ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA
VOL. XXIII, N 1, 5 SECTIO DDD 2010

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Hydroxyapatite biomaterials for a filling of bone defects

Biomatriały hydroksyapatytowe jako wypełnienie ubytków kości

HYDROXYAPATITE AS BONE REPLACEMENT MATERIAL

Hydroxyapatite HAp ($Ca_{10}(PO4)_6(OH)_2$) – Calcium phosphate is a bioceramic material of chemical and mineralogical similarity to the non-organic component of bones and teeth. HAp is a well known material for filling bone defects. Current biomedical applications of bioceramics include replacements of hips, knees, teeth, tendons and ligaments, as well as repair in periodontal disease, maxillofacial reconstruction, augmentation and stabilization of the jawbone, spinal fusion, and bone fillers after tumor surgery. Potential future applications of bioceramics will include drug-delivery systems, as well as carriers of growth factors, bioactive peptides and various types of cells for tissue engineering purposes [10]. HAp is used for skeletal tissue engineering because of its biocompatibility and osteoconductive properties. The interconnected porous structure, good mechanical properties, biocompatibility of biodegradable composite provide a suitable microenvironment to promote osteoblast proliferation and osteogenesis. HAp caramics has no carcinogenic properties and does not cause trigger on allergic reactions. Nevertheless, despite its advantages, the application of this ceramics is limited due to poor resorption, a substantially high Young modulus and low fracture toughness [23]. Although HAp does not possess acceptable mechanical properties compared to other biomaterials, it demonstrates a significant potential is being used as a coating on orthopedic prosthesis and in dental surgery, e.g. in filling the dental alveolus after tooth extraction [26].

The bioactive HAp ceramics used as a material for filling defect is available in powder, granulate and block forms. Although powder ceramics is preferred in filling small irregular defects, bigger irregular defects become a problem in filling, because such a material has no so-called surgical handiness. This problem could be solved by preparing a responsible composite material consisting of the HAp phase and a natural polymer [33]. These could be biopolymers such as collagen, fibrin glue, starch-based material, gelatin, chitosan [28].

HAp combined with collagen fibers, extracted from equine tendon, is used in orthopedic and maxifacial surgery [25]. The obtained diphase material was X-rayed subjected to diffractometric, microscopic SEM, TEM, spectroscopic FTIR analyses to highlight the likeness of the artificial biomimetic composite with natural bone tissue [6]. Another way to obtain a more handy hard HAp biomaterial (in the form of powder and granulate) is fusion with plastic fibryn gel. This fusion can result in obtaining a formative and solidcomposite biomaterial [18].

Moreover, a new material consisting of porous hydroxyapatite granules and saccharic polymer was obtained [2, 3]. The obtained material allows easy processing and indicates good adaptation to the shape and bone defect size. Additionally, because of the possibility to get soaked it can serve as a drug carrier [2, 3].

HAp is currently applied for coating metallic implants. More frequently, a successfully used method for coating implant is plasma spraying. This material is expected to increase the bonding strength between the bone and the implant [36]. Although artificial HAp possesses many advantageous properties, its surface, especially in HAp coating, is known as a possible target for adhesion of bacteria and fungi. It happens even though HAp coatings are thought to reduce the bacterial adhesion to metallic surfaces as was reported, for example, for hydroxyapatite-coated stainless steel screws. After the implantation, HAp surface may be therefore colonized by numerous bacteria species, thus becoming the infection center [2, 3]. In order to reduce the incidence of implant-associated bacterial infections, antibiotics and different forms of Ag are proposed [30, 37]. Antibiotic-loaded hydroxyapatite is therefore frequently applied for filling bone defects with simultaneous prevention and treatment of prosthesis-related infections. It was reported that Ag can cause bacterial inactivation by binding both to microbial DNA preventing bacterial replication and to the sulfhydryl groups (SH) of bacterial protein of the bacterial electron transport chain [9].

