Original Article

Evaluation and comparison of hydroxyapatite nanoparticles' cytotoxicity on L929 fibroblast and human peripheral blood mononuclear cells: an in vitro study

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Abstract

Background and Objectives: Hydroxyapatite is the best bio-active material, which is compatible with the bone tissue. Degradation products of nano materials are potentially cytotoxic. Thus, it is essential to assess biocompatibility before their usage in clinical applications. The purpose of this research was to evaluate and compare the biocompatibility of nHA on human peripheral blood mononuclear cells (HPBMCs) and L929 fibroblast cells.

Material & methods: HPBMCs and fibroblast cells were cultured on a 96-well plate. Cells were exposed to nanocrystalline hydroxyapatite (nHA) at the following concentrations: 15.5, 31.25, 62.5, 125, 250, 500, 1000, 2000, 4000, 8000 ppm after 2, 24, 48 & 72 hours. Then, MTT method was utilised for measuring the biocompatibility.

Results: None of the nHA experimented concentrations were toxic.

Conclusion: "nHA" biomaterial has acceptable compatibility with HPBMCs and L929 fibroblast cells.

Key words: Nanoparticles; biomaterials; cytotoxicty; hydroxyapatite.

Introduction

Hydroxyapatites (HA) represent a family of bone grafting materials with a high degree of biocompatibility which makes up the majority of the inorganic components of human bones and teeth. However, one of the major drawbacks was that HA-based biomaterials required high-temperature and high-pressure processing, which resulted in higher density and decreased porosity [1]. Therefore, the HA bone-grafting materials exhibited decreased osteoconductivity and poor degradation characteristics [2]. In pursuit of improving these shortcomings, a novel fully synthetic nano crystalline hydroxyapatite (nHA) has been introduced for augmentation procedures in osseous defects [3-5]. The "nHA" materials are considered to be biocompatible. However, some reports suggest that they can be toxic and may inhibit proliferation [6]. So because of these contradictory data evaluations, the present study was designed to evaluate and compare the cytotoxicity of rod-like nHA particles on the human peripheral blood mononuclear cells (HPBMCs) and L929 fibroblast cells by using the MTT assay.

Materials and methods

Preparation and sterilisation of nHA- In this study, nano sized, rod- like hydroxyapatite particles, were

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provided from NANOSHEL Corporation (Batch No #290090621) and precisely sterilised by UV for 24 hours.

Isolation and culture of HPBMCs- Heparinised venous blood was collected from a healthy volunteer after taking written consent. HPBMCs were isolated by density gradient centrifugation on histopaque (Sigma) with 500 G and for 20 minutes. Cells from the interphase were harvested, washed and re-suspended at 100,000 cells/mL RPMI-1640(Gibco). The cell suspension was distributed in each well in triplicate on a 96-well culture plate and cultured at 37^0C in humidified air containing 5% carbon dioxide.

Preparation and culture of fibroblast cells grade L929- Murine L929 fibroblast cells were prepared from Iran-Pastoor institute, after defreezing the cells, they were stored in special flasks. We have used DMEM (Grand Island, NY) medium, in order to cultivating the cells. We also added 100 IU/mL Penicillin (Sigma, USA) and 100 IU/mL Streptomycin (Sigma, USA) to sterilise the medium. To enrich the cultivating medium, 10% FBS (GIBCO, USA) was added. The cell suspension was distributed in each well in triplicate on a 96-well culture plate and cultured at 37^0C in humidified air containing 5% carbon dioxide.

Exposure of HPBMCs and L929 fibroblast cells to nHA- One million HPBMCs and 10,000 fibroblast cells were exposed to nHA at the following concentrations: 15, 31, 62.5, 125, 250, 500, 1000, 2000, 4000, 8000 ppm. For measuring the cytotoxicity of materials, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay was utilised after 2, 24, 48 and 72 hours.

Cell viability assay- The viability of HPBMCs and fibroblast cells was assessed using the MTT assay. This method outlines a simple assay to determine the viability/number of coloured product (in a mitochondria-dependent reaction) to which the cell membrane is impermeable.

