Acta Biomaterialia xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

In vivo performance of novel soybean/gelatin-based bioactive and injectable hydroxyapatite foams

Anna Kovtun [a,1], Melanie J. Goeckelmann [a,1], Antje A. Niclas [b], Edgar B. Montufar [c], Maria-Pau Ginebra [c], Josep A. Planell [c,d], Matteo Santin [e], Anita Ignatius [a,*]

[a] Institute of Orthopaedic Research and Biomechanics, University of Ulm, Helmholtzstrasse 14, D-89081 Ulm, Germany
[b] Military Hospital Ulm, Oberer Eselsberg 40, D-89081 Ulm, Germany
[c] Biomaterials, Biomechanics and Tissue Engineering Group, Department of Materials Science and Metallurgical Engineering, Technical University of Catalonia, Av. Diagonal 647, E08028 Barcelona, Spain
[d] Institute for Bioengineering of Catalonia (IBEC), Baldiri Reixac 15-21, 08028 Barcelona, Spain
[e] School of Pharmacy and Biomolecular Sciences, University of Brighton, Cockcroft Building, Lewes Road, Brighton BN2 4GJ, UK

ARTICLE INFO

Article history:
Received 15 August 2014
Received in revised form 16 October 2014
Accepted 23 October 2014
Available online xxxx

Keywords:
Calcium phosphate cement
Gelatine
Soybean
Bone regeneration
Rabbit model

ABSTRACT

Major limitations of calcium phosphate cements (CPCs) are their relatively slow degradation rate and the lack of macropores allowing the ingrowth of bone tissue. The development of self-setting cement foams has been proposed as a suitable strategy to overcome these limitations. In previous work we developed a gelatine-based hydroxyapatite foam (G-foam), which exhibited good injectability and cohesion, interconnected porosity and good biocompatibility in vitro. In the present study we evaluated the in vivo performance of the G-foam. Furthermore, we investigated whether enrichment of the foam with soybean extract (SG-foam) increased its bioactivity. G-foam, SG-foam and non-foamed CPC were implanted in a critical-size bone defect in the distal femoral condyle of New Zealand white rabbits. Bone formation and degradation of the materials were investigated after 4, 12 and 20 weeks using histological and biomechanical methods. The foams maintained their macroporosity after injection and setting in vivo. Compared to non-foamed CPC, cellular degradation of the foams was considerably increased and accompanied by new bone formation. The additional functionalization with soybean extract in the SG-foam slightly reduced the degradation rate and positively influenced bone formation in the defect. Furthermore, both foams exhibited excellent biocompatibility, implying that these novel materials may be promising for clinical application in non-loaded bone defects.

1. Introduction

Calcium phosphate ceramics are frequently used as bone substitutes. In general, they are biocompatible, bioactive and integrate well in bony host tissue [1]. They are used as granules, blocks or cements either to reconstruct bone defects after trauma or to augment weak bone prior to implant placement. The advantages of using moldable calcium phosphate cements (CPCs) instead of pre-formed blocks or granules are the option to apply them using a minimally invasive surgical procedure and better adaptability to the defect geometry.

However, a major disadvantage of CPC is the lack of macropores to allow cell colonization and vessel formation. This hinders cell-mediated material resorption, which is particularly required for apatite cements given their low physicochemical solubility [2]. The development of self-setting cement foams has been proposed as a suitable strategy to overcome this limitation. Mechanical foaming of the cement paste by incorporating a biocompatible foaming agent has been demonstrated to be a promising approach [2]. Our group was the first to use albumen (i.e. egg white) as a foaming agent [3]. We confirmed the suitability of the resulting macroporous self-setting foam as a bone filler in vivo in rabbits, clearly demonstrating that the macroporous structure was maintained after implantation and that the foam resorbed significantly more rapidly compared to non-foamed cements [4]. Although the albumen foam was replaced by newly formed bone, the albumen did, however, evoke a moderate immunogenic reaction [4]. More recently, our group demonstrated that gelatine could also be used efficiently as a foaming agent for CPC [2,5]. Gelatine is denatured collagen, the main protein in bone extracellular matrix, and has

* Corresponding author. Tel.: +49 731 500 55301; fax: +49 731 500 55302.
E-mail address: anita.ignatius@uni-ulm.de (A. Ignatius).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.actbio.2014.10.034
1742-7061/

been shown to be biocompatible [6]. Perut et al. demonstrated that novel calcium phosphate foams containing gelatine exhibited good osteogenic properties in vitro [7]. Therefore, one aim of the present study was to evaluate the performance of this novel foam under in vivo conditions.

Taking a further developmental step, we also combined gelatine-based foams with soybean-derived compounds [7]. The rationales behind the application of soybean extract were its good foaming capabilities and the improvement of injectability of the cements [7]. Even more important is that soybean extract contains the isoflavones genistin and daidzin, which in contact with plasma are activated to genistein and daidzein [8,9]. These activated isoflavones, so-called phytoestrogens, are considered to provoke a beneficial effect on bone metabolism by acting similarly to estrogen [10]. They also elicit anti-inflammatory effects, including the inhibition of the T- and B-cell responses and natural killer cell cytotoxic activity [10,11]. These effects could potentially decrease the inflammatory response to an implant and stimulate bone formation. Santin et al. confirmed this by demonstrating decreased osteoclast formation and activity, and increased osteoblast differentiation after stimulating these cells with soybean-based biomaterial granules [11]. In agreement with these results, our group demonstrated that the enrichment of the novel hydroxyapatite/gelatine foam with soybean extract favored osteoblast activity and differentiation in vitro [7]. It was also demonstrated in a rabbit model that polymeric hydrogels which were functionalized with soybean extract showed good biocompatibility and high bone regeneration potential [12,13].

