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Research Article

Osteoblast and Fibroblast Culture Proliferation on Injectable Calcium Ceramics Polymer Composite

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Abstract Considerable demand for the repair of bone defects cannot be met solely by using biological donor materials. Hence, the use of biocomposites will most likely be increased in reconstructive surgery in future. Metallic implants cannot be used in all cases, because the defects in the skeleton vary greatly, differing from each other in their shape, size, anatomic location, and physiological weight-bearing in the anatomic location. Therefore, more sophisticated orthopaedic materials (e.g. non-metallic fibre-reinforced composites and particulate filler composites) should be available for clinical practice. Our studies have focused on the development of injectable composites of biostable bone cements, *i.e. in situ* curable resin systems containing impregnated calcium ceramics particles. The properties of the bone cement composites aspire to simulate as closely as possible the mechanical and structural properties of bone. The purpose of this study was to evaluate the *in vitro* cell proliferation on the experimental injectable biostable polymer modified with calcium ceramics. In the course of proliferation, the cell activity on the calcium ceramics containing biostable polymer composite increased throughout the experiment. As a conclusion, this cell proliferation study indicated that the studied biocomposite has a good potential to promote cell interaction.

Keywords bone cement composites; calcium ceramics; cell proliferation; human gingival fibroblasts; bone-marrow derived osteoblast-like cells

1 Introduction

The need for reconstructive surgery of bones is continuously increasing along with the degeneration diseases of the population, as well as the increase in bone tumors and traumatic injuries. Large trauma or disease-based hard tissue defects in bone and cartilage are normally repaired using metallic reconstructive implants and scaffolds (e.g. hip and knee prosthesis) that are often fixed by using acrylic bone cement [2] or metallic screws. In the craniofacial area, metallic implants cannot always be used and then the only relevant choice is to use moldable plastic biostable, i.e. non-resorbable structures. In fact, if metallic implants are used, the diagnostic imaging by means of MRI is limited or completely contraindicated. In addition, metal prosthesis and cortical bone are structurally different from the biomechanical point of view, e.g. metals are much more rigid than bone. Therefore, metal prostheses do not stimulate the regeneration process of bone. On the contrary, they can even cause the resorption of bone by the stress-shielding effect and increase the likelihood of adverse bone remodelling [5]. Based on these facts, clinicians still consider autografts as optimal bone substitutes [1, 3]. However, modern material technology has all the know-how for preparing excellent synthetic orthopaedic materials. Therefore, the focus of our studies is to modify biostable bone cements and non-metallic implants in order to improve their biological properties, for example, by creating porosity and bioactive structure in the cement matrix [7,9]. Clinically, injectable and mouldable bone filling materials are desired for filling bone defects. The aim of this study was to evaluate the *in vitro* cell proliferation on the experimental injectable biostable bone cements modified with calcium ceramics particles.

2 Materials and methods

To prepare the test specimens, the inorganic component was mixed with the organic monomer system. The inorganic compound (50 wt%) was dispersed in the viscous monomers of bisphenol-A-type, where after the specimens were allowed to cure under NTP conditions. Equally, the specimens of control group were prepared from the same organic polymer. The 3D structure of the composite was evaluated by high-resolution x-ray computed tomography (micro-CT). The micro-CT images were obtained by using

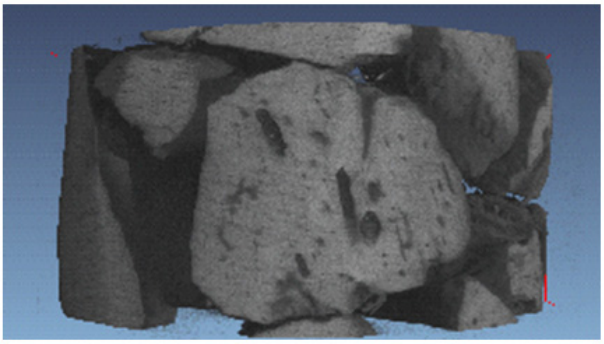
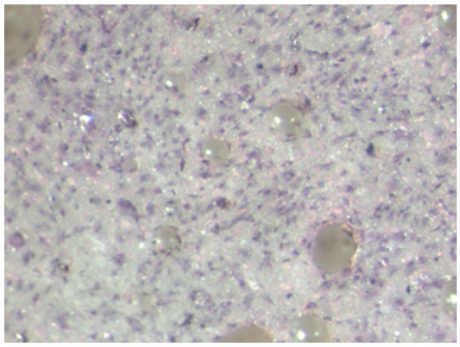

