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Evaluation of Hydroxyapatite Nanoparticles' Biocompatibility at Different Concentrations on the Human Peripheral Blood Mononuclear Cells: An *in vitro* Study

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Abstract: Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is the major inorganic component of hard tissues, the best bio-active materials which is compatible with the bone tissue. In addition, Hydroxyapatite nanoparticles (nHA) have received enormous national attention in medical and dental applications recently, the ultimate fate of the nHA within the body is still unknown. Degradation products of nanomaterials are potentially cytotoxic. Thus, it is essential to assess biocompatibility before their usage in clinical applications. The purpose of this research was to evaluate the biocompatibility of nHA on Human Peripheral Blood Mononuclear Cells (HPBMCs). To evaluation of the biocompatibility of nano-sized, rod-like hydroxyapatite particles, HPBMCs were isolated and cultured on a 96 well plate. Cells were exposed to nHA at the following: 15.5, 31.25, 62.5, 125, 250, 500, 1000, 2000, 4000 and 8000 ppm after 2, 24, 48 and 72 h later for measuring the biocompatibility of material, MTT method was utilized. Measuring the photo, absorption was done by ELISA reader system at 570 nm which assigne the vitality of cell by the value of MTT absorption cells. None of the nHA experimented concentrations were toxic so, it seems that nHA biomaterial has acceptable compatiblity with HPBMCs.

Key words: Hydroxyapatite nano particles, human peripheral blood mononuclear cells, photo absorption, biocompatibility, bone tissue, inorganic component, Iran

INTRODUCTION

Hydroxyapatites (HAs) represent a family of bone grafting materials with a high degree of biocompatibility which is largely attributable to its presence in natural calcified tissue (Thein-Han *et al*., 2009; Puvvada *et al*., 2010; Nam *et al*., 2011; Shahoon *et al*., 2010). HA, $Ca_{10}(PO_4)_6(OH)_2$ is a calcium phosphate-based bioceramic material which makes up the majority of the inorganic components of human bones and teeth. The composition has a Ca/P mole ratio of 1.67 and is formed by precipitation of calcium nitrate and ammonium dihydrogen phosphate (Heinz and Kasaj, 2009).

When HA is implanted into a bony site, it has been shown that it is slowly resorbed, providing calcium and phosphate that is needed for the process of biomineralization and new bone formation (Tamai *et al*., 2002). 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 (Puvvada *et al*., 2010; Tamai *et al*., 2002). Therefore, the HA bone-grafting materials exhibited decreased osteoconductivity and poor degradation characteristics (Suchanek and Masahiro, 2004).

In pursuit of improving these shortcomings, a novel fully synthetic nanocrystalline Hydroxyapatite (nHA) has been introduced for augmentation procedures in osseous defects (Mateus *et al*., 2008; Thorwarth *et al*., 2005; Kim *et al*., 2011; Webster *et al*., 2000). The nHA particles has already been used for treatment of of human periodontal bony defects (Kasaj *et al*., 2008; Krejci *et al*., 1987) and various types of metaphyseal fractures such as the calcaneus and tibia in orthopedic surgery (Huber *et al*., 2006) as well as tooth perforations (Grigor'ian *et al*., 2000), jaw cysts (Gerlach and Niehues, 2007) and periimplantitis lesions (Schwarz *et al*., 2006; Sheikh *et al*., 2010). Also, nHA particles are currently being investigated to be used as delivery vehicles in various medical applications including the

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delivery of growth factors antibiotics (Ferraz *et al*., 2008), anticancer drugs (Matsumoto *et al*., 2004; Uchida *et al*., 1992). Thus, it is imperative to assess biocompatibility before their usage in clinical applications. nHA materials are considered to be biocompatible. However, some reports suggest that they can be toxic and may inhibit proliferation (Zhou *et al*., 2007). So because of these contradictory data evaluations, the present study was designed to evaluate the biocompatibility of rod-like nHA particles on the Human Peripheral Blood Mononuclear Cells (HPBMCs) by using the MTT assay.

MATERIALS AND METHODS

Preparation and sterilization of nHA: In this study, nano sized, rod-like hydroxyapatite particles (Fig. 1) provided from Nanoshel corporation (Batch No. 290090621) and were precisely sterilized by UV for 24 h.

