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Amikacin-modified hybrid biomaterial biological properties

Właściwości biologiczne hybrydowego biomateriału modyfikowanego amikacyną

INTRODUCTION

Bone defects represent major clinical problems in the practice of reconstructive orthopaedic and craniofacial surgery. Treatment for these applications, such as autogenous or allogenous bone grafting have some limitations, so new approaches for bone tissue repair are required. Calcium and phosphate ceramics are being increasingly used as bone substitutes in orthopaedic, oral and maxillo-facial surgery. Such ceramics are biocompatible and lead to local osteogenesis in anosseous site. However, in cases involving large bone defects, there is still a need to reduce the time necessary to establish the ceramic-bone interface and bony ingrowth [1,7]. Recently, many biological substances such as bone morphogenetic proteins (BMPs) [2], transforming growth factor-*beta* (∃-TGF) [3] or keratin [10] have been investigated for their bone-inducing properties. Some antimicrobial substances combined with hydroxyapatite (HAp) influence on human bone were also studied [9]. However, this idea of connection HAp with various biological substances needs to answer such questions as the dose, the suitable carrier acting as a slow release delivery system, the mode of impregnation and the activity *in situ* of these substances. Lack of control of one of these parameters might lead to cytotoxicity, osteosarcoma formation or ectopic bone formation [1].

Experimental verification of amikacin-modified hybrid biomaterial influence on osteoblast cell culture was the main aim of this study.

MATERIALS AND METHODS

Biomaterials

Hydroxyapatite (HAp) was made in Department of Technology of Ceramics and Refractories, AGH-University of Science and Technology, Cracow, Poland. Hydroxyapatite parameters were: diameter: 0.3-0.5 mm, open porosity: 67%, sintering temperature: 800ºC.

Immobilization process

Portion of HAp was covered by γ-aminopropyltriethoxysilane and divide into three parts. Two parts of silanized-HAp were chemically modified by two kinds of protein (porcine gelatin or keratin derived from human hair) according to Weetall [11] procedure in authors own modification. So, three types of matrix were obtained (silanized-HAp, gelatin-HAp and keratin-HAp). Amikacin (Biodacyna®, Bioton, Poland; 250 mg/ml) was immobilized according to the Polish Patent [4] and its concentration was estimated spectrophotometrically according Ginalska et al. method [5].

Cell culture

Human fetal osteoblasts cell line hFOB 1.19 from ATTC (American Type Culture Collection) was used in our *in vitro* research. Cell cultures were incubated in 34°C in a humidified atmosphere consists of 95% air and 5% CO_2. Day before the beginning of experiment HAp granules were stabilized in culture medium. Next HAp granules were inoculated with hFOB cells (5×10^4) suspended in fresh culture medium. Osteoblasts growth and division in presence of HAp granules were observed during this experiment. Cell growth was estimated after 72, 120, 168, 216, 264, 312, 360 and 408 hours. The growth medium was replaced every two days. Cell viability was determined by 0.4% Trypan Blue exclusion.

LDH activity test

Substances, which have toxic influence on cells can caused their membrane damage. It leads to some enzymes release. Lactic dehydrogenase (LDH) is one of them. LDH concentration in medium samples was assayed to define whereas HAp granules indicate cytotoxity against human osteoblasts. The more toxic biomaterial is, the higher level of LDH in medium samples is detected. As a control we used osteoblasts growing in culture medium directly on polystyrene plates. LDH activity was measured after 24h of hFOB growth on HAp granules using LDH Cytotoxicity Detection Kit (Roche Diagnostic, Switzerland).

Confocal microscopy

Firstly, hFOB cells were cultured on HAp granules in LabTek chambers (Nunc, Denmark) during 360 hours. Next granules covered by hFOB cells were washed twice in PBS and then stained with 3,3'-dihexyloxacarbo-cyanine iodine ($DIO_{3(6)}$) for 10 minutes in dark. Samples were analyzed using confocal microscope (LSM-5, Zeiss, Germany) at 514 nm.

RESULTS

Cell viability

New hybrid biomaterial influence on growth and division of human osteoblasts was tested. Data presented on FIGURE 1 showed that during the experiment, cell number increased with time reaching the highest value at 312 h. HAp granules did not inhibit osteoblasts growth. Cell viability exceeded 95% what indicates that modified carriers were non toxic to osteoblasts. These results proved that our biomaterials modified by amikacin could be used as implants in orthopaedic, oral and maxillo-facial surgery in the future.

