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In vivo release of rhBMP-2 loaded porous calcium phosphate cement pretreated with albumin

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Abstract In this study, the release of rhBMP-2 loaded porous Ca-P cement was studied *in vitro* and *in vivo*. We hypothesized that adsorption sites of Ca-P ceramic with high affinity for rhBMP-2 can be blocked by pretreatment of the ceramic with albumin prior to rhBMP-2 loading, which would result in weaker rhBMP-2 binding and enhanced release of rhBMP-2. Preset porous Ca-P cement discs with a diameter of 6.35 mm (volume: 75 mm^3) were pretreated by incubation in a solution of 10% rat serum albumin for 24 h or in ddH_2O (control group) prior to administration of 5 μg radiolabeled ^{131}I-rhBMP-2. Release was assessed *in vitro* in phosphate buffered saline (PBS) and fetal calf serum and *in vivo* by longitudinal scintigraphic imaging of radiolabeled ^{131}I-rhBMP-2 and gamma counting of dissected implants.

In vitro release from pretreated discs was higher during the first day. For both formulations, release in PBS was limited compared to release in serum. *In vivo* release considerably exceeded *in vitro* release. *In vivo* release kinetics showed no significant difference of half-lives between pretreated and control discs. Both formulations showed sustained release during at least 4 weeks. *Ex vivo* gamma counting of retrieved samples confirmed scintigraphic results and showed that the capsule and surrounding tissues only contained a minor fraction rhBMP-2. We conclude that 1. scintigraphy of ^{131}I-labeled rhBMP-2 provides a reliable method for longitudinal measurement of rhBMP-2 release kinetics *in vivo*. 2. albumin pretreatment of porous Ca-P cement does not results in relevant increase of initial release of rhBMP-2 *in vivo,* and 3. preset rhBMP-2 loaded porous Ca-P cement discs exhibit one phase exponential release kinetics in the rat ectopic model, characterized by a retention of 20–30% after 4 weeks.

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1. Introduction

Calcium phosphate (Ca-P) ceramics form a distinct group among the diverse range of bone replacing biomaterials, and are generally known for their intrinsic osteophilic properties. Ca-P ceramics are commercially available in several chemico-physical forms and have also been used as scaffolding material for growth factor-based bone engineering. [1–16] Most of these studies reported an osteoinductive effect of the Bone Morphogenetic Protein (BMP) loaded construct, but were not designed to investigate the relation between release kinetics and bioactivity of the loaded growth factor. For example, the biomaterial might potentiate the activity of BMP by binding the protein and presenting it to target cells in a "bound" form. [17, 18] On the other hand, the ceramic can also act as a delivery vehicle and released protein may

provide a supra-physiological concentration of free protein in the vicinity of the implant, which may attract target cells to the implant-site by chemotaxis. [19] Consequently, protein binding to the biomaterial may play an important role in protein bioactivity. Column chromatography separation techniques have shown that Ca-P ceramics exhibit a high binding affinity for proteins. Unfortunately, the exact binding mechanism of rhBMP-2 to a Ca-P ceramic surface is not exactly known. Several adsorption mechanisms have been proposed for macromolecules with apatitic substrates, [20] such as (1) physical adsorption that takes place in the electrical double layer, [21] (2) chemisorption that involves direct bond formation between the adsorbing molecule and apatite, [22] (3) specific adsorption of calcium ions that may act as a bridge to bind anionic species to phosphate sites of the hydroxyapatite lattice, [22] (4) H-bonding that enables interactions with phosphate, hydroxyl and/or fluor groups of hydroxyapatite, [22] and (5) formation of multilayers on the surface. [23] Although elucidation of the precise binding mechanisms is hindered by the complexity of the structures and the diversity of functional groups in these macromolecules, maximum numbers of adsorption sites and affinity constants to various Ca-P substrates have been described for several adsorbates [20].

Until now, a limited number of studies focused on the *in vivo* release kinetics of growth factors loaded onto Ca-P ceramics. [2, 3, 8, 16] The outcome of these studies varied considerably, most likely due to differences in surgical site, dose, adsorption procedures, material surface area and differences in chemico-physical interactions between material and growth factor. Overall, ceramics exhibited one or two phasic release kinetics with a certain retention for several weeks. Uludag *et al.* concluded that growth factor retention depended on carrier- as well as on protein characteristics. [2, 3] Carriers exhibiting higher retention of BMP yielded higher osteoinductive activity. However, chemico-physical differences between carriers confounded any correlation between protein pharmacokinetics and protein bioactivity.