Figure 1: On the left, the 3D structure of *ex vivo* prepared calcium ceramics containing biostable polymer composite, the micro-CT image shows only the inorganic phases of composites. The width of the part shown is *ca*. 8 mm. On the right, osteoblasts spread over the composite material after the 14-day proliferation period. The optical magnification of the image is 40 times.

the nanotom 160 NF (phoenix x-ray, Germany) with 100 kV x-ray tube voltage and 160 μA anode current. 1440 images were collected and the voxelsize of the reconstructed volume was 4.4 μm^3/vox.

The specimens (diameter: 12 mm and height: 4 mm, $n = 4$/proliferation experiment) for cell culturing were first cleaned in an ultrasound bath for 10 m and sterilized by soaking in ethanol (70 vol%)/H_2O for 5 min. The specimens were also immersed in phosphate buffered saline (PBS) and culture medium for 1 h each. First, a preliminary proliferation study was performed on the specimens of calcium ceramics polymer composite and control group using human gingival fibroblasts. The fibroblasts were cultured in Dulbecco's modified Eagle Medium (DMEM, Gibco, The Netherlands) supplemented with 10% fetal bovine serum (FBS, EuroClone, Italy), 1% nonessential amino acids, 100 μg/mL streptomycin and 100 IU/mL penicillin (Gibco) at 37 °C and 5% CO_2. The fibroblasts were seeded on the test substrates at a density of 20,000 cells/cm^2 and the culture was continued for 2 weeks with medium replacement every 2–3 days.

In the case of the primary proliferation study, the employed rat bone marrow stromal cells were harvested from two 6-week-old male Sprague-Dawley rats and cultured according to Maniatopoulos et al. [6]. The cells were cultured in α-MEM (Sigma Chemical Co., USA) pH 7.3 containing 10 mM Na-β-glycerophosphate (Merck, Germany), 10% FBS, 100 μg/mL streptomycin, 100 IU/mL penicillin (Gibco), 10 mM dexamethasone and 50 μg/mL ascorbic acid (Sigma) at 37 °C and 5% CO_2. After 7 days of primary culture, the cells were trypsinized and seeded on the test specimens at a density of 20,000 cells/cm^2. After seeding, the culture was continued for 2 weeks with medium replacement every 2–3 days.

The increase in the number of cells as a result of cell proliferation was investigated. The amounts of cultured cells were determined using AlamarBlue™ (AB) assay (BioSource International, USA) in colorimetric format. 10% AB reagent in phenol red-free DMEM was added on the test specimens and incubated for 3 h. Absorbance values of the solution were taken at 560 nm and 595 nm using an ELISA plate reader and the values were used to calculate the reduction of AB reagent according to the manufacturer's instructions. The cell activities were normalized in relation to the activity of the control group (i.e. plain bisphenol-A-based matrix polymer) at the first time point. The specimens were fixed with 2% glutaraldehyde in PBS, stained with hematoxylin and eosin (H&E) and dehydrated in an increasing ethanol series. Stereomicroscopy was used to visualize the cell proliferation on the test specimens.

3 Results and discussion

According to our previous *in vitro* biocompatibility studies, the studied injectable biostable bone cement composites containing Ca ceramics seem to hold some osteoconductivity. Namely, the measured Ca/P molar ratio of the mineral, obtained from the formed calcium phosphate layer, was *ca.* 1.68 [8]. According to the images of micro-CT, Ca ceramics in the biostable polymer network seemed to be relatively homogenously distributed (Figure 1). In addition, histological staining showed very good cell proliferation and spreading over the material surfaces.

