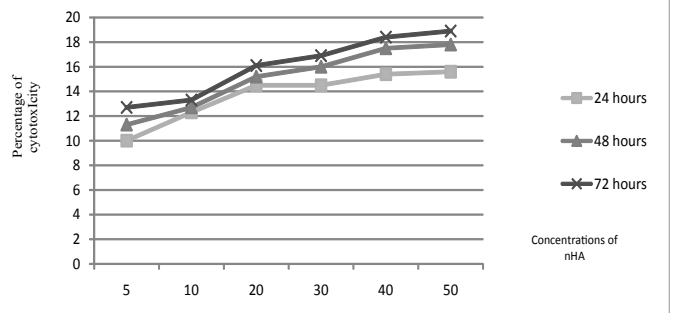

Figure 4: Comparisions of percentage of fibroblast cells' mortality exposing to nHA after 24, 48 and 72 hrs.

time / Concentrations of nano silver	24 hours	48 hours	72 hours
5	0.16 ± 0.003	0.18 ± 0.003	0.21 ± 0.016
10	0.13 ± 0.012	0.16± 0.002	0.2 ± 0.022
20	0.12 ± 0.024	0.16 ± 0.013	0.2 ± 0.0005
30	0.0 5 ± 0.001	0.15± 0.02	0.18 ± 0.04
40	0.0 6 ± 0.0004	0.15 ± 0.02	0.19 ± 0.005
50	0.0 3 ± 0.0003	0.13 ± 0.01	0.19 ± 0.043

Table 2: Mean and standard deviations of vitality of L929 fibroblast cells after exposure to different concentrations of nano silver particles at 24, 48 and 72 hrs.

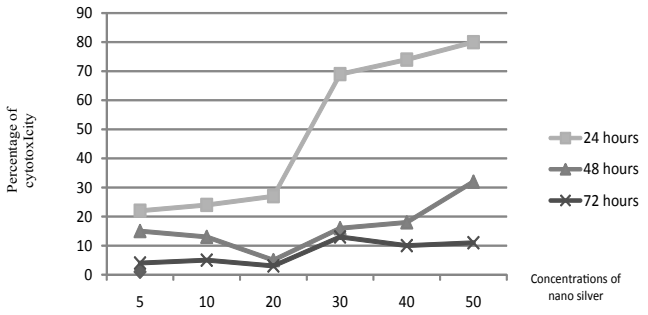

Figure 5: Comparisions of percentage of L929 fibroblast cells' mortality exposing to nano silver particles after 24, 48 and 72 hrs.

silver particles at 50 ppm concentration after 24 hrs had the maximum percentage of cell's mortality (80%).

Discussion

The results showed that the percentage of cells' mortality was elevated by increasing the concentration and duration of nHA exposure, no statistically significant difference was found between the groups ($p>0.05$) but the result showed nano silver particles at concentrations more than 20 ppm and before 72 hours had toxic effects and it was significant ($p<0.02$).

Zhao et al. [25] studied the influence of HA nanocrystal at 10-100 ppm on osteoblasts' proliferation after 24 hrs by MTT method and found that this materials exhibit good biocompatibility and would be safe to be used. Also, Hsieh et al. [26] used culture of MC3T3-E1 osteoblast cells for evaluating toxicity of nHA particles and figured out that nHA particles have minimal toxicity on osteoblast cells. Our findings confirm the results of these studies and in the present study L929 fibroblast cells were used as samples.

Motskin et al. [27] studied the cytotoxicity of synthetic colloid and gel nHA at 31, 62, 125, 250 and 500 ppm concentrations on Human Monocytes'-derived Macrophages (HMMs) by MTT assay and found gel preparation being the most toxic. Other preparations were also toxic but only at higher concentrations (>250 ppm). In this study we used the suspension of nHA and the cytotoxity was evaluated on L929 fibroblast cells [27].

Scheel et al. [28] evaluated the cytotoxicity of nHAparticles at 50, 100, 500, 1000 and 5000 ppm concentrations on RAW 264.7 macrophages and cells were analyzed for viability (MTT-test) after 18 and 42 hrs. Their results showed that up to concentrations of 500 ppm cell viability was not considerably impaired by the test samples at both time points. The results of mentioned reports suggest nHA materials can be toxic and may inhibit proliferation.

Cao et al. [29] had done a research about biocompatibility of nano composite silver on L929 fibroblast cells, by use of 3-4-5 dimethyl thiazole test and 2-5 diphenyl tetras volume, and they found that nano composite silver is useful for cells division and increasing the cohesiveness of fibroblast cells.

The result from this research was different from our study that could result from the use of nano particles at Cao et al. [29] study as nano composite. Nano composite is two phase's material with polymer or metal or ceramic base, lead to change in their mechanical properties.

Zhang et al. [30] studied the citotoxicity of 6 types of nano particles with silver base, on L929 fibroblast cells, by MTT assay and result of this study showed that these particles didn't have any toxic effect in concentrations less than 25 ppm. Our findings confirm the results of this study and showed that nano silver concentration above 20 ppm are toxic on fibroblast cells too.

Thus, nHA were more biocompatible rather than nanosilver particles on L929 fibroblast cells. But the main cause of nHA cytotoxicity on macrophages at concentrations up to 125 ppm is probably phagocytosis of particles and releasing of calcium in cytoplasm of cells but fibroblast cells cannot phagocytosis the particles, so we can adjudicate that the degree of toxicity correlated strongly with the degree of uptake and it highly strongly suggests that cellular particle load is the main cause of cytotoxicity of nHA. However, differences in the physicochemical and structural characteristics between the various forms of nHA and nano silver particles may lead to differences in the properties and surface geometry, and surface chemistry which play a determinant role in biocompatibility. Therefore, development of novel nanoparticles for pharmacology, therapeutics and diagnostics must proceed in tandem with assessment of any toxicological and environmental side effects of these particles and further studies including histological and biological evidences, molecule reactions are required to determine the ultimate fate of the nHA and nano silver particles within the body.

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