In view of the studies mentioned above, we carried out a study where rhBMP-2 was lyophilized on the surface of porous Ca-P cement discs. Ten weeks after subcutaneous implantation in rabbits, significant osteoinduction was seen inside the disc porosity. [7] Nevertheless, *in vitro* analysis revealed a very limited release of rhBMP-2 from the Ca-P cement, which suggested an avid interaction between the Ca-P cement and the loaded protein, but provided no answer to the question whether in this study optimal release kinetics and maximal bioactivity of the growth factor were obtained. [10] Therefore, in the present study we focused on the *in vivo* release of rhBMP-2 loaded porous Ca-P cement and aimed for a method to enhance release kinetics without vital alterations in chemico-physical material properties. We hypothesized, that *in vivo* rhBMP-2 release kinetics can be assessed reliably and longitudinally by scintigraphic imaging of radiolabeled rhBMP-2. We further hypothesized that the high affinity adsorption sites of the Ca-P ceramic can be blocked by pretreatment of Ca-P with another protein prior to rhBMP-2 administration. Blocking of the high binding affinity sites would result in a less avid protein binding of subsequently administered rhBMP-2. We assume that weaker interaction of rhBMP-2 and Ca-P cement will cause an enhanced release of rhBMP-2. In this study, albumin was used for pretreatment as this is the most abundant body protein and known for its limited specific activity. Consequently, albumin pretreatment would result in enhanced growth factor release without changing the Ca-P material properties.

2. Materials and methods

2.1. Materials

The Ca-P cement (Calcibon,® Biomet Merck, Darmstadt, Germany) consisted of a mixture of 62.5 wt% tricalcium phosphate (α-TCP), 26.8 wt% dicalcium phosphate anhydrous (DCPA), 8.9 wt% calcium carbonate ($CaCO_3$) and 1.8 wt% hydroxyapatite (HA). $NaHCO_3$ was added to the cement powder for production of CO_2 macroporosity in the cement. [24] Sterile aqueous solutions of Na_2HPO_4 (2 wt%) and NaH_2PO_4 (8 wt%) were used as the liquid component. Rat serum albumin (RSA), derived from Sprague Dawley rats and bovine serum albumin were obtained from Sigma-Aldrich Co. (St. Louis, MO). Human recombinant BMP-2 (rhBMP-2) was kindly provided by Wyeth Europe Ltd. (Berks, UK) and radiolabeled with ^{131}I (Perkin Elmer, Milano, Italy).

2.2. Preparation of porous Ca-P cement discs

A 2 ml syringe was closed at the tip with a plastic stopper and filled with a mixture of 500 mg Ca-P cement and 50 mg $NaHCO_3$. After mixing for 15 s with Na_2HPO_4 (2%) in a mixing device (Silamat, Vivadent, Schaan, Liechtenstein), NaH_2PO_4 (8%) was added and the syringe was shaken again for 2 s. The well-mixed Ca-P material was then injected into Teflon molds to ensure a standardized shape of the scaffolds (discs of 6.35 mm in diameter, 2.4 mm thickness and 75 mm^3 volume). The discs were removed from the molds after setting of the cement and sterilized by autoclaving at 121°C for 15 min. In this way, fifty-eight sterile discs were prepared with an average weight of 63.2 ± 3.7 mg. To visualize the porous structure within the Ca-P cement, one disc was reconstructed by microcomputed tomography (μCT) (SkyScan 1072, SkyScan, Aartselaar, Belgium) (Fig. 1).

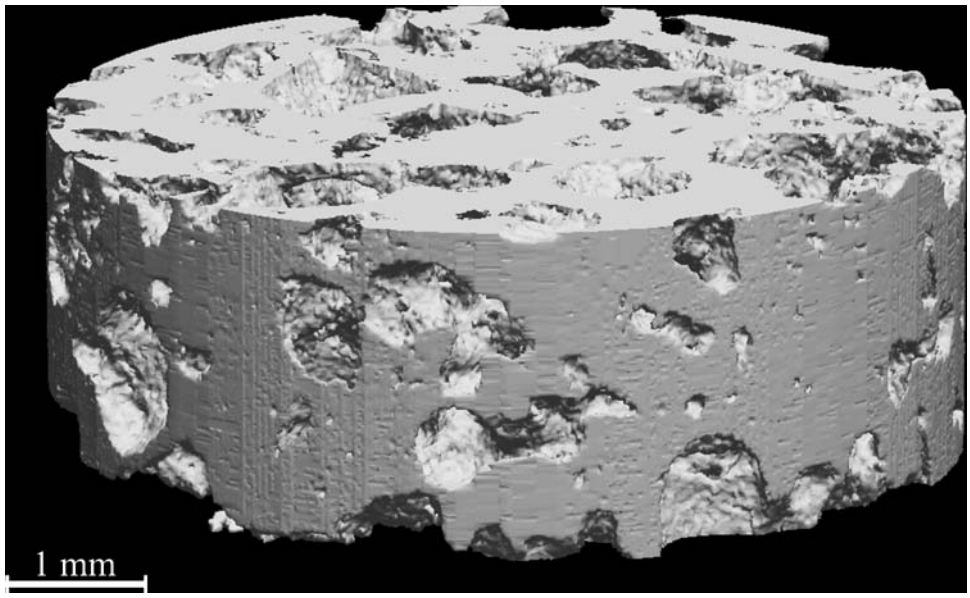

Fig. 1 Microcomputed tomography image of preset porous Ca-P cement disc with irregular formed macropores. Bar represents 1 mm.