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 hours at 37^0C. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes. Cells were rinsed with PBS (Phosphate buffered saline) and 500 mL of extracting solution (0.04 M hydrochloric acid in isopropanol) was added to each well so the water insoluble formazan can be solubilised. Plates were incubated for 15 minutes 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.

Quantitative analysis- In this study, we compared biocompatibility of 10 different concentrations of nHA particles and their cytotoxicity to HPBMCs and fibroblast cells after 2, 24, 48 and 72 hours which were assessed by MTT assay. The absorption in each of the 10 densities was calculated, mean and standard deviation (SD) were registered (table 1 and 2). Also, mean and SD of concentrations was measured in each time space separately (2/24/48/72 hours) and each concentration was measured in all mentioned time durations. The statistical ANOVA test was used in this study.

Results

Results of this study showed that the mean count of HPBMCs and L929 fibroblast cells' vitality did not vary significantly with increasing concentrations and time elongation (tables 1 and 2, ANOVA, $p > 0.05$).

Cytotoxicity percentages of HPBMCs and fibroblast cells are shown in Figures 1 and 2 for each concentration in all time durations. The maximum percentages of cell's mortality were 24.6% and 22.4% for HPBMCs and fibroblast cells, respectively.

Discussion

The nHA particles have already been used for treatment of human periodontal bony defects [5] and various types of metaphyseal fractures such as the calcaneus and tibia in orthopaedic surgery [7], as well as tooth perforations [8], jaw cysts [9], and peri-implantitis lesions [10]. Also nHA particles are currently being investigated to be used as delivery vehicles in various medical applications, including the delivery of growth factors antibiotics [11] and anticancer drugs [12,13].

Thus, it is imperative to assess biocompatibility

Table 1- Mean and standard deviations of vitality of HPBMCs after exposure to 10 different concentrations of nHA at 2, 24, 48 and 72 hrs.

Concentrations of nHA (ppm)	2 hours	24 hours	48 hours	72 hours
15.5	0.327±0.003	0.299±0.005	0.295±0.009	0.259±0.007
31	0.337± 0.003	0.306±0.01	0.300±0.005	0.267±0.006
62.5	0.334±0.005	0.309±0.002	0.297±0.003	0.262±0.002
125	0.330±0.006	0.298±0.009	0.299±0.009	0.256±0.002
250	0.340±0.002	0.304±0.004	0.295±0.007	0.238±0.002
500	0.336±0.002	0.308±0.009	0.295±0.005	0.236±0.004
1000	0.339±0.001	0.302±0.007	0.290±0.0015	0.236±0.006
2000	0.332±0.003	0.290±0.006	0.281±0.002	0.234±0.004
4000	0.336±0.002	0.300±0.005	0.280±0.008	0.230±0.002
8000	0.338±0.003	0.269±0.009	0.261±0.003	0.220±0.004

Table 2- Mean and standard deviations of vitality of L929 fibroblast cells after exposure to 10 different concentrations of nHA at 2, 24, 48 and 72 hours.

Concentrations of nHA (ppm)	2 hours	24 hours	48 hours	72 hours
15.5	0.06±0. 65	0.03±0.256	0.005±0.16	0.012±0.14
31	0.045±0.6	0.015±0.23	0.006±0.14	0.002±0.122
62.5	0.009±0.59	0.007±0.224	0.006±0.139	0.007±0.121
125	0.012±0.59	0.01±0.219	0.008±0.135	0.006±0.117
250	0.006±0.58	0.011±0.218	0.007±0.134	0.01±0.116
500	0.007±0.57	0.009±0.216	0.003±0.132	0.002±0.114
1000	0.006±0.56	0.004±0.216	0.005±0.131	0.009±0.113
2000	0.01±0.55	0.005±0.215	0.002±0.13	0.001±0.113
4000	0.007±0.55	0.010±0.213	0.002±0.128	0.008±0.112
8000	0.012±0.54	0.006±0.212	0.005±0.127	0.003±0.11