Isolation and culture of HPBMCs: Heparinized venous blood was collected from a healthy volunteer. HPBMCs were isolated by density gradient centrifugation on histopaque (sigma) with 500 G and for 20 min. Cells from the interphase were harvested, washed and resuspended at 100,000 cells mL^{-1} RPMI-1640 (Gibco). 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 of HPBMCs to nHA: About 100,000 cells were exposed to nHA at the following concentrations; 15, 31, 62.5, 125, 250, 500, 1000, 2000, 4000 and 8000 ppm. For measuring the cytotoxicity of materials, MTT method was utilized after 2, 24, 48 and 72 h.

Cell viability assay: The viability of HPBMCs 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 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^{-1} 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 (Fig. 2). 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.

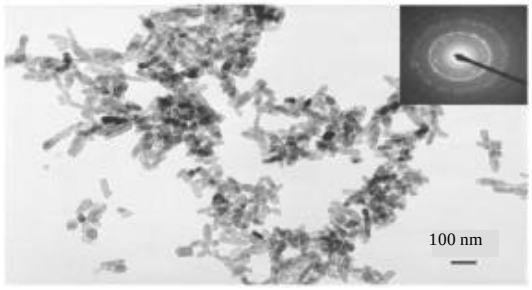

Fig. 1: Transmission electron micrograph of nHA particles

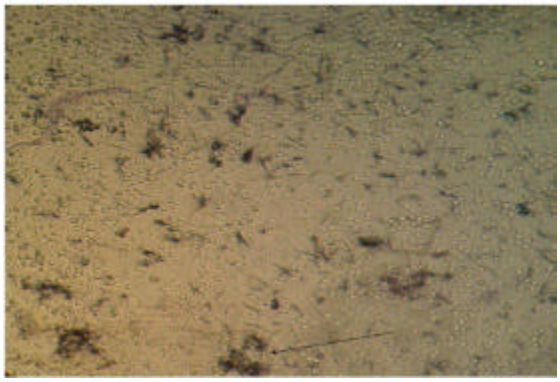

Fig. 2: Formation of formazan crystals (arrow head) adjusent to HPBMCs

Table 1: Mean and standard deviations of 10 different concentrations of nHA after 2, 24, 48 and 72 h

Concentrations of nHA	Time (h) 2	24	48	72
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±0.010	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	0.29±0.0015	0.236±0.006
2000	0.332±0.003	0.29±0.0060	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

Statistical analysis: In this study, we compared biocompatibility of 10 different concentrations of nHA particles and their cytotoxicity to HPMNCs after 2, 24, 48 and 72 h which was assessed by MTT assay. The absorption in each 3 densities was calculated, mean and Standard Deviation (SD) were registered (Table 1). Mean while, mean and SDs of concentrations was measured in each time space separatedly (2, 24, 48 and 72 h). Also each concentration was measured in all

mentianed time durations. The statistical ANOVA test was used in this study. The statistical analysis revealed that there was no significant difference among the groups ($p>0.05$).

RESULTS AND DISCUSSION

Results of this study showed that although, the mean of cell's vitality was decreased by increasing concentration and time elongation but ANOVA analyze indicated that there was non-significant difference between groups ($p>0.05$). Cytotoxicity percentage was measured for each concentration in all time durations (Fig. 3-7) nHA at 8000 ppm concentration after 72 h had the maximum percentage of cell's mortality (24.6%). Has represent a family of bone grafting materials with a high degree of biocompatibility. However, one of the major drawbacks was that HA-based b iomaterials required high-temperature and pressure processing which resulted in higher density and decreased porosity and all available data indicate that alloplastic grafts act primarily as biocompatible space fillers and support repair rather than regeneration. Therefore, the HA bone-grafting materials exhibited decreased osteoconductivity and poor degradation characteristics (Tamai *et al*., 2002; Suchanek and Masahiro, 2004). In pursuit of improving these shortcomings, a novel fully synthetic nanocrystalline Hydroxyapatite (nHA) has been introduced. A special feature of the nanostructured materials was the existence of an extremely high number of molecules on the surface in comparison with the bulk material. Because of this special feature, nHA materials received enormous national attention in medical and dental applications, especially for augmentation procedures in osseous defects (Ferraz *et al*., 2008). Thus, it is imperative to assess biocompatibility before their being used in clinical applications.