2.3. RSA pretreatment of porous Ca-P cement discs

A 10% solution of rat serum albumin (RSA) was prepared in double distilled water (ddH_2O) and filtered through a 0.2 μm cellulose acetate membrane filter (Omnilabo International BV, Breda, The Netherlands) to assure sterility. Under sterile conditions, twenty-nine Ca-P cement discs were soaked in 14.5 ml RSA solution and placed on an orbital shaker table at low rotational speed (40 rpm) at room temperature for 24 h. Subsequently, the RSA solution was removed and the discs were carefully rinsed with 29 ml ddH_2O to remove free RSA. The discs were then lyophilized and stored dry at room temperature until use. Non-pretreated (control) Ca-P cement discs were prepared by incubation in ddH_2O instead of RSA.

2.4. Radioiodination of rhBMP-2

rhBMP-2 was labeled with ^{131}I according to the iodogen method. [25] Briefly, 0.6 mg rhBMP-2 in 200 μl 500 mM phosphate buffer, pH 7.4 and 862 MBq ^{131}I (862 MBq/ml) were added to a glass vial pre-coated with 500 μg 1, 3, 4, 6-tetrachloro-3α, 6α-diphenylglucouril (Pierce, Rockford, IL). The reaction was allowed to proceed for 10 min at room temperature and then the reaction mixture was eluted on a disposable Sephadex G25M column (PD10; Pharmacia, Uppsala, Sweden) to separate labeled rhBMP-2 from free ^{131}I. The void fractions were pooled and the ^{131}I-rhBMP-2 was concentrated to a final concentration of 70 MBq/ml using a YM-10 filter device with a cut-off of 10,000 Da (Millipore, Bedford, MA).

The radiochemical purity of the ^{131}I-labeled rhBMP-2 was determined by instant thin-layer chromatography (ITLC) on Gelman ITLC-SG strips (Gelman Laboratories, Ann Arbor, MI) with 0.1 M citrate, pH 5.0 as the mobile phase. The radiochemical purity of the ^{131}I-labeled rhBMP-2 preparation exceeded 99%, which indicates that more than 99% of the ^{131}I-label was covalently linked to rhBMP-2. The radiolabeled product was further characterized on a TSK G3000SW gelfiltration column (7.8 × 300 mm) using a Bio-Rad Bio-Logic Duo-Flow FPLC system. The column was eluted with PBS at a flow rate of 1.0 ml/min. FPLC analysis revealed a monomeric peak co-eluting with unlabeled rhBMP-2 at 22 min, demonstrating that the radioiodinated preparation did not contain rhBMP-2 aggregates or degradation products.

The specific activity of the labeled protein was 10 μCi/μg. Hot/cold solutions were prepared in ratios of 3:2 for the *in vivo* release study and 1:20 for the *in vitro* release study. The final rhBMP-2 concentration was adjusted to 0.2 mg/ml in PBS/BSA[0.1%].

2.5. ^{131}I-rhBMP-2 implant loading

^{131}I-rhBMP-2 was administered to twenty-nine albumin pretreated and twenty-nine control porous Ca-P cement discs. A volume of 12.5 μl ^{131}I-rhBMP-2 solution was carefully loaded onto the surface of each side of the discs. In this way, all discs were loaded with 5 μg ^{131}I-rhBMP-2. Thereafter, the discs were frozen and lyophilized. Subsequent gamma emission of the samples was measured in a shielded well-type gamma counter (Wizard, Pharmacia-LKB, Sweden) simultaneously with aliquots of the administered dose (standards) to determine the initial dosage.

2.6. *In vitro* release assay

Twenty discs were placed in 10 ml glass vials for evaluation of *in vitro* rhBMP-2 release kinetics. Samples were incubated

in 2 ml PBS or 2 ml fetal calf serum (FCS) at 37°C on an orbital shaker at low rotational speed (50 rpm) for 21 days ($n = 5$ for each formulation and release medium). Both release media were supplemented with 120 μg/ml gentamycin as antibiotic prophylaxis. At 1, 3, 7, 14, and 21 days, the samples were carefully transferred to new vials with fresh release medium. At each time point, activity in the discs was measured in a gamma counter prior to ongoing incubation at low rotational speed. Standards were counted simultaneously to correct for radioactive decay. The remaining activity in discs was expressed as percentage of the initial dose.

2.7. In vivo release assay

Fifteen healthy male Sprague Dawley rats (Harlan, Horst, The Netherlands) with an approximate weight of 250 g were used as experimental animals. National guidelines for the care and use of laboratory animals were respected. Anesthesia was induced and maintained using Isoflurane inhalation. The ^{131}I-rhBMP-2 loaded discs were implanted subcutaneously in the back of the animals. For the insertion of the implants, the animals were immobilized and placed in a ventral position. The back of the animals was shaved and disinfected with povidone-iodine. Subcutaneous pockets were created by blunt dissection at each side of longitudinal dermal incisions at thoracic and lumbar vertebrae. In this way, each animal received two subcutaneous implants of both formulations placed according to a statistical randomization scheme. After insertion of the implants the skin was closed using agraven suture material.

