Limitation of biocompatibility of hydrated nanocrystalline hydroxyapatite

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Abstract. Nanostructured hydroxyapatite (HA) in the form of hydrated paste is considered to be a promising material for a minor-invasive surgical curing of bone tissue injury. However questions about adhesion of cells on this material and its biocompatibility still remain. In this study biocompatibility of paste-formed nanosized HA (nano-HA) by in vitro methods is investigated. Nano-HA (particles sized about 20 nm) was synthesized under conditions of mechano-acoustic activation of an aqueous reaction mixture of ammonium hydrophosphate and calcium nitrate. It was ascertained that nanocrystalline paste was not cytotoxic although limitation of adhesion, spreading and growth of the cells on its surface was revealed. The results obtained point on the need of modification of hydrated nano-HA in the aims of increasing its biocompatibility and osteoplastic potential.

1. Introduction

Artificial constructions made of composite materials combining organic and mineral phase – hydroxyapatite evoke a high interest in creation of biomaterials out of them. These materials could provide regeneration of bone tissue in the area of its damage. A plenty of results showed high biocompatibility potential of the composites made from HA and organic matrix for instance collagen. Stimulation of osteogenesis was also demonstrated as the material played role of matrix for a newly forming bone [1-4].

However, in the creation of artificial biocomposites there remain unsolved problems connected with controlling of forming of material structure and its composition which are able to provide efficient regeneration of bone tissue. Excessive ratio of HA in the material can inhibit the process of osteogenesis [5-7]. Significant influence can be caused by level of dispersion of composite’s mineral component [8, 9].
Several investigations point that synthetic HA which particles have high dispersion level looks more like a mineral constituent of bone tissue and is more likely to activate osteoblastic differentiation of mesenchymal cells than HA with bigger particles [10]. Paste-formed nano-HA is of practical interest as a material for less-invasive surgical intervention in the treating of bone defects but from time to time it appears that surgeons have contrasting views on the possibilities of this material [11]. Some of them think consider it to be a beneficial material but others point on its insufficient biocompatibility lack of its resorption and regeneration of bone tissue in the site of implantation.

Objective reasons of this situation remain unclear. In the current study we attempted to estimate whether these reasons could be adhesive properties and biocompatibility of nano-HA in relation to cells of mesenchymal origin.

2. Materials and methods

2.1 Synthesis of nano-HA

The process of receiving HA was held in the rotary pulsation apparatus (RPA) (Scientific production enterprise ‘Aviatekhnika’, LLC) [12] using a well-known reaction [13]

$$6(NH_4)_2HPO_4 + 10Ca(NO_3)_2 \times 4H_2O + 8NH_4OH \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 46H_2O$$

For the ion exchange reaction and receiving HA, two solutions were used: (1) calcium nitrate tetrahydrate (450.6 g were dissolved in 5 l of distilled water and the pH was adjusted to pH 10.1 by adding a concentrated ammonia solution) and (2) diammonium phosphate (151 g were dissolved in 2 l of distilled water and the pH adjusted to 10 by adding a concentrated ammonia solution). Solution 1 in cooling conditions was placed in the loading chamber of the RPA, the mechano-acoustic activation mode (3000 min⁻¹) was switched on and then solution 2 was added into the chamber at a rate of 0.5 l/min. The mixture was treated in this regime for 2 minutes. Purification of HA in the resulting pulp from ammonium nitrate was carried out by washing with distilled water after centrifugation (2500 g, 15 min, 4 cycles). The content of mineralized calcium in the paste-formed material after the final washing was 100 mg/g of paste weight.

2.2. Analysis of nano-HA

The particle size of the dispersed phase was evaluated by dynamic light scattering using device Zetasizer Nano «Malvern».

IR spectra were recorded in the reflection mode on a Nicolet 380 spectrometer, ZnSe glass.

The ζ-potential of particles dispersed in an aqueous medium was measured by Zetasizer Nano device.

Elemental analysis was carried out on a CNE analyzer Eager 300.

Calorimetric curves were obtained on a Netzsch instrument, model DSC-204 F1.

X-ray analysis was performed on an Emma diffractometer, GBC Scientific Equipment Pty Ltd.

2.3. Cell culture

Mouse embryonic fibroblasts NIH/3T3 were obtained from the Russian Cell Culture Collection (Institute of Cytology RAS, St. Petersburg). The cells were cultured in a DMEM/F12 culture medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 80 μg/ml gentamicin sulphate (Sigma-Aldrich, USA) at 37°C under conditions 5% CO₂ in the air.