The present study was to evaluate the cytotoxicity of nHA particles (nearly rod-like, ranging size from 10-100 nm in diameter) on the Human Peripheral Blood Mononuclear Cells (HPBMCs) using the MTT assay. The results showed that the cell's viability was decreased at all tested concentrations (15.5-8000 ppm) after 2, 24, 48 and 72 h but the reduction extent was not statistically significant difference in all groups ($p>0.05$). Despite this, 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 ($p>0.05$). Zhao *et al*. (2009) studied the influence of HA nanocrystal morphology (rod-like

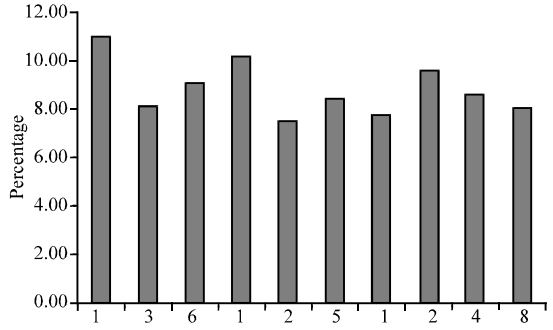

Fig. 3: Percentage of cell's mortality after 2 h

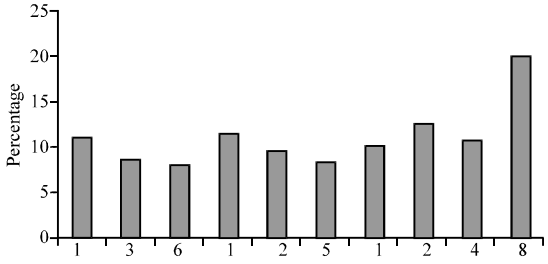

Fig. 4: Percentage of cell's mortality after 24 h

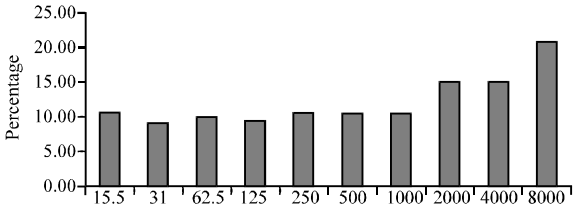

Fig. 5: Percentage of cell's mortality after 48 h

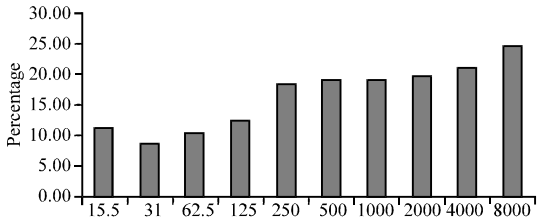

Fig. 6: Percentage of cell's mortality after 72 h

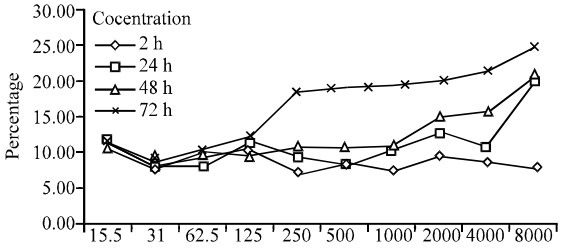

Fig. 7: Comparisions of percentage of cell's mortality after 2, 24, 48 and 72 h

and spherical crystals) at 10-100 ppm on osteoblasts proliferation after 24 h by MTT method and found that this materials exhibit good biocompatibility and would be safe to be used (Zhao *et al*., 2009). Also, Hsieh *et al*. (2009) 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 (Hsieh *et al*., 2009). The findings confirm the results of this study. In the present study, HPBMCs were used as samples and different concentrations of nHA were evaluated too.

Motskin *et al*. (2009) 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) (Scheel *et al*., 2009). In this study, we used the suspension of nHA and the cytotoxity was evaluated on HPBMCs. Scheel *et al*. (2009) evaluated the cytotoxicity of nHA particles at 50, 100, 500, 1000 and 5000 ppm concentrations on RAW 264.7 macrophages and cells were analyzed for viability (XTT-test) after 18 and 42 h. Their results showed that up to concentrations of 500 ppm cell viability was not considerably impaired by the test samples at both time-points (Scheel *et al*., 2009).

Albrecht *et al*. (2009) 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 (Albrecht *et al*., 2009).

The results of mentioned reports suggest nHA materials can be toxic and may inhibit proliferation (Motskin *et al*., 2009; Albrecht *et al*., 2009). 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 and peripheral blood mononuclear 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. 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. However, the results of a recent *in vitro* study demonstrated better compatibility of nHA at extra cellular forms incomparison with intracellular forms.

CONCLUSION

According to this study, it seems that nHA biomaterial is compatible with the human blood mononuclear cells and is known as a safe bone grafting sub-stitude. 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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