One of the methods of HAp coating on titanium implant surfaces is „magnetron sputtering". This method allows the mechanical properties of titanium to be preserved maintaining the bioactivity of the thin layer of the HAp coating. Compared to uncoated titanium implants, the thin layer of HAp covering an implant was shown to achieve an equal or higher bone implant bond strength and an equal or higher percentage bone contact at bone implant interface [21].

Chen et al. [8] suggest that the contact angles for surfaces coated with HAp and Ag-HAp (without no significant differences in the surface roughness), were observed to be significantly smaller as compared to titanium surface. The analysis of bacterial adsorption *in vitro* indicated a significantly reduced number of *Streptococcus epidermidis* and *Streptococcus aureus* on hydroxyapatite surface covered with Ag (Ag-HAp) ions. No significant difference in the *in vitro* cytotoxicity was observed between HAp and Ag-HAp surface. Investigators concluded that the creation of a multifunctional surface could be achieved by the „co-sputtering" method joining the osteoconductive HA properties with antibacterial Ag [8].

Charles et al. [7] showed the effects of HAp to PCL (hydroxyapatite polyε-caprolactone) ratio and molding temperature on the flexural mechanical properties. The molding temperatures in relation HAp:PCL decreased, while the flexural modulus and strength was increased. The process successfully produced composites with similar flexural mechanical properties close to the bone. Such composites may have a clinical application for load bearing bone fixation [7].

MICROARRAY AS A TOOL FOR GENE EXPRESSION ANALYSIS OF HUMAN OSTEOBLASTS IN RESPONSE TO HAP BIOMATERIALS

By using bioactive ceramic materials that mimic the mineral composition of natural bone, stimulation of a gene expression which is involved in the commitment of mesodermal progenitor cells (MPCs) to osteoblasts was noted by microarray technology.

Osteogenesis is a strictly controlled developmental process in which numerous extrinsic factors, including hormones and growth factors, activate osteoblast-specific signaling proteins and transcription factors (TFs) required for osteoblast differentiation [34]. Human bone marrow-derived MPCs differentiate into osteoblasts, chondrocytes, adipocytes, myocytes, and endothelial cells. Qi et al. [24] identified genes involved in the commitment of MPCs to osteoblasts and examined the expressed gene profile of undifferentiated MPCs and MPCs induced to the osteoblast lineage for 1–7 days by cDNA microarray analysis. These studies showed that *in vitro* differentiation cultures in which MPCs are induced to one of multiple cell fates should be very useful for defining signals important for lineage-specific differentiation [24].

Song et al. [29] found that the extracellular signal-regulated kinase – ERK (extracellular-signal-regulated protein kinases) signaling molecule, is activated in response to HAp. Using the analysis of expression profiles, they found eleven genes, including those involved in calcium regulation and bone matrix formation and showed a greater than 2.0-fold change in the expression level in response to HAp. Among those genes upregulated by HAp was the gene encoding SOX9 (transcription factor), whose expression was confirmed by real-time PCR analysis with a 5.7-fold increase in expression. Their results suggest that the activation of ERK and SOX9 by HAp may have important implications for understanding the mechanisms by which cells respond on a molecular level to HAp [29].

Bombonato-Prado et al. [5] tried to answer the question how biomaterials alter osteoblast gene expression. By using a cDNA microarray containing 687 human IMAGE sequences, they identified, in primary cultures of osteoblastic cells differentiated from human bone marrow and exposed to these biomaterials the genes whose expression was significantly upregulated or downregulated. They found genes involved with cell cycle regulation, cell differentiation and proliferation, apoptosis, cell adhesion, bone mineralization and skeletal development [5].

