BIOACTIVE HYDROXYAPATITE CERAMICS AS SCAFFOLDS FOR BONE TISSUE ENGINEERING - A PRELIMINARY STUDY

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Introduction

Unlike currently used allografts and autografts in clinical practice, which are associated with problems such as limited supply, potential immune rejection and pathogen transfer, synthetic materials show promising results in tissue engineering. Tissue engineering has been defined as an interdisciplinary field which utilizes methods of material engineering and life sciences to develop and create a substitute with similar function and morphology to achieve tissue or organ repair [1]. To create tissue replacements, tissue engineering utilizes cells on artificial material carriers, i.e. scaffolds. In advanced tissue engineering, the scaffold materials should mimic the natural extracellular matrix, i.e. they should provide a place for growth and proliferation, differentiation and metabolism, and should control these processes, e.g. by carrying signal molecules.

The architecture of a good scaffold should be similar to the natural bone, resulting in good biocompatibility and osteoconductivity. Bone porosity is about 50-90%, with the average pore size up to 1 mm. This kind of porosity and pore size are essential for scaffolds. If the pores are too large, the fibrous tissue will cover the inside of the pores. If they are too small (i.e., less than 70 μm), the cells cannot permeate the inside of the scaffold, leading to limited bone formation to the surface layer (for a review, see [2,3]). On the other hand, micropores are required as the superbonding space for cells, and they are also a better substrate or microenvironment for osteoclast formation or maintenance of osteoclast activity, causing the resorption of artificial materials [4]. In addition, micropores promote the formation of capillaries and thus the creation of the blood supply in the scaffolds (for a review, see [5]). Macropores of an appropriate size (usually several hundreds μm in diameter) influence the tissue function, providing a comfortable space for cells to penetrate inside the scaffolds. Moreover, the interconnectivity of the pores plays an important role in bioactivity, allowing deeper tissue ingrowth. Interconnected pores are of a suitable size for supporting cell migration and proliferation, as well as exchange of nutrients and excretion of waste through the whole material pore volume. In addition, the scaffold materials should have appropriate mechanical strength, offering temporary support for the formation of a new tissue.

In this study, we investigated five types of hydroxyapatite (HAp) scaffolds differing from one another in their physical properties, such as the open porosity percentage, closed porosity, bulk density, and also the size, distribution and shape of the pores. The biological properties were examined in vitro using osteoblast-like cells of the line MG 63.

Material and methods

Preparation and characterization of material samples

Pure HAp, i.e., $Ca_{10}(PO_4)_6(OH)_2$, was synthesized by a simple wet chemical method using analytical grade reagents at the Institute of Chemical Technology in Prague, Department of Glass and Ceramics. Samples cylindrical in shape, 1.5 cm in diameter and 0.7 cm in length were prepared using various pore foaming methods and various sintering temperatures, leading to changes in various crystalline parameters (TABLE 1).

Porosity of the samples was measured by a method based on Archimedes Law. The pore size was obtained from SEM (Scanning Electron Microscope) micrographs.

TABLE 1. Characteristics of the hydroxyapatite samples.

Name of samples	Sintering temperature [°C]	Porosity [%]	Pore forming agents	Pore size and distribution	Compression strength [kPa]
HAp1	900	78	Hydrogen peroxide	20-200 μm (66%) 50-100 μm (62%)	40
HAp2	900	65	Wax spheres	50-100 μm (84%)	100
HAp3	1100	58	Psyllium	100-500 μm (81%) 200-500 μm (48%) 100-200 μm (34%) 50-100 μm (1%)	In progress
HAp4	900	67	Psyllium	50-100 μm (84%)	In progress
HAp5	1100	30	Wax spheres	50-200 μm (78%) 50-100 μm (71%)	64

Culture of bone cells on material samples

For the cell culture experiments, the samples were sterilized in a hot air sterilizer at 120°C for 2 hours.

