Cytotoxic Effects of Granulated Hydroxyapatite with Various Particle Size Distribution

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Abstract. Hydroxyapatite-based materials show promise in such applications as reconstructive surgery, stomatology and cosmetology, they can also be used as components of toothpaste, disinfectant products and dietary supplements (DS). However, cytotoxicity of hydroxyapatite powders synthesized via various methods towards a variety of biological cells requires a thorough study.

Cytotoxicity of hydroxyapatite powder samples with spherical particles in three size ranges: 5-25 μm, 25-45 μm and 40-125 μm, synthesized by hydrothermal synthesis with further ultrasonic and spray-drying treatment, was studied on human blood cells and on gram-negative bacteria E.coli. The examined hydroxyapatite samples have no toxic effect on human blood leukocytes (in concentration 40 mg per 1 ml H₂O or normal saline) and on gram-negative bacteria E.coli. (in concentration 40 mg per 1 ml H₂O or normal saline) regardless of the contact time and particle size. The obtained data demonstrate safety of the examined materials and absence of toxic effects towards the test objects. This allows us to regard the studied hydroxyapatite samples as biocompatible.

The present study results could be used to develop products and medicinal drugs based on hydroxyapatite granules synthesized by means of hydrothermal synthesis technique with further sonication and spray drying.

1. Introduction

Nanostructured apatite-based coatings are widely used on metal implants to improve their biocompatibility, advance osteointegration, promote bone fixation and accelerate bone growth. Development of new biocompatible and nontoxic materials with possibility of achieving direct osteointegration is an important task. Hydroxyapatite (Ca₁₀(PO₄)₆(OH)₂) is a promising material for performing the presented task. The hydroxyapatite-based could be successfully employed in reconstructive surgery (diseases and injuries of the human musculoskeletal system, maxillofacial surgery, filling damaged and diseased bones), stomatology, as components of toothpaste, cosmetological and disinfectant products and as dietary supplements [1-3] due to their high biocompatibility, wear resistance and non-toxicity. Hydroxyapatite-based materials are readily available, inexpensive and easy-to-use. Hydroxyapatite, as one of the major inorganic components of human bones and teeth, is widely studied as a filler and implant coating material [2].

In addition, hydroxyapatites have also garnered attention in soft tissue regeneration due to their excellent biocompatibility with soft tissues such as skin, muscle, and other. Numerous studies have
shown that hydroxyapatite can activate fibroblasts and accumulate vessel endothelial cells and thereby support the healing of skin wounds [4].

Hydroxyapatite bioceramics have sealing contact and strong adhesion with skin tissue to prevent exit-site and tunnel bacterial infection, which suggests that hydroxyapatite-based materials might be utilized as percutaneous devices [5]. Composite products with a hydroxyapatite component have been successfully developed for soft-tissue augmentation [6], and a study has demonstrated that hydroxyapatite nanoparticles could stimulate the axonal outgrowth, suggesting that hydroxyapatite might provide a new approach for therapy or prevention of nerve injury [7].

Biocompatibility examination is the most important step in developing new biomaterials. Two major aspects have to be taken into consideration while performing biocompatibility tests on novel medicinal materials, i.e. the material can produce toxic effects on the cells in immediate contact and on the body as a whole; and the material itself can undergo degradation or lose its functionality under the influence of the internal environment.

The cytotoxicity test allows evaluation of the toxic potential of the material at the cellular level, i.e. in vitro. The method provides quantitative and comparable results, therefore, it allows one to quickly evaluate and select toxic materials before conducting animal studies. Either cell cultures or unicellular microorganisms (bacteria, microalgae) are usually used as test objects in assessing cytotoxicity. Initially the test objects’ survival following treatment with the material is assessed, and in the event of any negative effects the possible toxicity mechanisms are established.

This paper shows the results of cytotoxicity evaluation performed for spherical hydroxyapatite samples obtained via hydrothermal synthesis method with ultrasonic treatment and spray drying, human blood cells and gram-negative bacteria E.coli are used as test objects.

2. Materials and methods
The hydroxyapatite samples were synthesized by the hydrothermal synthesis method. Calcium nitrate tetrahydrate (Ca(NO$_3$)$_2$, AR Grade CAS 13477-34-4), diammonium hydrogen phosphate (NH$_4$)$_2$HPO$_4$, AR Grade) ammonium hydroxide (NH$_4$OH, AR Grade, CAS 7664-41-7) produced by Reachem Co (Russia) were used as precursors in reaction (1).

$$10\text{Ca(NO}_3\text{)}_2 + 6(\text{NH}_4\text{)}_2\text{HPO}_4 + 8\text{NH}_4\text{OH} \rightarrow \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 + 20\text{NH}_4\text{NO}_3$$ (1)

The resulting suspension was mixed by a mechanical mixer Heidolph RZR 2051. The Ca/P ratio was calculated as 1.67. The suspensions were sprayed with the spray dryer equipment Buchi Mini Spray Dryer B-290 with the ultrasonic atomizer Buchi B-290 Ultrasonic Package. As a result we obtained granulated hydroxyapatite samples with different particle size distributions (5 – 25 μm, 25 – 45 μm and 40 – 125 μm), depending on the spray drying process parameters.