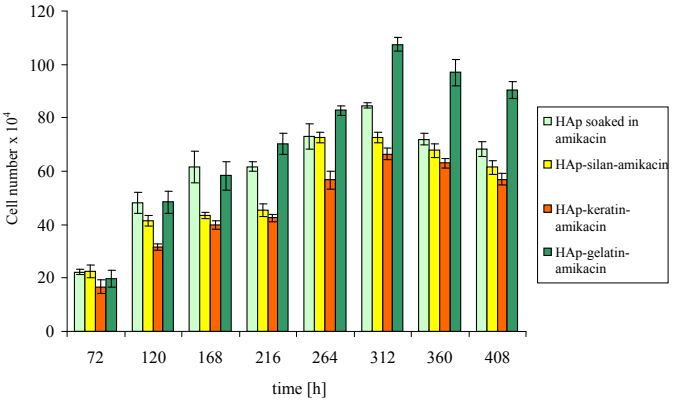

Fig. 1. Osteoblasts' growth on HAp granules depending on cell culture duration

LDH activity test

Assessment of HAp granules modified by amikacin potential cytotoxicity was the main purpose of experiment. To reach this aim LDH activity was measured in extracellular medium. Osteoblasts cell culture was a control in our study. Results presented in FIGURE 2 showed that none of HAp granules revealed cytotoxicity features. LDH activity was similar to control LDH activity (osteoblasts cell culture without any HAp granules type presence) for all types HAp granules. It was also observed that HAp granules with amikacin influenced on LDH activity reduction and gave evidence that smaller number of osteoblasts was damaged.

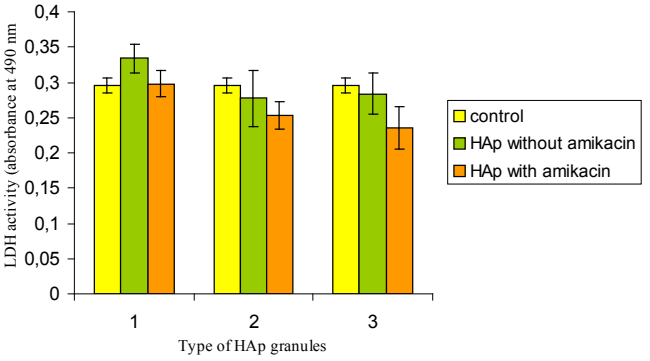

Fig. 2. Various HAp types influence on LDH activity.

Confocal microscopy

New hybrid biomaterial HAp-amikacin microphotographs were made using confocal microscope to visualize osteoblasts cells presence on amikacin modified HAp-granules. FIGURE 3 presented

HAp granules surface completely covered by osteoblasts what confirmed our previous experiments and proved that examined biomaterial did not show toxic effect towards human osteoblasts.

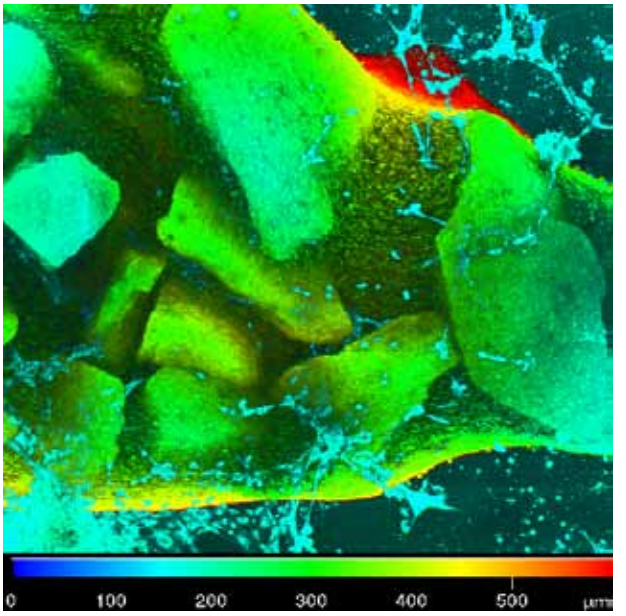

Fig 3. Micrograph (confocal microscopy) showing HAp-keratin-amikacin granules covered with hFOB cells (photograph with DepthCod option).
Magnification x 50

DISSCUSION

Hydroxyapatite bioceramics shows neither cytotoxicity nor carcinogenic effects after implantation because its chemical and mineralogical composition is similar to bones and teeth building substance. Bioceramics has also two main features: great biocompatibility towards soft and bone tissue and bioactivity responsible for direct connection with bone. Its porous structure gives possibility to use HAp as a various biological and chemical substances local delivery system. Such systems have to be investigated by scientists in order to check their potential cytotoxicity towards tissues.