In the first experiment, the samples were inserted in polystyrene 12-well plates (well diameter 2.1 mm, TPP Company, Switzerland) and were seeded with osteoblast-like MG 63 cell line (European Collection of Cell Cultures, Salisbury, UK; passage 162). Each well contained 60 000 cells (i.e., approx. 17 300 cells/cm^2) and 3 ml of Dulbecco's Modified Eagle Minimum Essential Medium (DMEM; Sigma, U.S.A., Cat. No. 10270-106) supplemented with 10% foetal bovine serum (FBS; Gibco, Cat. No. 10270-106) and gentamicin (40 μg/ml, LEK, Slovenia). Polystyrene wells (PS) served as a control material. The cells were incubated at 37°C in an air atmosphere with 5% of CO_2.

On days 1, 3 and 6 after seeding, the cells were fixed with 70% ethanol and stained with propidium iodide. The cell number was evaluated by cell counting on microphotographs taken under an Olympus IX 51 microscope equipped with a DP 70 digital camera. For each experimental group and time interval, 6 microphotographs were evaluated.

For an evaluation of the cell morphology, the cells were stained with propidium iodide and Alexa Fluor 488 (Molecular Probes, Invitrogen). Images were taken under an Olympus IX 51 microscope and under a Leica SP2 laser confocal microscope (Germany).

The cell viability was determined by the epifluorescence staining method using the LIVE/DEAD kit (Invitrogen, Molecular Probes, USA). The principle of this test involves staining the living cells in green with calcein AM, an indicator of esterase activity. At the same time, the dead cells are stained in red with ethidium homodimer-1, due to the permeability of the cytoplasmic membrane of these cells. The cell metabolic activity (namely the activity of the mitochondrial enzymes) was evaluated by the XTT assay kit (Roche Diagnostic GmbH, Mannheim, Germany) and by the WST-8 assay kit (Fluka).

At all observed cell culture intervals, the pH of the medium in wells with the tested samples was also measured (inoLab level 1 pH meter).

In the second experiment, samples of the same types and dimensions as in experiment 1 were used, and the same experimental procedures as those described above were performed. Only the volume of the culture medium was increased to 10 ml/well. For this purpose, the samples were inserted into polystyrene 6-well plates (well diameter 3.4 cm, TPP Company, Switzerland). In addition, the number of seeded cells was increased to 500 000 cells/well (approx. 55 100 cells/cm^2).

Statistical analysis

The quantitative data was presented as mean ± S.E.M. (Standard Error of the Mean). The statistical analyses were performed using SigmaStat (Jandel Corporation, U.S.A.). The multiple comparison procedures were carried out by the ANOVA, Student-Newman-Keuls Method. The value $p \leq 0.05$ was considered significant.

Results and discussion

In the first experiment, i.e. an experiment performed in relatively small volumes of cell culture medium and with low numbers of seeded cells, the cells adhered to all tested samples in densities that were usually close to the seeding density of the cells. Only on HAp5 was the number of cells very high, remarkably (more than twice) exceeding the cell seeding density. The cell numbers on samples HAp1 and HAp2 were similar to those found on the control PS wells, while the cell numbers on HAp3 and on particularly HAp4 were significantly lower (TABLE 2).

From day 1 to day 3 the numbers of cells decreased significantly, and on day 3 the numbers were significantly lower than on day 1 (TABLE 3). The average cell density on all samples was at least six times lower than on PS, which could be explained by rapid release of Ca^{2+} ions from HAp. High levels of Ca^{2+} ions cause cytotoxicity [6,7]. The decrease in cell number was relatively less apparent on HAp 5. On day 3, this sample still contained the highest number of cells among the HAp samples, and this density still exceeded the number of seeded cells. A possible explanation is the lowest porosity of HAp5, which led to the smallest specific surface of the sample, and thus to the slowest release of Ca^{2+} ions. Another negative factor was an increase in the pH of the medium in the presence of HAp samples (TABLE 4), which was also manifested by the violet colour of phenol red in the medium. The physiological pH of the culture medium is 7.35.

On day 6, the cell number on all HAp samples was very low (FIG. 1), and the cells were mostly rounded, non-spread. As revealed by staining with the LIVE/DEAD kit, all cells were practically dead, supposedly due to cytotoxic levels of Ca^{2+} ions. These results were confirmed by the XTT and WST-8 assays, which revealed very low metabolic activity of the cells, or no metabolic activity at all.

