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Monitoring the biological effect of BMP-2 release on bone healing by PET/CT

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ABSTRACT

Bone morphogenetic protein-2 (BMP-2) releasing scaffolds are routinely used in bone tissue engineering to accelerate regeneration. Thus, assessing growth factor activity, and tissue regeneration around a biomaterial delivery system is extremely important to optimize the stages of bone healing. Such assessment commonly relies on histology. A non-invasive method would allow longitudinal monitoring of the regeneration process. The present study sought to explore the feasibility of Positron Emission Tomography (PET) as a non-invasive method to monitor the osteogenic potential of a BMP-2 releasing calcium phosphate cement (CPC) bone substitute. In a calvarial defect model in rats, ^{18}F-Fluoride PET was used to quantify ^{18}F-Fluoride uptake around the BMP-2/CPC material. As controls, non-loaded porous CPC, dense CPC and autograft samples were used. PET was performed every two weeks, during a period of 8 weeks post-implantation. In parallel, computed tomography (CT) imaging was performed for anatomical reference. Four specimens were evaluated per group per time point. The highest ^{18}F-Fluoride signal intensity was measured in the BMP-2 group (approximately 5 times higher than the baseline value), followed by the controls in the order, from high to low, porous, dense and autograft. The same trend was maintained during the entire experimental period. Histology and measurements of the bone volume percentage confirmed the significantly higher new bone formation in the BMP-2 releasing group (about 25%), compared to the controls. Moreover, ^{18}F-Fluoride PET uptake showed a statistically significant positive correlation ($r = 0.6038$, $P = 0.0171$) with the newly formed bone volume. In conclusion, our results support the use of a PET/CT method for the longitudinal monitoring of BMP-2 releasing scaffolds, *in vivo*.

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1. Introduction

The release of osteoinductive growth factors plays an important role in bone regenerative medicinal strategies [1,2]. A pre-requisite for the release of such proteins is a suitable scaffold material, as delivery vector. Among many candidate materials especially porous ceramics, like calcium phosphate cement (CPC) [3,4], are promising because of the good characterization and proven beneficial effects on bone healing. To introduce osteogenic properties, mostly proteins from the Transforming Growth Factor β (TGFβ) super family are chosen, like Bone Morphogenic Protein 2 (BMP-2). BMP-2 can be incorporated into CPC *via* mixing into the ceramic starting powders, as the hardening takes place at ambient temperature, and thus the risk of thermal denaturation or loss of activity during preparation is minimal. Alternatively, BMP-2 can be adsorbed onto the surface after the cement has set [5,6]. Several studies have already shown that both of these incorporation modes result in a rather similar release profile [7–9]: *i.e.* an initial burst release followed by a stable sustained release. Blom et al. attributed the initial burst to the release of non-bound proteins from the surface of the material, while the steady stage was attributed to the strong binding affinity of TGF family proteins to CPC [10]. Such data were also confirmed by the van de Watering et al. using a highly porous CPC formulation. Also in this study, surface-loaded BMP-2 showed an initial burst release within 1 day (~40%), followed by a sustained release up to day 28. Moreover, despite the similar release profiles, surface-loaded CPC produced better outcomes in terms of osteogenic capacity [11]. Further evidence about the use of surface-loaded CPCs as a local BMP-2 delivery system, has been reported, both *in vitro* [12] and *in vivo* [13–16].

Investigation of the protein release profile from CPCs is usually performed with scintigraphic imaging of radiolabeled proteins. For such purpose, BMP-2 is labeled with Iodine-125 (^{125}I) or Iodine-131 (^{131}I) to trace its release longitudinally. However, besides pure spatial information no data on the biological effect of BMP-2 (*i.e.* bone

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formation) can be extrapolated, and thus histological evaluation at various time points is always required. Although histology still remains the gold standard to provide decisive evidence, it is also obvious that early and non-destructive detection methods would be desirable. Those detection methods would not only allow to reduce the number of experimental animals, but also improve the statistical power of the study as each animal would act as its own control, while reducing an inter-animal variability. Therefore, we postulated that the metabolic activity measurement provided by the Positron Emission Tomography (PET), would be optimal, especially since our previous studies showed that neither Computed Tomography (CT), nor Magnetic Resonance (MR) imaging are suitable to visualize and quantify newly formed bone in the presence of CPC material [10].

We propose that ^{18}F-Fluoride PET scanning could be a valid alternative to monitor the effectiveness of BMP-2-releasing CPC scaffolds over time. ^{18}F-Fluoride PET is routinely used for clinical bone scans, as specific bone-seeking tracers are available. The specific and rapid bone uptake of ^{18}F-Fluoride results in a high bone-to-background ratio after intravenous administration [17], thus, areas of increased ^{18}F-Fluoride uptake reflect the sites of enhanced osteoblast activity and osteoid production [18]. Moreover, our previous study showed that PET is a valuable tool for the qualitative and quantitative follow-up of bone healing around radiopaque bone substitutes *in vivo* [19]. The present study sought to follow the osteogenic performance of one of a frequently used and well-characterized BMP-2 releasing system (*i.e.* the surface-loaded CPC) with non-invasive ^{18}F-Fluoride PET. Pre-set CPCs, either dense or highly porous, were used as negative and positive controls, respectively. Furthermore, autograft implants were also included as control, as such and not morsalized, as also described in [20,21]. Following *in vitro* characterization, the scaffolds were implanted in a bilateral calvarial defect model in rats and monitored by PET once every two weeks, up to 8 weeks post-implantation. CT was performed simultaneously for anatomical reference, and further ^{18}F-Fluoride PET activity was correlated to the efficacy (*i.e.* bone volume percentage) measured by histology.