In total, forty-eight implants were placed in twelve rats. Three rats only received a paravertebral subcutaneous injection of 5 μg ^{131}I-rhBMP-2 in 100 μl PBS/BSA[0.1%] at each side. These animals served as control to evaluate the clearance of a depot of rhBMP-2 and did not receive implants to prevent the risk of adsorption of rhBMP-2 onto the Ca-P cement.

A single-head gamma camera (Orbiter, Siemens Medical Systems Inc., Hoffman Estates, IL) equipped with a parallel-hole high-energy collimator was used to measure longitudinal release in three rats (n = 6 for each formulation) under general inhalation anaesthesia at 0, 16 h, 2, 3, 7, 9, 12, 14, 17, 21 and 28 days. Additionally, activity distribution in the three rats with subcutaneous rhBMP-2 depots (n = 6) was imaged using the gamma camera after 0, 2, 6, 24 and 48 h. Images (50,000–300,000 counts per image) were obtained and digitally stored in 256 × 256 matrices. The scintigraphic images were analyzed quantitatively by drawing standardized regions of interest (ROI) over the implant. Activity measurements in the ROIs were corrected for radioactive decay, acquisition time and background radiation. The measured activity in implants was expressed as percentage of the initial dose. At the end of the study, all rats used for scintigraphy were euthanized by CO_2/O_2 suffocation.

After 3, 7 and 21 days, three rats were euthanized by CO_2/O_2 suffocation for retrieval of implants and their surrounding tissues. Subsequently, radioactivity was counted in a shielded well-type gamma counter (Wizard, Pharmacia-LKB, Sweden). Eight implants retrieved after 3 and 7 days were separated carefully from their surrounding tissue by dissection of the thin fibrous capsule. Both parts were counted independently to determine the amount of rhBMP-2 present in the surrounding tissue. Standards were counted simultaneously to correct for radioactive decay. The measured activity in implants and surrounding tissue were expressed as percentage of the initial dose.

2.8. Statistical analysis

All measurements were statistically evaluated with GraphPad® Instat 3.05 software (GraphPad Software Inc., San Diego, CA), using an unpaired T-test with Welch correction. Pharmacokinetic analysis of rhBMP-2 release *in vitro* (gamma counter analysis) and *in vivo* (scintigraphic measurements) was done by nonlinear regression analysis using GraphPad® Prism 4.02 software (GraphPad Software Inc.). Data were weighted reciprocally and fitted according to a one phase exponential release model. From this model half-life ($T_{1/2}$) and plateau (P) were calculated, in which $T_{1/2}$ represents the average time required for a release of 50% of the total released dose and, P is the value to which the fitted curve decays, representing the finally retained dose.

3. Results

3.1. *In vitro* release assay

Fifty-eight sterile porous Ca-P cement discs (Fig. 1) with an average weight of 63.2 ± 3.7 mg were prepared, pretreated with albumin, and loaded with rhBMP-2 according to the described protocol. *In vitro* release kinetics of rhBMP-2 for rhBMP-2 loaded porous Ca-P cement discs with albumin pretreatment (initial loading 4.8 ± 0.2 μg rhBMP-2/scaffold) and control discs without albumin pretreatment (initial loading 4.8 ± 0.2 μg rhBMP-2/scaffold) were evaluated in PBS and serum over the course of 21 days (n = 5 for each formulation). All discs maintained their integrity during this period. Pharmacokinetic analysis revealed that a single phase exponential release model could only be fitted appropriately to the data between day 1 and day 21. Pharmacokinetics could not be analyzed separately for the rapid initial release during the first day, due to the lack of data in this phase. Retention of the activity in the samples is depicted in Fig. 2. Albumin pretreated Ca-P discs in serum showed an initial