2.4. Investigation of biocompatibility of hydrated HA in vitro

To investigate the biocompatibility of nanocrystalline paste-formed hydroxyapatite in vitro, a 0.5-mm HA paste layer was placed on one half of the cover glass and the other half was uncovered. The glass was placed in a 35 mm diameter Petri dish, and a cell suspension (1×10^4 cells/cm²) was added. After 24 hours and 72 hours after seeding a cytotoxic effect, mitotic activity and cell microphotography were performed. As a control sample commercial ReproBone novo paste (Ceramisys, England) was used.
2.5 Cytotoxicity analysis. For the cytotoxicity analysis the number of live and dead cells was determined by fluorescence microscopy. Cells were stained for 20 minutes at 37°C in a culture medium with adding of 1 μg/ml calcein AM and 2 μg/ml propidium iodide (Sigma-Aldrich, USA). Using a confocal fluorescence microscope TCS SP5 (Leica, Germany) at least 500 cells were analyzed.

2.6. Analysis of mitotic activity. The analysis of mitotic activity was performed by fluorescence microscopy using a vital staining with fluorescent nuclear dye Hoechst 33342 (Sigma-Aldrich, USA) at a concentration of 1 μg/ml and 2 μg/ml of propidium iodide. Mitotic cells were detected by the chromatin distribution characteristic of prophase, metaphase, anaphase, and telophase using a fluorescent microscope DM 6000 (Leica, Germany). At least 500 cells were analysed. The mitotic index (MI) was calculated from the formula MI = (P+M+A+T)/N * 100% where (P+M+A+T) is the sum of cells in the prophase, metaphase, anaphase and telophase, respectively, and N is the total number of cells analyzed.

2.7. Statistical analysis
The results of the experiments were presented as the mean ± standard error (M±SEM). The experiments were performed in at least three replicates (n≥3). The statistical significance of the difference was determined using the Mann-Whitney test.

3. Results and discussion
Mechano-acoustic activation of the exchange reaction between diammonium phosphate and calcium nitrate allows the formation of nanosized particles of HA. Reduction of the feed rate of the solution of diammonium phosphate to the reactor and increase in the mixing speed of the reactants lead to increase in dispersion. A HA dispersion characterized by particles having an average diameter of 20 nm was obtained at a rotation RPA rotor speed of 3000 min⁻¹ and the rate of introduction of 0.5 l/min solution of diammonium phosphate into the reactor. The mass concentration of such particles is ≈90% in the reaction medium (Figure 1).

An important stage of the HA synthesis is its purification from the ammonium nitrate. Centrifugation can significantly increase the speed of the cleaning process in comparison with decantation. Thus filtering the pulp, washing the retentate once with distilled water, drying it at 120°C for 3 hours leads to a sample of technical HA contaminated with ammonium nitrate (absorption bands

![Size Distribution by Volume](image)

**Figure 1.** The distribution of particle sizes of HA obtained under mechano-acoustic treatment conditions. RPA rotor rotation speed is 3000 min⁻¹. The rate of introduction of a solution of diammonium phosphate into the reactor is 0.5 l/min.
1330, 820 cm$^{-1}$, Figure 2b). Four-time washing with centrifugation and subsequent drying (120°C for 3 hours) successfully removes ammonium nitrate as well as heat treatment of HA (350°C for 30 min). After these treatments these absorption bands are not detected in the samples (Figure 2a).

The elemental analysis of purified HA confirms the complete absence of nitrogen-containing impurities in four-time washed with centrifugation as well as in heat-treated HA (reference/ammonium nitrate).

Additional evidence of the absence of ammonium nitrate in purified samples of HA was the results of calorimetric analysis (Figure 3). Calorimetric curves after 4 times washing and after heat treatment (300°C) coincide.

The $\zeta$-potential of the particles of the non-washed hydroxyapatite dispersed in distilled water was +30 mV (Figure 4a) but it was reduced to about +5 mV (Figure 4b) after 4 times washing.

Figure 5 shows the diffractogram of a heat-treated purified HA sample. The crystalline phase of HA has been identified, which has an elementary hexagonal cell with dimensions (Å): $a=9.4257$, $c=6.8853$, space group P63/m. The experimental characteristics of the X-ray spectrum of HA are given in Table 1. The results indicate the presence of phases of not only crystalline but also amorphous HA.