This approach provides one with a possibility to control solubility of materials used for implant manufacturing from rapidly soluble to insoluble (calcium tetraphosphate > α-tricalcium phosphate dehydrate > anhydrous dicalcium phosphate > octocalcium phosphate tricalcium phosphate > hydroxyapatite) via controlling the ratio between hydroxyapatite and calcium phosphate substances.

The cytotoxicity analysis was performed on human blood cells (mononuclear leukocytes) and gram-negative bacteria E.coli.

The intact donor blood (n = 6) stabilized with 25 unit/ml of heparin was suspended with sterile normal saline (isotonic sodium chloride solution, 0.9 % solution of NaCl, PanEco, Russian Federation), in a 1:2 ratio. The mononuclear leukocytes (ML) were separated with the one-step ficoll gradient (PanEco, Russian Federation, density 1.077g/cm$^3$) with centrifugation at 400 g for 30 minutes. The diluted blood was carefully layered on a tube wall with a pipette above 5 cm$^3$ of ficoll – verographin poured into centrifuge tubes (V = 50 ml).

The mononuclear leukocytes forming the interphase ring were collected with a pipette and triply washed with Roswell Park Memorial Institute medium (RPMI - 1640). The nutrient medium was prepared on the RPMI-1640 basis according to the following procedure: 10 % of fetal calf serum, 2 mM of glutamine and 5000 ME/ml of streptomycin and penicillin at 5000 ME/ml were added to
RPMI-1640 under sterile conditions. After the final rinsing from ficoll the samples were resuspended in the nutrient-rich medium.

The cell viability in the suspensions was analyzed by the trypan blue dye exclusion method using optical microscopy. The cell concentration was calculated in the Goryaev chamber. The live mononuclear leukocytes concentration in the biological medium corresponded to 3.2 – 3.4 • 10^6 cells per 1 ml of the medium.

The resulting suspension was added, to the wells of a standard plate, 1 ml per well, containing 40 mg of hydroxyapatite, gently stirred and incubated for 30 min at 37 °C in a reciprocating shaker (ELMI ST–3L, RF) at 350 rpm. The control leukocytes suspension was incubated under the similar conditions, with no hydroxyapatite added.

The cytotoxicity analysis was carried out using the colorimetric assay for assessing cell metabolic activity (MTT colorimetric assay). After incubation 100 μl of 5 % MTT solution in normal saline was added to each well with further incubation for 4 h in a CO₂ incubator at 37 °C and CO 5 %.

The volume of 800 μl of the suspension was moved from each well to 2 ml centrifugal microtest-tubes (Eppendorf) and centrifuged for 10 h at 1800 rpm/min in an Eppendorf S 415 R centrifuge. The supernatant fluid was carefully removed with a pipet leaving the cell precipitate in the tubes.

Dimethyl sulfoxide (DMSO) was added to every test tube (500 μl/tube). The samples were incubated in a rotary shaker at room temperature for 30 minutes at 350 rpm/min. When the formazan was diluted, the dyed DMSO solution was pipetted into the wells of a 96-well plate (100μl/well). The effect of hydroxyapatite on the cells from each donor was determined via characterization of the cell lysate in parallel wells. The intensity of purple staining was determined by characterization of the optical density of formazan solution in DMSO using a Multiskan MS spectrophotometer (Labsystem, Finland) at 540 nm wavelength.

The index cytotoxicity (IC) of the hydroxyapatite samples was calculated using the equation (2)

\[
\text{IC}, \% = 100 - \left( \frac{OAs}{OAc} \cdot 100 \right)
\]

where OAs – optical absorption of the obtained hydroxyapatite sample, OAc – optical absorption of the intact control.

The cytotoxicity analysis on bacterial cells was performed according to the bioluminescence method [8-10] on *Escherichia coli* with the cloned luxCDAEB genes of *Photobacterium leiognathi*. The toxic effect produced by the studied nanomaterial on the bacteria was determined by inhibition of their bioluminescence. The measurements were performed on the specialized luminometer Biotox-10 (Russian Federation). The first stage of the test was performed immediately after the sample preparation and the second stage was performed after a 24-hour ageing period.