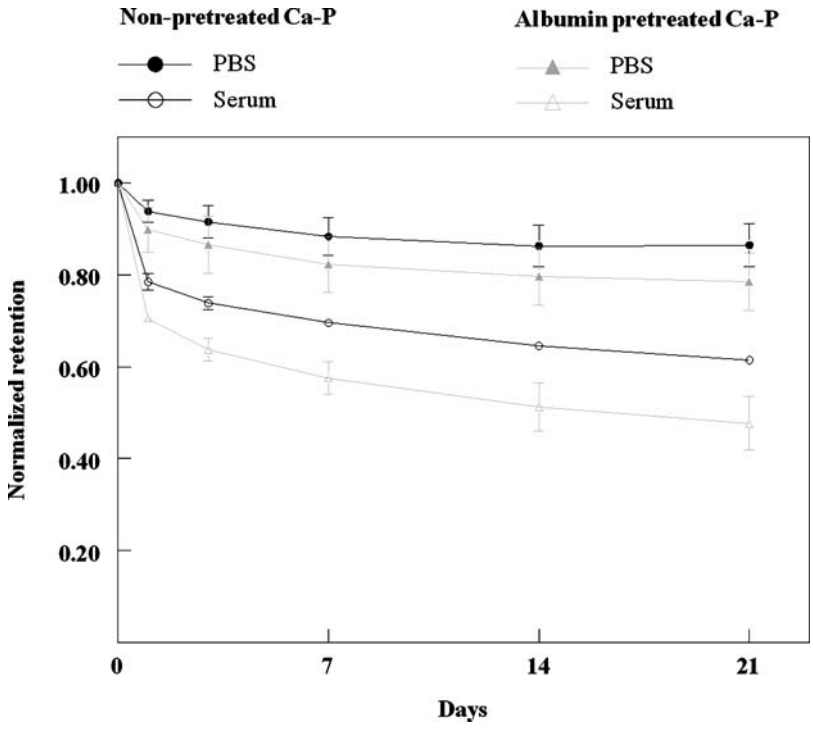

Fig. 2 *In vitro* retention of rhBMP-2 loaded porous Ca-P cement discs with or without albumin pretreatment in PBS or FCS under continuous shaking at 37°C. Retention is expressed as the ratio of total mass of rhBMP-2 retained at each time point to total mass of rhBMP-2 loaded. Error bars represent the mean and standard deviation for five samples.

release of $30 \pm 1\%$ during the first 24 h, followed by a one phase exponential release ($T_{1/2} = 6 \pm 1$ days, $P = 46 \pm 5\%$). Without albumin pretreatment, Ca-P cement discs in serum showed an initial release of $22 \pm 2\%$ during the first 24 h, followed by a similar exponential release ($T_{1/2} = 7 \pm 1$ days, $P = 58 \pm 2\%$). In PBS, initial release was slower for both formulations ($11 \pm 5\%$ and $6 \pm 2\%$ for pretreated and control discs, respectively). After the first day, release was very limited compared to similar formulations in serum ($T_{1/2} = 4 \pm 2$ days and 4 ± 1 days, $P = 75 \pm 8\%$ and $86 \pm 4\%$ for pretreated and control discs, respectively). Calculated half-lives after the first day were not significantly different among the various groups. Due to differences in initial release, plateaus were significantly lower for albumin pretreated discs. ($p < 0.05$ for release in PBS, $p < 0.005$ for release in serum).

3.2. *In vivo* release assay

In vivo release kinetics was assessed in the rat ectopic model by scintigraphic imaging of radiolabeled scaffolds *in vivo* and *ex vivo* gamma counting of retrieved scaffolds. All animals, except one, remained healthy during the experiment without any sign of complication in wound healing; one rat died after gamma camera imaging on day 21, probably due to an overdose of anaesthesia. All implants remained intact over the course of 28 days.

Scintigraphy of rhBMP-2 injected as subcutaneous depot in the back of the rats revealed one phase exponential release kinetics over a time course of 48 h ($T_{1/2} = 0.3$ day). Retention of subcutaneously injected ^{131}I-rhBMP-2 was limited to $8 \pm 12\%$ after 24 h (Fig. 3). Retention of rhBMP-2 in the porous Ca-P cement implants is shown in Fig. 4. The scatter plots show the retention measured by longitudinal *in vivo* scintigraphic imaging and *ex vivo* gamma counting of retrieved implants. The line graph represents the kinetic release model fitted on the scintigraphic imaging data. Scintigraphy determined an initial rhBMP-2 release from control and albumin pretreated discs of $19 \pm 8\%$ and $13 \pm 10\%$ after 16 h, followed by sustained release phase. After 28 days, $27 \pm 5\%$ of the initial activity in the control discs and $19 \pm 5\%$ of the initial activity in the albumin pretreated discs was retained. Release kinetics revealed no significant difference in half-lives between control and albumin pretreated discs ($T_{1/2} = 3 \pm 1$ days and 6 ± 3 days, $p > 0.05$). The extrapolated plateaus (P) at the terminal phase of the release profile model were significantly lower for pretreated discs than for control discs ($P = 16 \pm 10\%$ and $31 \pm 5\%$, $p < 0.005$).

Gamma camera measurements were confirmed by *ex vivo* gamma counting of retrieved samples with an average overestimation of gamma camera measurements of $8 \pm 4\%$ for each formulation and time period (Fig. 4). Further, *ex vivo* gamma counting showed that the capsule and surrounding tissues only contained a minor fraction rhBMP-2 compared to the implant ($3 \pm 1\%$ for samples retrieved after 3 days, $3 \pm 2\%$ for samples retrieved after 7 days; no significant difference among groups) (Fig. 5).