2. Materials and methods

2.1. Scaffold preparation

Acid-terminated poly(DL-Lactic-co-glycolic acid) (PLGA, PURASORB, Purac, Gorinchem, the Netherlands) with a Lactic-to-Glycolic acid ratio of 50:50 and an average molecular weight of 4.55 ± 0.03 kDa, was used for microparticle preparation using a double-emulsion solvent-extraction technique as described previously [22]. The morphology and size distribution of the PLGA microspheres were determined by light microscopy. Spheres were suspended in H_2O and optical micrographs were made (Leica/Leitz DM RBE Microscope system, Leica Microsystems AG, Wetzlar, Germany). The size distribution of the microspheres was determined by digital image software (Leica Qwin®, Leica Microsystems AG, Wetzlar, Germany) using a sample size of 200 microspheres and resulted in an average size of 175 ± 69 μm. PLGA microparticles were mixed with the CaP cement powder in a proportion of 40% wt/wt.

Thereafter calcium phosphate cement was prepared, consisting of 85% α-Tricalcium Phosphate, 10% Dicalcium Phosphate Dihydrate and 5% Hydroxy Apatite [23]. The cement was created by adding a filtered sterilized (0.2 μm filter) 2% aqueous solution of sodium phosphate (Na_2HPO_4) to the CaP powder mixture (with or without PLGA particles) using a 2-mL syringe (Plastipak, Becton Dickinson, Madrid, Spain) with a closed tip. The components were shaken for 20 s using a Silamat mixing apparatus (Vivadent, Schaan, Liechtenstein) and the composite was injected into a 4 mm diameter × 1 mm height cylindrical plastic mold. After setting, a constant temperature of 650 °C for 2 h was used to burn off the PLGA from the scaffold. The rate of temperature increase and decrease during the process was 1.5 °C/min.

2.2. Loading of BMP-2

BMP-2 (Pfizer, InductOs® former Wyeth Europe Ltd., Berkshire, UK), containing glycine, sucrose and glutamic acid, was reconstituted in sterile water. A stock solution of BMP-2 at a concentration of 1.5 mg/mL was prepared and stored at 4 °C. Following the method also described by van de Watering et al. [11], a volume of 15 μL water solution of growth factor, containing 5 μg BMP-2 was applied to the surface of each side of the pre-set porous scaffolds to obtain 10 μg BMP-2/scaffold. Thereafter, the scaffolds were lyophilized.

2.3. Characterization of scaffold material

The scaffolds ($n = 5$ per group) were placed vertically into a micro-CT imaging system (Skyscan 1072, Kontich, Belgium) and recorded at a 11.09 μm resolution (X-ray Source 100 kV/98SA; Exposure Time 3.9 s; 1 mm filter). Using NRecon V1.4 (SkyScan), a cone beam reconstruction was performed on the projected files. Reconstructed files were analyzed using CTAnalyser software (v1.10.1.0; SkyScan). Finally, 3D-reconstructions of the samples were also assessed in 3D-DOCTOR (V4.0, Able Software Corp, Lexington, MA) on porosity, pore size and Euler number, as an indication of pore interconnectivity.

2.4. Animal model

All *in vivo* procedures were performed in accordance with the standards and protocols of the Radboud University Medical Centre, Nijmegen, the Netherlands. National guidelines for care and use of laboratory animals were obeyed and approval of the Experimental Animal Ethical Committee was obtained (RU-DEC 2012-289). A bilateral calvarial defect was made in 32 adult male 250–300 g Wistar rats (10–12 weeks old). Besides the experimental CPC/BMP-2 material, three controls were included in the study, *i.e.* a porous CPC scaffolds without BMP-2, a dense CPC scaffold, and autograft bone. Sample size calculation was performed in line with the existing literature and previous experience of our research group. Power calculations [24], resulted in $n = 4$ repetitions per group necessary to obtain statistically significant data. During the surgical procedure animals were anesthetized by intubation with Isoflurane inhalation anesthesia, in order to prevent any eventual respiratory depressions that may occur during the procedures. The skin of the head was shaved and disinfected and a midline longitudinal incision was made from the nasofrontal area to the external occipital protuberance along the mid-sagittal suture. Skin and underlying tissues were reflected bilaterally to expose the calvaria and two symmetrical 4 mm wide full-thickness bone defects were created in both parietal bones lateral to the midsagittal suture using a dental drill (ELCOMED 100, W&H Dental Work Bürmoos GmbH, Bürmoos, Austria) and a trephine bur (ACE Dental Implant System, Brockton, MA) under constant saline coolant irrigation. Special care was taken to prevent damage to the dura mater. For the autograft group, the removed 4 mm bone disks were placed back, as such, into their original position. The remaining defects were filled with the scaffolds depending on the group, according to a Latin Square randomization scheme. Finally, the periosteum and the scalp were closed with 3.0 and 4.0 Vicryl® resorbable sutures (Johnson & Johnson, St. Stevens-Woluwe, Belgium).

2.5. In vivo imaging

PET/CT imaging was performed before surgery, at day 2, and then every two weeks, starting from week 2 up to week 8 post-surgery. ^{18}F-Fluoride PET/CT data were acquired 1 h after injecting ~15 MBq/rat of ^{18}F-Fluoride in 0.9% NaCl (200 μL/rat) (BV Cyclotron VU, Amsterdam, The Netherlands), with an Inveon small-animal PET/CT scanner (Siemens Preclinical Solutions, Knoxville, TN). PET emission scans were acquired for 15 min, followed by CT acquisitions for

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