Based on our previous work, the aim of this study was to evaluate the in vivo performance of injectable self-setting hydroxyapatite/gelatine foams with and without soybean-extract enrichment. Using a critical-size defect in the rabbit femur, we investigated the time course of material degradation and new bone formation in comparison with non-foamed CPC.

2. Materials and method

2.1. Preparation of materials

We synthesized three types of material: non-foamed CPC, hydroxyapatite/gelatine foam (G-foam) and soybean-enriched hydroxyapatite/gelatine foam (SG-foam) as we described previously in detail [7].

The calcium phosphate powder, which was the basis for all the materials, consisted of 98 wt.% of α-tricalcium phosphate (α-TCP) and 2 wt.% of precipitated hydroxyapatite (pHA, Merck, Darmstadt, Germany). The α-TCP was prepared by heat treatment of a stoichiometric mixture of $CaCO_3$ and $CaHPO_4$ (both Sigma-Aldrich, Gillingham, UK) at 1400 °C, followed by quenching in air, to avoid the unwanted beta phase, and finally milled to obtain the powder.

The liquid phase was a 2.5 wt.% Na_2HPO_4 water solution (Merck, Darmstadt, Germany). To prepare the G- and SG-foam, the liquid phase was prepared by dissolving respectively 15 and 5 wt.% of bovine type B gelatine (Bloom 250, Rousselot, Courbevoie, France) in the 2.5 wt.% Na_2HPO_4 (Merck, Germany) water solution at 50 °C in a water bath. The liquid phase of the SG-foam was additionally enriched with 20 wt.% of soybean extract, which was obtained from soybean flour (Infinity Foods, Brighton, UK) using a co-solvent defatting system at 50 °C according to a previously described process [7].

For the in vivo test, the cement powder and the Na_2HPO_4 salt were sterilized using 25 kGy gamma radiation, while the polymeric components of the liquid phase were sterilized as dried powders using 8 kGy gamma radiation to avoid their denaturation. To prepare the sterile liquid phase, all the sterile components were dissolved under a sterile hood in previously autoclaved distilled water. The cement foaming process was performed under sterile conditions in the operating theater using a water bath at 50 °C. First, 2 ml of the liquid phase were foamed for 1 min at 11,000 rpm using a customized hand mixer. Second, after foaming, the cement powder was incorporated in the liquid foam and further mixed using a sterile spatula, preventing foam disruption, until complete homogenization of the paste.

The liquid to powder (L/P) ratio for the preparation of the G- and SG-foam was adjusted to 0.75 ml g^{-1} and 0.55 ml g^{-1}, respectively. For the non-foamed CPC, the liquid phase consisted of a 2.5 wt.% Na_2HPO_4 water solution without any polymer incorporation and was mixed by spatula with the cement powder at an L/P ratio of 0.50 ml g^{-1}. At these L/P ratios, all three pastes presented complete injectability and good cohesion in distilled water.

Finally, for orthotopic implantation, the pastes were filled into sterile syringes and manually injected into the bone defect. In all cases, the time elapsed from the liquid-phase foaming until the implantation by injection was <5 min. In contrast, for subcutaneous implantation the pastes were injected in Teflon molds and allowed to set for 12 days in Ringer's solution (0.9% NaCl) at 37 °C to obtain disks of 5 mm diameter and 3 mm thickness. Prior to implantation, the pre-set disks were sterilized using 25 kGy gamma radiation.

2.2. Animal study design and surgery

The experimental procedures were performed in accordance with the international regulations for the care and use of laboratory animals, and were approved by the German government (Regierungspräsidium Tübingen, No. 837). 60 skeletally mature female New Zealand white rabbits (age: 28 weeks; mean weight: 3.8 ± 0.5 kg) were randomly divided into three groups (n = 20 per group), corresponding to the three tested materials: CPC, G-foam and SG-foam. Each material was implanted in both the left and right femur.

After premedication with a subcutaneous injection of atropine sulfate (Atropinsulfat Braun 0.5 mg®, B.Braun, Melsungen, Germany), the rabbits were anesthetized using an intravenous injection of a mixture of ketamine hydrochloride (Ketamin 10%®, WDT, Garbsen, Germany) and xylazine hydrochloride (Rompun®, 2%, Bayer Health Care, Grenzach, Germany). The lateral condyle of the left and right femur was exposed and a cylindrical defect of 5 mm diameter and 10 mm depth was drilled. Bone debris were removed from the drill hole by washing with sterile saline solution prior to injection of the materials. The muscle and the subcutaneous soft tissue were sutured. A pre-set cement disk (5 mm diameter, 3 mm thickness) of the same material composition was placed subcutaneously before closing the skin. Immediately after surgery, the animals were allowed full weight bearing and freedom of movement.

Six animals were euthanized after 4 weeks and seven animals after 12 and 20 weeks, respectively, per material group. Both femurs as well as the subcutaneous samples were recovered for analysis. One femur was evaluated histologically and the other by biomechanical testing as described below.

2.3. Assessment of the foam-setting reaction in vivo

The progress of the setting reaction in vivo was assessed by determining the crystalline phases present in the foams 1 month after orthotopic implantation. The samples, embedded in methyl methacrylate resin (Technovit VLC 7200®, Heraeus Kulzer GmbH, Wehrheim, Germany), were analyzed using X-ray diffraction (XRD; PANalytical, X'Pert PRO Alpha-1, Almelo, Netherlands) by scanning in Bragg–Brentano geometry using copper Kα radiation.

Please cite this article in press as: Kovtun A et al. In vivo performance of novel soybean/gelatin-based bioactive and injectable hydroxyapatite foams. Acta Biomater (2014), http://dx.doi.org/10.1016/j.actbio.2014.10.034