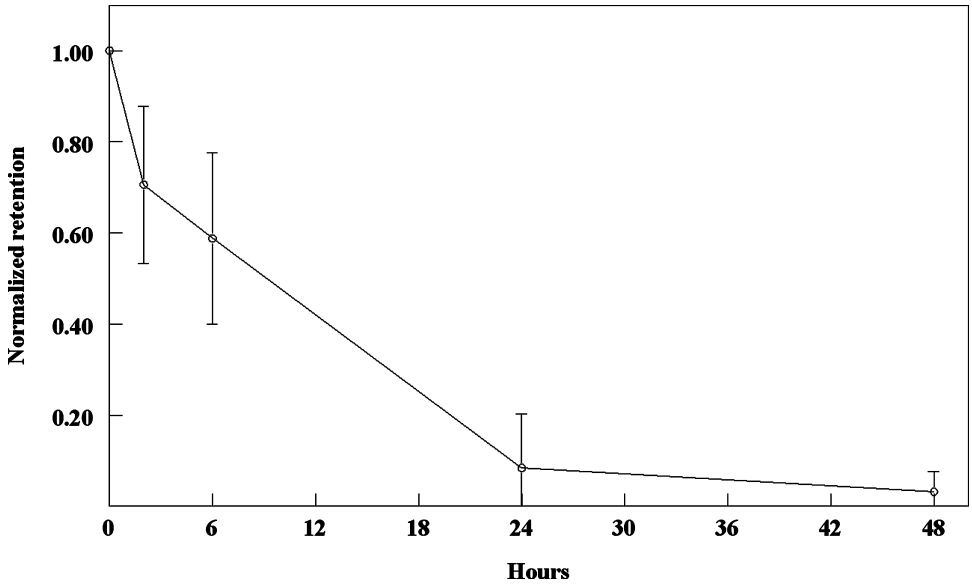

Fig. 3 *In vivo* retention of rhBMP-2 injected as subcutaneous depot in the back of rats. Retention is expressed as the ratio of total mass of rhBMP-2 retained to total mass of rhBMP-2 loaded. Error bars represent the mean and standard deviation for six depots.

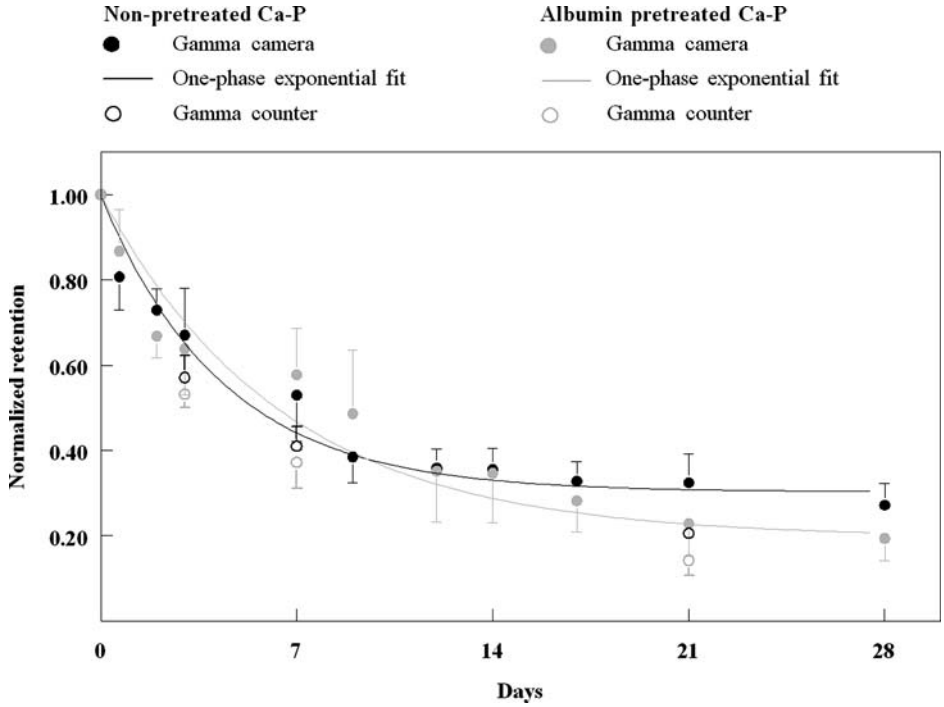

Fig. 4 *In vivo* retention of rhBMP-2 loaded porous Ca-P cement discs with or without albumin pretreatment implanted subcutaneously in the back of rats. The scatter plots show the results of longitudinal *in vivo* scintigraphic imaging (closed markers) and *ex vivo* gamma counting of retrieved implants (open markers). The line graph represents the one-phase exponential release model fitted on the scintigraphic imaging data. Retention is expressed as the ratio of total mass of rhBMP-2 retained to total mass of rhBMP-2 loaded. Error bars represent the mean and standard deviation for six samples.

4. Discussion

In this study, we investigated the *in vitro* and *in vivo* release kinetics of rhBMP-2 loaded porous Ca-P cement. Release of growth factor depends on the protein binding affinity of the biomaterial surface. We hypothesized that adsorption sites responsible for the high protein binding affinity of Ca-P ceramics can be blocked by adsorption of an inactive protein as albumin prior to rhBMP-2 adsorption, resulting in a lower rhBMP-2 binding affinity and enhanced release. Furthermore, we hypothesized that *in vivo* rhBMP-2 release kinetics can be assessed in a reliable and longitudinal fashion using ^{131}I-labeled rhBMP-2 in combination with gamma-camera scintigraphy.