TABLE 2. Population density of MG 63 cells on day 1 after seeding on hydroxyapatite samples (HAp) and polystyrene dishes (PS) in the first experiment. Mean ± S.E.M.

Material / Parameter	Number of cells/cm^2	Statistical significance
HAp1	15 740 ± 817	vs. HAp5
HAp2	14 103 ± 881	vs. HAp5
HAp3	12 211 ± 494	vs. PS, HAp5
HAp4	8 990 ± 609	vs. PS, HAp1, HAp5
HAp5	37 731 ± 2993	
PS	19 117 ± 1485	vs. HAp5

TABLE 3. Population density of MG 63 cells on day 3 after seeding on hydroxyapatite samples (HAp) and polystyrene dishes (PS) in the first experiment. Mean ± S.E.M.

Material / Parameter	Number of cells/cm^2	Statistical significance
HAp1	3 642 ± 318	vs. PS, HAp5
HAp2	2 809 ± 414	vs. PS, HAp5
HAp3	4 568 ± 888	vs. PS, HAp5
HAp4	3 122 ± 250	vs. PS, HAp5
HAp5	23 372 ± 1 609	vs. PS
PS	129 534 ± 14 673	

TABLE 4. pH of the cell culture medium in wells with the HAp samples and in the control polystyrene wells (PS) in the first experiment.

Material / pH	Day 1	Day 3	Day 6
HAp1	7.68	7.65	7.90
HAp2	8.22	8.20	8.24
HAp3	8.24	7.90	8.17
HAp4	7.88	8.12	8.10
HAp5	7.91	7.85	8.20
PS	7.67	7.61	7.50

The second experiment was carried out in larger volumes of cell culture medium and with a higher number of seeded cells. Similarly as in experiment 1, the cells adhered well to all tested HAp samples on day 1 after seeding. However, in contrast to the first experiment, the cell population increased in numbers from day 1 to day 4. On day 4, the cell population was confluent, with almost 100% viability and with similar density as on PS. From day 4 to day 8, the cell number did not change significantly, which could be explained by entry into the stationary phase of cell growth, which is defined as the phase in which the number of cells does not change, or even decreases. The cell populations still remained confluent on samples HAp4 and HAp5, but samples HAp2, HAp3 and especially HAp1 showed lower numbers of cells (FIG. 2). Nevertheless, the cells on HAp1 showed a well-spread polygonal or spindle-shaped morphology. In addition, the cell viability on all HAp samples was very high, approaching 100%, as determined using the LIVE/DEAD kit. The pH of the culture medium in the wells with HAp samples mostly decreased below the physiological value (7.35), which suggests intensive metabolic activity of the cells. Only in the wells with samples HAp2 and HAp4 did the pH still remain elevated above the physiological value (TABLE 5).