![Figure 2. IR spectra: a – HA after heat treatment at 300 °C, b – technical HA, c – ammonium nitrate](image-url)
Figure 3. Calorimetric curves: a – HA heated at 300 °C, b – technical HA after a single wash, c – ammonium nitrate

Figure 4. ζ-potential of dispersed HA, crude (a) and purified (b) from impurities
Figure 5. Diffractogram of purified and heat-treated HA (a). For comparison, the diffractogram of HA presented in [14] is shown (b).

| № reflection | 2θ gon | I₀ | d/n, Å |
|--------------|--------|----|--------|
| 1            | 25.8541| 60 | 3.443  |
| 2            | 31.73  | 96 | 2.818  |
| 3            | 31.8108| 100| 2.811  |
| 4            | 31.88  | 91 | 2.805  |
| 5            | 34.20  | 21 | 2.62   |
| 6            | 39.4575| 9  | 2.282  |
| 7            | 46.64  | 27 | 1.946  |
| 8            | 49.42  | 32 | 1.843  |
| 9            | 51.82  | 18 | 1.763  |

Table 1. The experimental values of the angles (2θ), the relative intensities (I₀) and the interplanar distances (d/n) for the diffractogram of the purified and heat-treated HA.

A study of the cytotoxicity of HA paste was carried out 24 and 72 hours after cell seeding. It was established that the HA paste and the ReproBone novo paste didn’t have a cytotoxic effect. Figure 6 shows the results of studying cytotoxicity 72 hours after cell seeding. The number of dead cells on these samples of HA didn’t exceed 5% and didn’t differ from the number of dead cells when cultivated on culture dish surface.
The mitotic activity of cells cultivated on glasses coated with HA paste was studied 72 hours after the start of the cultivation. The data presented in Figure 7 show that the cells on the surface of the HA paste and the ReproBone novo paste are incapable of dividing. The mitotic index after 72 hours of cultivation was 4.1±0.8% when the cells were cultured on culture plastic.

Taking into account the obtained data on the absence of the cytotoxic effect of the test paste and the suppression of the mitotic activity of the cells we assumed a violation of adhesion and cell spreading on their surface. We carried out a morphological analysis of the adhesion and cell spreading on the surface of nanocrystalline HA and ReproBone novo pastes to verify this assumption.
It has been established that the cells attach to the paste surface however the spreading of cells on the pastes is limited in comparison with cells on the culture plastic. Only 35±5% of the cells were spread on the HA while on the culture plastic 90±6% of the cells were spread. In Figure 8 photomicrographs are presented illustrating this effect. On micrographs the cells spread on the HA had a spindle-shaped elongated shape and were more "compressed" than on the plastic (Figure 8A). On HA and ReproBone novo pastes were observed many attached but not spreaded cells having a rounded shape (indicated with rows on Figure 8A and B). On culture plastic almost all cells were spreaded and had a spindle-shaped or elongated triangular shape (Figure 8C).

Figure 8. Microphotographs of NIH/3T3 cells after 72 hours of cultivation on a layer of nanocrystalline HA paste (A), ReproBone novo (B) paste and culture plastic (C). Fluorescence microscopy, coloration of acridine orange. The arrows indicate the cells attached but not spread on the paste. Magnification: objective x20, ocular x10

4. Conclusion
The obtained results make it possible to suggest limiting biocompatibility of hydrated nanocrystalline hydroxyapatite and disordering of cell adhesion to this material. Cell adhesion disorder to nanocrystalline HA will inevitably be a limiting factor for the migration of cells into it and as a consequence will limit its osteoconductive properties. A modification of the hydrated HA paste with osteoconductive components which ensures the adhesion of cells and their migration into the material is necessary to overcome this difficulty. Another possible reason for limiting cell adhesion to hydrated nanocrystalline HA can be the sorption of the components of the intercellular fluid (the culture medium in our studies) and as a result the ionic, amino acid and other compositions in the material zone is disrupted. In order to determine which of these reasons can be decisive and how to overcome them additional studies of cell response to hydrated nanocrystalline HA modified by osteoconductive and osteoinductive activities in accordance with the proposed operational hypotheses are needed to be carried out in vitro and in vivo. This work is actual since the creation of hydrated HA paste which has
a high osteoplastic potential and possesses osteoconductive properties is urgently needed for minimally invasive bone tissue repair surgery.

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