Scintigraphy of ^{131}I is generally used in nuclear medicine and allows accurate quantification of radiolabeled molecules. Radioiodination of rhBMP-2 is usually done with isotope ^{125}I, but essentially identical as labeling with ^{131}I, which has more appropriate physical characteristics for gamma camera measurements, mainly because the energy of the gamma emission is more suitable for scintigraphic imaging. A limitation of gamma camera measurements, however, is that the

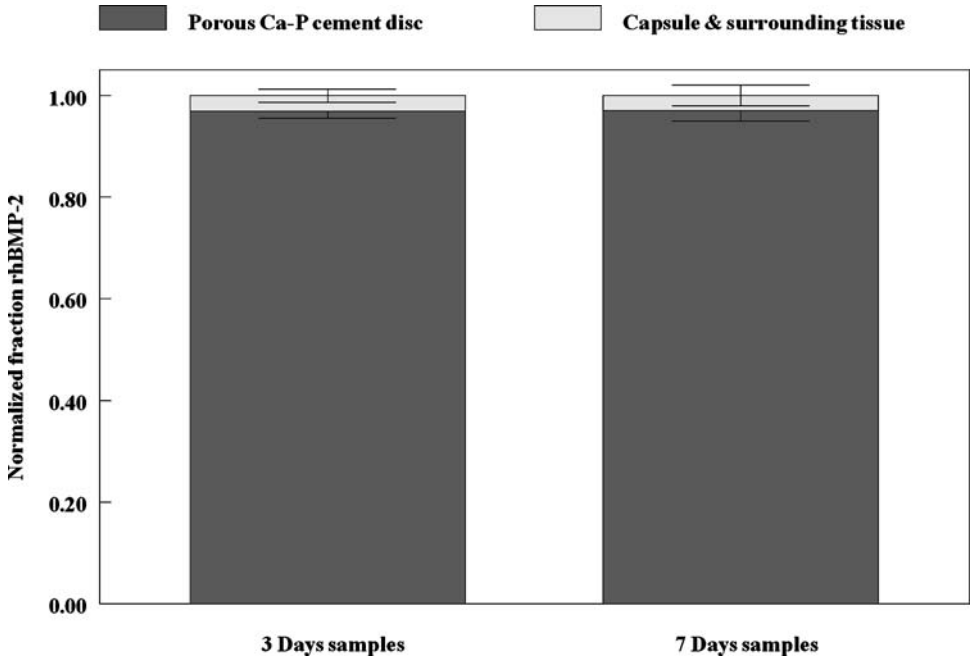

Fig. 5 Distribution of rhBMP-2 retained in porous Ca-P cement discs and rhBMP-2 present in capsules and surrounding tissues for samples retrieved after 3 and 7 days of implantation, expressed as the ratio of rhBMP-2 mass to total cumulative rhBMP-2 mass of disc, capsule and surrounding tissue. Error bars represent the mean and standard deviation for eight samples. No significant differences among groups are observed ($p > 0.05$).

camera resolution is relatively low and that the activity in the implant and the surrounding tissue can not be distinguished. Consequently, overestimation of the retained rhBMP-2 is a potential risk, as released protein in the vicinity of the implant is also counted and considered as retained protein. To investigate the residence time of rhBMP-2 in subcutaneous tissue, three animals received two subcutaneous depots of ^{131}I-labeled rhBMP-2. Scintigraphy showed, that only $8 \pm 12\%$ of the injected free rhBMP-2 was retained at the injection site after 24 h ($T_{1/2} = 0.3$ days). This indicates, that released protein will only reside in the vicinity during a short period before being cleared from the tissue. On the other hand, clearance within a capsule may be different from clearance from a subcutaneous injected depot. Therefore, additional rats were included in this study for separate *ex vivo* counting of the implant and the capsule with surrounding tissue. These measurements showed that the capsule and surrounding tissues contained $3.1 \pm 1.6\%$ of total rhBMP-2 present in implant and surrounding tissue, indicating that overestimation of scintigraphy due to released protein will be less than this percentage.

The retention of rhBMP-2 measured by scintigraphic imaging in the current study was $12 \pm 4\%$ higher for each formulation and time period than that determined by conventional *ex vivo* counting. Most likely, scattering of radioactive sources in the vicinity of the measured object is responsible for this limited overestimation in the scintigraphic analysis. Limitation of the number of implants per animal, and increase of distances between implants can reduce the effect of scattering and further enhance the accuracy of the measurements. Obviously, this will increase the number of animals required for sufficient statistical power. Nevertheless, we conclude that scintigraphy of ^{131}I-labeled rhBMP-2 provides a reliable and straightforward method for longitudinal measurement of rhBMP-2 release kinetics. This longitudinal technique reduces the amount of experimental animals and growth factors required as compared to *ex vivo* counting of retrieved specimens.