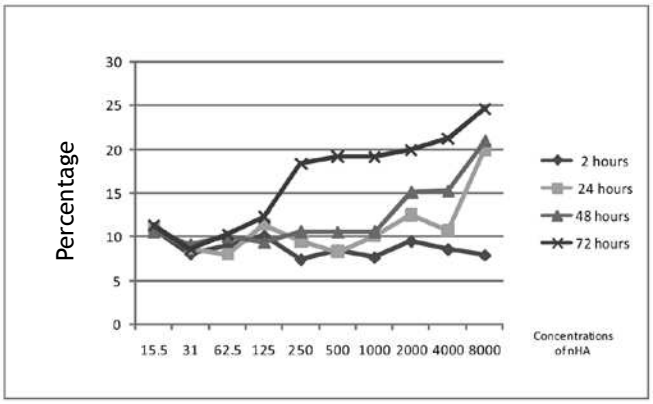

Figure 1- Comparision of percentage of HPBMCs' mortality after 2, 24, 48 and 72 hours.

before their usage in clinical applications. The present study was designed to evaluate the cytotoxicity of nHA particles (nearly rod-like, ranging size from 10 to 100 nm in diameter), on the human peripheral blood mononuclear cells (HPBMCs) and L929 fibroblast cells by using the MTT assay. The results showed that the cells' viability was decreased at all tested concentrations (15.5-8000 ppm) after 2, 24, 48 & 72 hours but there was no statistically significant difference in all groups ($p > 0.05$). Although, the percentage of cells' mortality was elevated by increasing the concentration and duration of nHA exposure, but no statistically significant difference was found between the groups ($p > 0.05$).

Some researchers studied the influence of HA nanocrystal morphology (rod-like and spherical crystals) at 10-100 ppm on osteoblasts proliferation after 24 hours by MTT method and found that these materials exhibit good biocompatibility and would be safe to be used [14].Also, other researchers, 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 [15]. Our findings confirm the results of these studies. Although, HPBMCs and fibroblast cells were used as samples and different concentrations of nHA were evaluated too.

Another study focused on 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

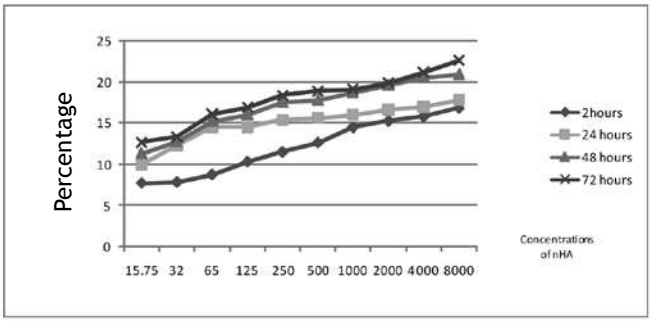

Figure 2- Comparison of percentage of fibroblast cells' mortality after 2, 24, 48 and 72 hours.

(>250 ppm) [16].

Also, the cytotoxicity of nHA particles at 50, 100, 500, 1000 and 5000 ppm concentrations were evaluated on RAW 264.7 macrophages and cells which were analysed for viability (XTT-test) after 18 and 42 hours. Their results showed that up to concentrations of 500 ppm, cell viability was not considerably impaired by the test samples at both time points [17]. However, different method was considered in their study.

Other researchers studied the biocompatibility of five hydroxyapatite materials of different morphology, i.e., rod-like, needle-shaped and plate-like on primary alveolar macrophages by LDH assays and concluded that no cytotoxicity was observed with all samples up to 300 ppm [18]. The results of mentioned reports suggest nHA materials can be toxic and may inhibit proliferation [16-18]. These contradictions appear to be related to the different characteristics of the nHA used and exposed cells' types. So, 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 osteoblast cells, peripheral blood mononuclear cells and fibroblast cells cannot phagocytosis the particles, so we can adjudicate that the degree of toxicity correlated strongly with the degree of uptake and it strongly suggests that cellular particle load is the main cause of cytotoxicity. However, differences in the physicochemical and structural characteristics between the various forms of nHA may lead to differences in the properties as well as in resorption characteristics, surface geometry, and surface chemistry which play a determinant role in biocompatibility. Although, the results of a recent in vitro study demonstrated better compatibility

of nHA at extracellular forms in comparison with intracellular forms.

The present study indicates that “nHA” biomaterial is compatible with the human blood mononuclear cells and fibroblast cells and thus appears to be a safe bone grafting substitute. Further studies including histological and biological evidences, molecule reactions are required to determine the ultimate fate of the nHA within the body.

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