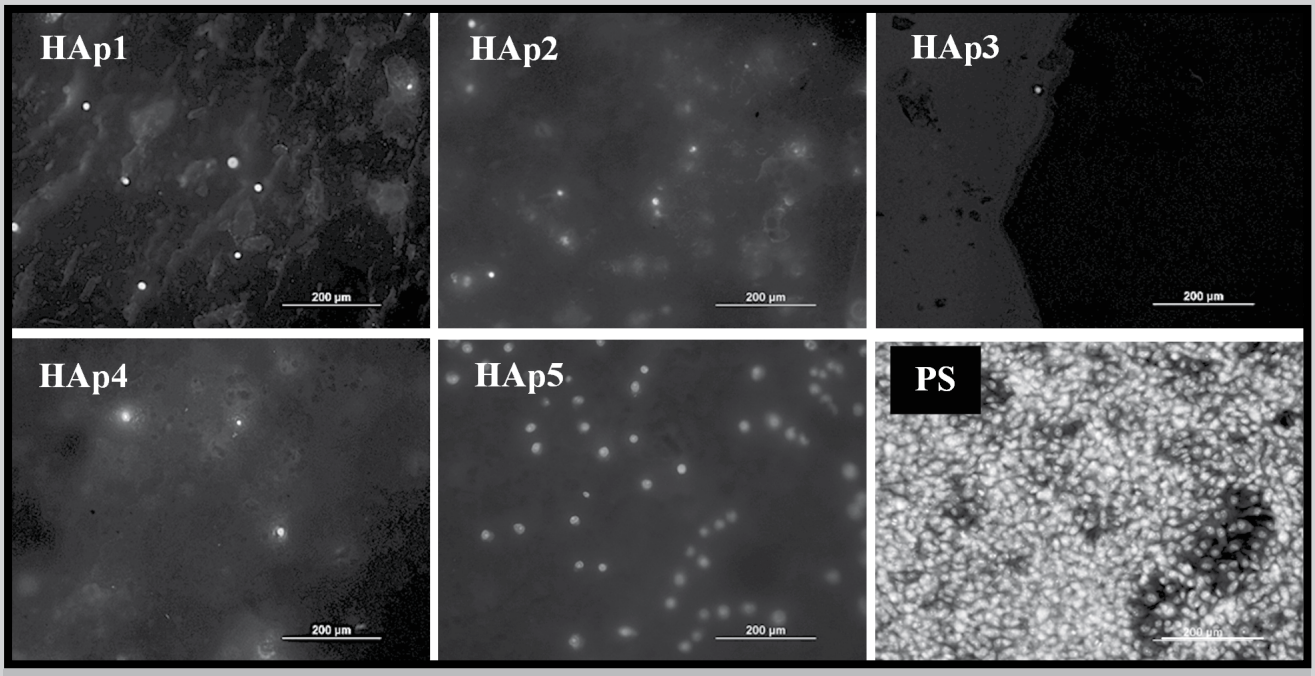

FIG. 1. Morphology of human osteoblast-like MG 63 on day 6 after seeding on hydroxyapatite samples (HAp1-HAp5) and the control polystyrene culture dish (PS) in the first experiment. Cells stained with propidium iodide. Olympus IX 51 microscope, obj. 10, DP 70 digital camera, bar = 200 µm.