The lyophilized bacterial culture was revived in distilled water and in normal saline (pH = 7.0 – 7.4). The hydroxyapatite suspensions were also prepared by dispersion in distilled water and in normal saline (pH = 7.1 ± 0.2). The quantity of 400 mg from each hydroxyapatite sample was accurately weighed on an analytical balance ViBRA HT (Shinko Denshi, Japan) with the accuracy ± 0.0001 g, poured into a 10 ml glass tube with the liquid medium and mixed by intensive shaking with further ultrasonic treatment.

The study was performed at different exposition times: 5 min, 30 min, 60 min and 120 min.

The descriptive primary analysis data are presented in the format sample mean (χ) ± standard deviation value (SD). Statistical analysis was carried out using Statistica 6.0, StatSoft software with the “Basic statistic/Tables” and “Non-parametric data” modules.

3. Results and discussion
The cytotoxicity index of the hydroxyapatite samples was calculated for each donor, based on the optical absorption values obtained after measuring formazan in DMSO solution extinction both for the test samples and control samples. The description analysis of the obtained primary data generalising the cytotoxicity index values of the tested and control samples was performed employing the statistical analysis software 6.0 with module “Basic statistic/Tables” (figure 1).
Figure 1. IC value distribution in the control before and after co-incubation with the hydroxyapatite samples in mononuclear leukocytes suspensions.

The presented data show that the leukocytes death intensity induced by the hydroxyapatite samples never exceeded 9% for all the tested donors. Moreover, the statistical comparison analysis of the IC indicators in the control group, where the leucocytes were subjected only to constant agitation in a shaker, and in the experimental group, where the leukocytes were in contact with the hydroxyapatite particles in addition to physical agitation revealed no significant differences.

The hydroxyapatite cytotoxicity analysis in an aqueous solution in relation to \textit{E.coli} bacteria was carried out after 5, 30, 60 and 120 min exposition. The observed results are presented in figure 2.

Figure 2. Test-object luminescence intensity under the influence of a hydroxyapatite sample in a water medium with different exposition times.
We observed an increase in bio-luminescence both in the control and experimental samples when water medium was replaced with normal saline. Also, a decrease in the standard deviation value was registered which can be an indication of higher stability of the bacterial suspension (figure 3). The primary results descriptive analysis revealed no significant differences between the luminescence intensity of the test object and the control group, regardless of the exposition time (figure 3).

![Figure 3](image-url)

*Figure 3. Luminescence intensity of the test subject treated with hydroxyapatite in sodium solution at different exposition times.*

The data show that the hydroxyapatite samples produced no toxic effect on *E. coli* both in water and in normal saline, regardless of the period of exposition of the cells to the material and of the particle size distribution. However, toxic effect of hydroxyapatite in the same concentration on different test subjects was observed by a group of authors. In [11] genotoxic and cytotoxic effects as well asoxidative damage from increased concentrations of hydroxyapatite (5, 10, 20, 50, 75, 100, 150, 300, 500 and 1000 ppm) were studied in primary human blood cell cultures. In paper [12] toxic effect of nanosized hydroxyapatite at various concentrations (0 – 1000 μg cm\(^{-2}\)) on a primary rat hepatocyte culture was evaluated. The author of [13] studied antimicrobial activity of hydroxyapatite nanoparticles against bacterial pathogens associated with implants. The mechanisms of nanoparticle effect on bacteria were studied by evaluating the generation of reactive oxygen species (ROS), DNA fragmentation, loss of lactate dehydrogenase (LDH), and cell interaction. Also, the cytotoxicity of hydroxyapatite particles was determined with the MTT test and fluorescence microscopic analysis. The difference in toxicity noted in these studies and in our work can be explained by the fact that we studied microgranular hydroxyapatite characterised by a lower cytotoxicity level than nanodispersed material examined by other research teams.
4. Conclusion

Thus, we have established that the studied hydroxyapatite samples with particle sizes of 5 – 25 μm, 25 – 45 μm and 40 – 125 μm have no toxic effect on human blood leukocytes (at a concentration of 40 mg per 1 ml of cell suspension), and on bacteria E. coli (at a concentration of 40 mg per 1 ml of water or normal saline), regardless of the time of contact of the cells with the material and the particle size distribution. The obtained data demonstrate safety of the examined materials and absence of toxic effects towards the test objects. This allows us to regard the studied hydroxyapatite samples as biocompatible.

Acknowledgments

The work was funded by the Ministry of Science and Higher Education (formerly, the Ministry of Education and Science) of the Russian Federation under Project No. RFMEFI57517X0168.

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