Hanagata et al. [12] compared the phenotype and gene expression pattern of osteoblast-like cells cultured on HAp and HAp with pre-adsorbed type-I collagen from neutral solutions (HAp/NCs) with those of tissue culture grade polystyrene (TCPS) and TCPS with collagen (TCPS/NCs). They suggest that basal substrates, i.e. (TCPS and HAp) influence osteoblast maturation even on the surfaces with pre-adsorbed collagen, since mineralization was induced by TCPS, but not by HAp. The gene expression pattern analyzed with DNA microarray also supports a greater influence of basal substrates than pre-adsorbed collagen [12].

TISSUE ENGINEERING

Tissue Engineering (TE) combines the principles of many biological disciplines (biology, biochemistry, chemistry) and medicine in order to get a biological substitute which regenerates damaged tissues, providing a mechanical support for growing tissues [19]. This technique relies on cell culturing on bioceramic material. Due to this technique a new tissue is obtained which is then implemented into chosen patients place. An ideal material for cell scaffold should be characterized by biocompatibility, bioresorption, a high interconnected porous structure, non-toxic and easy to remove waste product, helpful microenvironment for cell proliferation, as well as good mechanical properties [11, 22].

In tissue engineering hydroxyapatite ceramics are more frequently used because of their biocompatibility and osteoconductive properties [17]. Furthermore, toxicity deficit in immunological response and lack of hemolysis in the organism with implanted HAp lead to a wide application inspite of brittleness and fragility of this inorganic component. The most important thing in tissue engineering is permeability of untended material for cells scaffold.

Bignon et al. [4] studied the influence of pore size on building a scaffold for bone formation. They suggested that in the case of HAp with 2–8 μm microporosity the process of the scaffold building is faster than at 250–350 μm macroporosity. Macroporosity (pores > 50 μm) is thought to conribute to osteogenesis by facilitating cell and ion transport [4]. Studies suggest that microporosity (pores < 20μm) improves bone growth into scaffolds by increasing the surface area for protein adsorption, increasing ion solubility, and providing attachment points for osteoblasts [16]. Regular interconnections of pores provide spacing for the vasculature required to nourish new bones and remove waste products. Changes in macroporosity have been shown to affect the mechanical properties more than changes in microporosity [13]. Kim et al. [15] recommended a construct of a large surface area to provide a conduit for cell delivery to give a high cell density with the optimal pore size depending on the intended application of the matrix. Ishaug et al. [14] suggested a pore size range of 200–400 μm because of the optimum compression and tension on the osteoblast mechanoreceptors.

Cultures of osteogenic cells on porous HAp scaffolds offer a new solution to bone grafting using autologous human mesenchymal stem cells (hMSC) from patients. These scaffolds could be defined as perfectly fit replacements to reconstruct the patient’s skeleton. Before their use as bone replacements, HAp and its composites should be tested *in vivo* and *in vitro*. Mygind et al. [20] compared coralline hydroxyapatite scaffolds with pore sizes of 200 and 500 μm for expansion and differentiation of hMSCs. They suggested that the hMSC cultivated (pores size 200 μm) for 1, 7, 14, 21 days showed a faster rate of osteogenic differentiation, as shown by an alkaline phosphatase activity assay and a real-time reverse transcriptase polymerase chain reaction for 10 osteogenic markers. On this basis, they assumed that biomaterials could be used for hMSC/scaffold construction, on which hMSC differentiate into osteogenic cells [20].

Seebach et al. [27] indicated that various bone-graft substitutes influence cell seeding efficiency that could be important for metabolic activity and growth behavior of MSC in different ways. They detected a high variety of cellular integration of MSC *in vitro*, which may be important for bone integration in the clinical setting.

As Wamke et al. suggest [35], these scaffolds could be designed as perfectly fit replacements to reconstruct the patient’s skeleton. Before their use as bone replacement, HAp and its composites should be tested *in vivo* and *in vitro*.