Krisanapiboon et al. [6] investigated the biocompatibility of hydroxyapatite composite impregnated with gentamicin, fosfomycin, imipenem and amphotericin B. Extracts from all drugs showed good biocompatibility and no osteoblasts morphological changes were observed in all drug tests at any concentrations.

The purpose of study made by Rauschmann et al. [8] was to assess sulphate nanoparticulate HA composite material properties and to analyze its *in vitro* uptake and release of vancomycin and gentamicin. Furthermore, *in vitro* cytotoxic properties were also investigated – direct growth and adhesion of human osteoblasts onto the surface. This calcium sulphate nanoparticulate HA composite

material did not show any in vitro cytotoxicity and exhibited good biocompatibility compared. Loading with antibiotics was performed after hardening and sterilization of the pellets to prevent inactivation of atibiotics by these procedures. Loading is not limited to one specific antibiotic (e.g. tobramycin or gentamicin) but can be done according to antibiograms offering individual treatment options. The high porosity of this composite material revealed initial high antibiotic release with subsequent decline ensuring concentrations above MICs. Our studies confirmed these experiments. HAp granules did not affect osteoblast proliferation or cell morphology.

To increased cells adhesion to biomaterial some researchers used keratin which showes advantageous influence on osteoblasts growth and proliferation. Keratins are fibrillar proteins building hair, wool and nails. Cystein residues presence in keratin gives possibility to create disulfide bonds. It is important feature, because keratin modification leads to bioactive substances attachement to cystein residues of keratin. Moreover keratins possess sequences which facilitate cells adhesion: RGD (Arg-Gly-Asn) and LVD (Leu-Wal-Asn). Tachinaba et al. [10] investigated influence keratin-HAp sponge on osteoblast's proliferation. It was showed that preosteoblasts MC3T3–E1 grew correctly both on keratin-HAp sponge and control sponge (without keratin) but proliferation process began earlier on keratin sponge. This experiment confirmed profitable influence of keratin on osteoblasts growth and proliferation. In our research keratin also did not influenced negatively on cell growth and proliferation process.

CONCLUSION

Antibiotic-modified hybrid biomaterial do not show any cytotoxicity features towards hFOB in our *in vitro* experiments. Therefore, this biomaterial could function as scaffolds for bone regeneration and eradicate infection at the same time.

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SUMMARY

Hydroxyapatite is used in bone reconstruction because of its similar chemical structure compared to the inorganic composition of human bone. Bone is a complex material composed of proteins, mainly collagen, with hydroxyapatite. Therefore, now many investigations are focused on the hybrid biomaterials of hydroxyapatite with proteins and other synthetic polymers. We investigated HAp granules covered by two types of proteins (keratin or gelatin) in our study. This biomaterial was also modified by amikacin. In our previous paper [12] we proved that such hybrid biomaterial had antimicrobial properties. This paper gives evidence that HAp-modified granules did not influence on growth and proliferation of osteoblasts. So this new biomaterial could be used as an implant on orthopaedic, oral and maxillo-facial surgery in the future.

Keywords: hydroxyapatite, amikacin, antibiotic immobilization, implant infection, cytotoxicity

STRESZCZENIE

Hydroksyapatyt jest używany w rekonstrukcji kości z powodu jego struktury chemicznej podobnej do nieorganicznych składników kości ludzkich. Kość jest kompleksem złożonym z białek (głównie kolagenu) i hydroksyapatytu. Dlatego też obecnie wielu badaczy jest skupionych na tworzeniu materiałów hybrydowych. W naszych badaniach testowano granule HAp pokryte dwoma rodzajami białka (keratyną lub żelatyną). Biomateriał ten był też modyfikowany amikacyną. W poprzedniej publikacji [12] wykazano, że ten typ biomateriału hybrydowego posiada właściwości antybakteryjne. W obecnych badaniach wykazano, że modyfikowane granule HAp nie wpływają negatywnie na wzrost i różnicowanie osteoblastów. Tak więc nowy typ biomateriału w przyszłości może znaleźć zastosowanie jako materiał implantacyjny w chirurgii ortopedycznej, stomatologicznej i twarzoczaszki.

Słowa kluczowe: hydroksyapatyt, amikacyna, immobilizacja antybiotyków, zakażenia implantów, cytotoksyczność