Albumin can be considered as a relatively inert protein, which expresses its principle functions as carrier protein and regulator of osmotic blood pressure rather by its quantity than by its specificity. [26] Moreover, any implanted biomaterial is directly covered with a layer of extracellular proteins, of which albumin is the most abundant. [27–30] Therefore, we assumed that adsorption of serum albumin prior to implantation would not dramatically alter the tissue response compared to control material covered by albumin after implantation. Meanwhile, we assumed that the interaction between rhBMP-2 and the Ca-P cement would be weaker after albumin pretreatment, leading to enhanced release of rhBMP-2.

In previous studies, we carried out *in vitro* release assays in protein-free buffer solutions (e.g. PBS) as frequently done in investigations of release kinetics. Based on the results of those studies, we assumed that Ca-P cement showed a high retention of growth factors. The current *in vitro* release study appeared to support this aforementioned suggestion. In PBS, a limited release of rhBMP-2 from porous Ca-P cement was observed, comparable with our earlier experiment. On the other hand, substitution of the incubation medium with FCS resulted in a significantly higher release pattern. This finding corroborates with observations of Lind *et al.* [31] and Laffargue *et al.* [32] who observed similar differences

investigating release of various growth factors from Ca-P ceramic surfaces, and suggested that serum proteins could enhance desorption of growth factors. Further, the *in vitro* release of rhBMP-2 was somewhat accelerated by pretreatment of the porous Ca-P cement surface with serum albumin, which seemed to prove our initial hypothesis. On the other hand, we also investigated the *in vivo* release of rhBMP-2 and compared the results with the *in vitro* data. Striking differences in release kinetics were observed. Evidently, *in vitro* release in serum still underestimated the release rate occurring *in vivo*. In addition, differences observed among pretreated and control scaffolds were not found *in vivo*. This underlines that *in vitro* release studies of growth factors from Ca-P ceramics only represent the *in vivo* release kinetics to a limited degree. Similar observations were reported by Li *et al.*, who investigated release of rhBMP-2 mixed through α-BSM, a highly resorbable Ca-P cement [16] After a period of 21 days, they found an *in vitro* release in serum/DMEM of less than 10% and an *in vivo* release of more than 90% in a rabbit ulnar defect model. Based on the results of the current study, we strongly recommend that *in vitro* release studies with Ca-P ceramic materials should be performed in a protein-rich buffer solution similar to body fluids and that one should be reluctant when extrapolating the data to the *in vivo* situation.

Furthermore, we have to emphasize that not all the observations in the present *in vivo* study correspond with observations by others who carried out *in vivo* release studies with rhBMP-2 loaded Ca-P ceramics. For example, Louis-Ugbo *et al.* [8] observed an initial release of only 10% after 1 day from rhBMP-2 loaded biphasic calcium phosphate granules in a spinal fusion model in rabbits. On the other hand, Li *et al.* determined a burst release of rhBMP-2 loaded α-BSM of approximately 40% within 1 day in a rabbit ulnar defect [16], while Uludag *et al.* [2] even observed a burst release of more than 70% within 3 h from various rhBMP-2 loaded Ca-P ceramics implanted subcutaneously in rats. Also, the subsequent release phase observed in the present *in vivo* study was different from that reported in other *in vivo* studies. We observed a retention of 20–30% after 28 days, whereas the investigators mentioned above found retention of less than 10% within that time period. Moreover, pharmacokinetics were described by a two phase exponential release model by Uludag *et al.*, whereas a one-phase exponential release model fitted the data best in the current study. This illustrates that growth factor release kinetics are complex and may be influenced by many variables. A definite explanation for the discrepancy in results is difficult to give, because the current study was not designed to elucidate the protein-Ca-P binding mechanism. Moreover, the pharmacokinetic model used in this study does not describe the mechanism responsible for the rhBMP-2 release. Therefore, we can only hypothesize that competition of protein binding to the adsorption sites in combination with complex Ca-P dissolution and precipitation processes occurring *in vivo* can cause differences in protein release characteristics. [33, 34] The biological relevance of these differences in release profile is difficult to predict due to the diversity in chemico-physical properties of the used carriers, but should be a topic of future research.

The clinical importance of long term retention as observed in the current study remains unclear and can be studied in future investigation. Currently, we assume that the life cycle of rhBMP-2 does not exceed four weeks and that no additional bioactivity should be expected of proteins retaining for a longer time period. For that reason, we do not consider the differences in extrapolated plateaus between control Ca-P cement discs ($P = 31 \pm 5\%$) and albumin pretreated discs ($P = 16 \pm 10\%$) as biologically relevant.

5. Conclusions

Scintigraphy of ^{131}I-labeled rhBMP-2 provides a reliable and straightforward method for longitudinal measurement of rhBMP-2 release kinetics *in vivo*. Although albumin pretreatment of porous Ca-P cement results in increased initial release of rhBMP-2 *in vitro*, *in vivo* release kinetics are similar compared to non-pretreated porous Ca-P cement. Preset rhBMP-2 loaded porous Ca-P cement discs exhibit one phase exponential release kinetics in the rat ectopic model, characterized by a retention of 20–30% after 28 days.

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