In the course of proliferation, the cell activity on the calcium ceramics polymer composite and control group increased quite equally throughout the experiment (Figure 2). More precisely, during a 14-day period, fibroblast activity increased 10 times on both materials, whereas the osteoblast activity increased over 13 times on the tested composite and 17 times on the control group compared to the cell activity on the first proliferation day. According to the microscopical analysis, the surface of composite material was noticed to become porous in

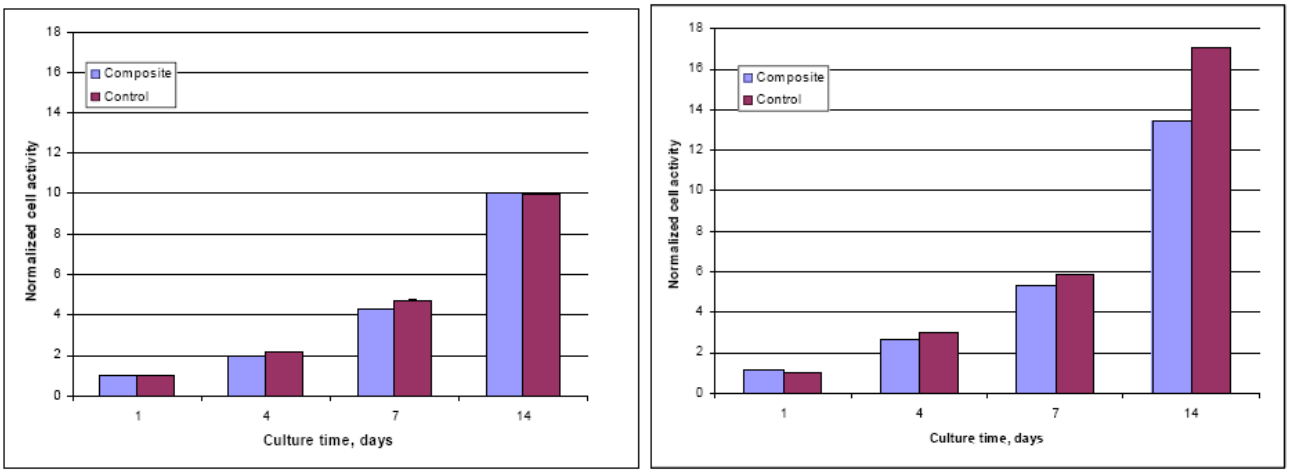

Figure 2: The diagrams show the normalized cell activity (fibroblasts on left and osteoblasts on right) on the calcium ceramics polymer composite and control group.

contact with water, which increased the surface area. The cell proliferations on both materials were only 2D measured from their outermost surfaces. Therefore, the cell activity of the composite might have been even higher than measured, if the whole outermost surface could have been evaluated as a function of 3D structure.

For bone and joint reconstruction, the non-metallic prostheses (i.e. bioactive composites) can be prepared relatively easily *ex vivo*. In fact, the biomechanics, biomimetics and shape of these composites could be tailor-made as a function of the defect. Diagnostic 3D imaging of bone defects can nowadays be performed clinically in a very detailed way using techniques such as extremely high-resolution MRI (μMRI), magnetic resonance microscopy, and x-ray CT. Therefore, for producing biostable bone cement composites containing Ca ceramics, it is a relevant concept to receive a material with more biocompatible properties. These injectable composites have the possibility to be utilized for the manufacturing of *ex vivo* implants, in which case the leaching of toxic substances (e.g. the residual monomers) could also be minimized. In the future, the studies of these biostable bone cements containing Ca ceramics will be continued for improving their biomechanical properties using fiber-reinforcement. In fact, the biomechanics fulfilling the demands of cortical bone are possible to be achieved [4]. The biomechanical properties of orthopedic materials should be both strong enough to withstand the physiological stresses of the body, but they should also have identical elasticity to bone.

4 Conclusion

Cell proliferation study indicated that the calcium ceramics polymer composite had a good potential to promote the cell interaction of both soft and hard tissues. Therefore, these materials have potential to be clinically used for bone and joint reconstruction.

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