Experiments with HAp i TCP (*tricalcium phosphate*) showed that more human osteoblasts were seen on HAp than TCP scaffold. Biocompatibility was assessed by standard tests for cell proliferation

MTT (tetrazolium salt-cell viability assay), WST (cell viability and proliferation Assay Kit) and fluorescence microscopy. The osteoblast growth and vitality in the case of HAp was better than in the case of TCP, supporting the possibility of HAp applications for bone tissue engineering [31, 35]. A cell viability assay (MTS) on HAp -chitosan complex showed that over 90% cell survived [1].

Tu at al. [32] suggested using bone marrow stromal cells (BMSCs) cultured in coralline hydroxyapatite (CHAp) in bone transplantation. The composite microsphere such as coralline hydroxyapatite is a suitable material to replace autologous bone graft (iliac crest autograft). CHAp was successfully used to fill a defect in the femoral trochlea of goats and in the treatment of a bone loss in the proximal tibia of the rabbit [32].

Zigang et al. [38] demostrated the potential of HAp-chitin matrixes as a good candidate for tissue engineered bone application by providing the environment for osteoblast to attach, migrate, proliferate and differentiate. The most important thing is non-cytotoxicity promotion of calcification, degradation *in vitro*. The ability of the cell to proliferate on the HAp-chitin indicates that biomaterial is a good choice for temporary bone healing devices.

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SUMMARY

Calcium phosphate ceramic materials such as hydroxyapatite HAp ($Ca_{10}(PO4)_6(OH)_2$) are used for skeletal tissue engineering because of their biocompatibility and osteoconductive properties. The interconnected porous structure, good mechanical properties, biocompatibility of biodegradable composite provide a suitable microenvironment to promote osteoblast proliferation and osteogenesis. Culturing of osteogenic cells on a porous HAp scaffold offers a new solution to bone grafting using autologous human mesenchymal stem cells (hMSC) from the patient. These scaffolds could be designed as a perfectly fit replacements to reconstruct the patient's skeleton. Before their use as bone replacement, HAp and its composites should be tested *in vivo* and *in vitro*. Microarray is a tool for gene expresion analysis of human osteoblasts in response to HAp biomaterials. By using bioactive ceramic materials that mimic the mineral composition of natural bones, the stimulation of a gene expression which is involved in the commitment of MPCs to osteoblasts was noted by microarray technology. Among up-regulated eleven genes were the genes encoding SOX9 (transcription factor SOX-9) and ERK (extracellular-signal-regulated protein kinases).

STRESZCZENIE

Ceramika fosforanowo-wapniowa w postaci hydroksyapatytu (HAp) ($Ca_{10}(PO4)_6(OH)_2$) używana jest w inżynierii tkankowej ze względu na biokompatybilność i właściwości osteokondukcyjne. Struktura połączonych porów, dobre właściwości mechaniczne i biokompatybilność tego biodegradowalnego kompozytu stanowi odpowiednie mikrośrodowisko do zapoczątkowania proliferacji osteoblastów i procesu osteogenezy. Kolonie osteogenicznych komórek na rusztowaniu HAp oferują nowe rozwiązanie w przeszczepach kostnych z użyciem autologicznych mezenchymalnych komórek pierwotnych (hMSC) pacjenta. Rusztowanie powinno być tak zaprojektowane, żeby doskonale pasowało do szkieletu pacjenta i służyło jego rekonstrukcji. HAp i jego kompozyty, przed użyciem jako materiał kościozastępczy, są testowane *in vivo* i *in vitro*. Mikromacierze są najnowocześniejszym narzędziem przy badaniu ekspresji genów ludzkich osteoblastów w odpowiedzi na stosowany HAp. Stosując bioaktywne materiały ceramiczne, które składem mineralnym przypominają naturalne kości, stwierdzono przy użyciu wymienionej techniki stymulację ekspresji genów determinujących przejście MPCs w osteoblasty. Wśród genów, których ekspresja była stymulowana, są geny SOX9 (czynnik transkrypcji SOX9) i ERK (kinaza regulowana sygnałem zewnątrzkomórkowym).