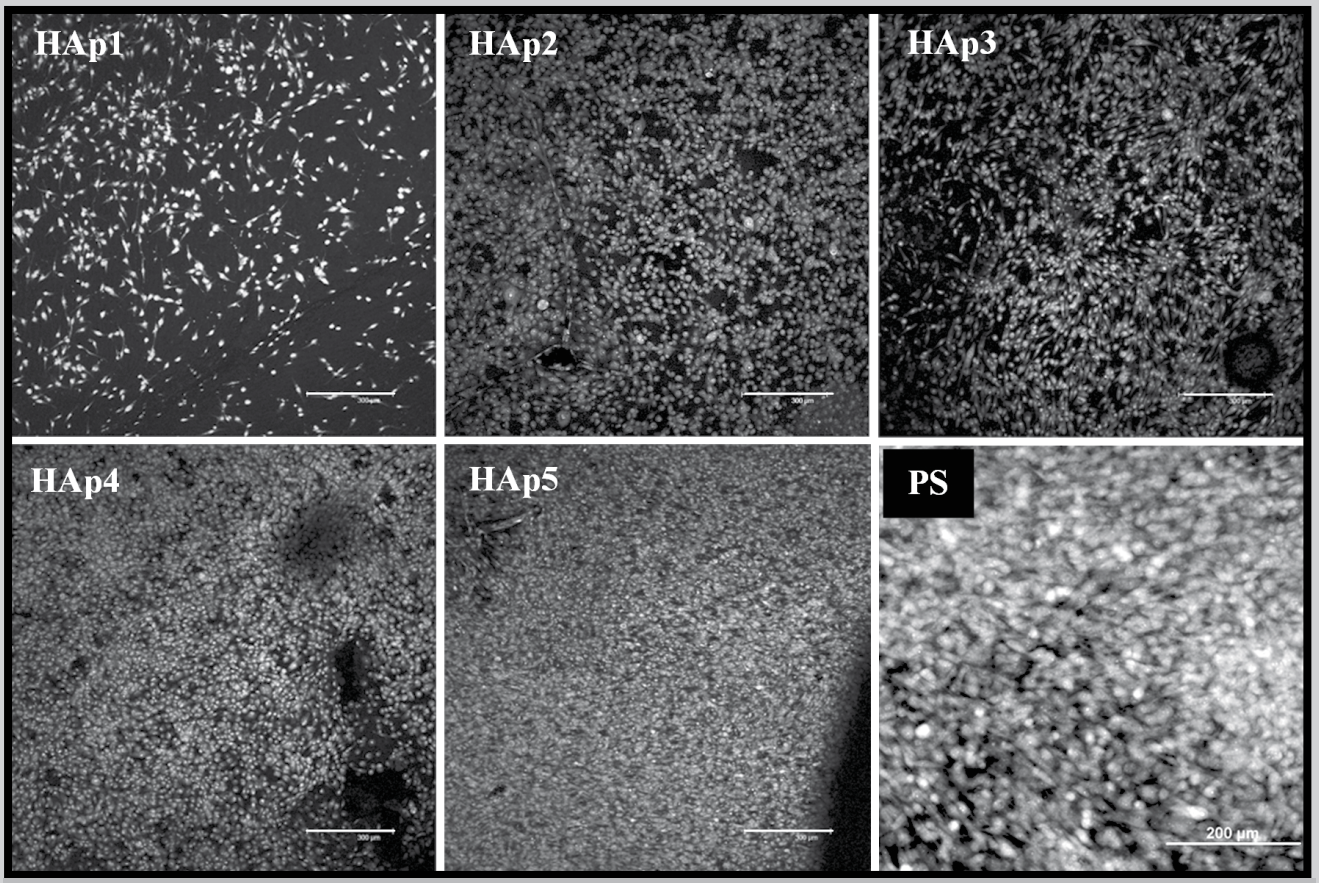

FIG. 2. Morphology of human osteoblast-like MG 63 on day 8 after seeding on hydroxyapatite samples (HAp1-HAp5) and on the control polystyrene culture dish (PS) in the second experiment. Cells stained with Alexa Fluor 488 and propidium iodide. Leica SP2 Confocal Inverse Laser Scanning Microscope, obj. 10x, bar = 300 µm (HAp1-HAp5); Olympus IX 51 microscope, obj. 10, DP 70 digital camera, bar = 200 µm (PS).

TABLE 5. pH of the cell culture medium in wells with HAp samples and in the control polystyrene wells (PS) in the second experiment.

Material / pH	Day 8
HAp1	7.25
HAp2	7.50
HAp3	7.26
HAp4	7.65
HAp5	7.18
PS	7.12

It can be concluded that in experiment 1 and in experiment 2, the preliminary biocompatibility tests proved favourably that hydroxyapatite enables and promotes cell adhesion, which could be explained by high availability of Ca^{2+} ions that are indispensable for integrin-mediated cell adhesion. Another explanation might be improved adsorption of cell adhesion-mediating proteins to HAp compared to other materials. For example, HAp adsorbed more vitronectin and fibronectin from the serum of the culture medium than other materials used for bone implantation, namely commercially pure titanium, and 316L stainless steel [8]. When the adsorption of fibronectin was compared on HAp and on gold, this protein was adsorbed on HAp in a more active geometrical conformation, allowing better accessibility of oligopeptidic ligands (e.g. RGD) in fibronectin molecules for cell adhesion receptors [9]. However, in experiment 1, the tested HAp samples showed cytotoxic activity on days 3 and 6 after seeding, which could be due to excessive and rapid release of Ca^{2+} ions. We came to this conclusion with experiment 2, where cytotoxicity of the HAp samples was not observed in large volumes of cell culture medium and after higher numbers of cells had been seeded, i.e. in conditions in which Ca^{2+} could be more diluted and more rapidly metabolized by the cells. It should also be pointed out that all the tests in our study were conducted with a static method. This does not entirely simulate the real bone tissue surrounding, which is more dynamic from the point of view of blood circulation and mechanical stimulation. Nevertheless, it is suggested that the tested HAp materials be used in orthopaedics and in dentistry only for filling small cavities.

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