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Guided tissue engineering for healing of cancellous and cortical bone using a combination of biomaterial based scaffolding and local bone active molecule delivery

Deepak Bushan Raina\textsuperscript{a,*}, Irfan Qayoom\textsuperscript{b}, David Larsson\textsuperscript{a,c}, Ming Hao Zheng\textsuperscript{d}, Ashok Kumar\textsuperscript{b}, Hanna Isaksson\textsuperscript{a,e}, Lars Lidgren\textsuperscript{a}, Magnus Täg\textsuperscript{a}

\textsuperscript{a} Lund University, Faculty of Medicine, Department of Clinical Sciences Lund, Orthopedics, Lund 22185, Sweden
\textsuperscript{b} Indian Institute of Technology Kanpur, Department of Biological Sciences and Bioengineering, Kanpur, UP 208016, India
\textsuperscript{c} Umeå University, Faculty of Medicine, Umeå 90187, Sweden
\textsuperscript{d} University of Western Australia, Faculty of Health and Medical Sciences, Crawley, WA 6009, Australia
\textsuperscript{e} Lund University, Department of Biomedical Engineering, Lund 22100, Sweden

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**ABSTRACT**

A metaphyseal bone defect due to infection, tumor or fracture leads to loss of cancellous and cortical bone. An animal model separating the cancellous and cortical healing was used with a combination of a macroporous gelatin-calcium sulphate-hydroxyapatite (Gel-CaS-HA) biomaterial as a cancellous defect filler, and a thin collagen membrane (CM) guiding cortical bone regeneration. The membrane was immobilized with bone morphogenetic protein-2 (rhBMP-2) to enhance the osteoinductive properties. The Gel-CaS-HA cancellous defect filler contained both rhBMP-2 and a bisphosphonate, (zoledronate = ZA) to prevent premature calus resorption induced by the pro-osteoclast effect of rhBMP-2 alone. In the first part of the study, the CM delivering both rhBMP-2 and ZA was tested in a muscle pouch model in rats and the co-delivery of rhBMP-2 and ZA via the CM resulted in higher amounts of bone compared to rhBMP-2 alone. Secondly, an established tibia defect model in rats was used to study cortical and cancellous bone regeneration. The defect was left empty, filled with Gel-CaS-HA alone, Gel-CaS-HA immobilized with ZA or Gel-CaS-HA immobilized with rhBMP-2 + ZA. Functionalization of the Gel-CaS-HA scaffold with bioactive molecules produced significantly more bone in the cancellous defect and its surroundings but cortical defect healing was delayed likely due to the protrusion of the Gel-CaS-HA into the cortical bone. To guide cortical regeneration, the cortical defect was sealed endosteally by a CM with or without rhBMP-2. Subsequently, the cancellous defect was filled with Gel-CaS-HA containing ZA and rhBMP-2 + ZA. In the groups where the CM was doped with rhBMP-2, significantly higher number of cortices bridged. The approach to guide cancellous as well as cortical bone regeneration separately in a metaphyseal defect using two bioactive molecule immobilized biomaterials is promising and could improve the clinical care of patients with metaphyseal defects.

1. Introduction

Bone tissue engineering involves an interplay of cells, biomaterials, bone active proteins and drugs to regenerate viable bone tissue \cite{1}. Different cell types and healing stages orchestrate bone regeneration \cite{2,3}. Biomaterials provide initial scaffolding in a bone void, which may or may not be suitable for load bearing, depending on their inherent mechanical properties \cite{4,5}. These scaffolds also provide a template for cells to migrate onto and start the repair process. Despite large progress in scaffold development, the osteoinductivity is limited.

Bone active proteins and drugs are required to provide cells with sufficient stimulus to regenerate large volumes of bone in humans and for achieving performance on par with autografts. The approved osteoinductive proteins include bone morphogenetic proteins-2 and 7 (BMP-2 and 7). After a brief successful stint in clinical application, their usage has been debated \cite{6} due to sub-optimal carrier systems, the use of supraphysiological doses, rebound osteoclast activity with concomitant premature resorption of bone and harmful side effects. An increase in incidence of cancer in patients treated with rhBMP-2 was reported \cite{7}, although a recent study analyzing a large patient
population treated with rhBMP-2 has shown contradictory findings [8]. Attempts have been made to prevent some of the side effects using bisphosphonates or anti-RANKL antibodies, focusing on silencing the osteoclast activity [9,10]. We recently demonstrated that a higher volume of mineralized tissue was induced by local co-delivery of BMP-2 and a bisphosphonate, zoledronic acid (ZA) using a porous biomaterial, and also allowed us to reduce the minimal effective local rhBMP-2 dose [11].

In recent years, metaphyseal bone defects have gained interest as a preclinical research focus especially due to bone cavities caused by resection of malignant tumors and infections. In a tibial metaphyseal defect in rats, we used a biphasic, microporous, slow release, calcium sulphate (CaS)/hydroxyapatite (HA) biomaterial to locally deliver ZA and a bisphosphonate, zoledronic acid (ZA) using a porous biomaterial, focusing on silencing the RANKL expression of the fabrication process of the collagen membrane is provided elsewhere [20]. Briefly, porcine connective tissue rich in collagen type I was cleaned of the fat, followed by denaturing the non-collagenous proteins using a mixture of 1% (v/v) sodium dodecyl sulphate and 0.2% (v/v) LiCl overnight at 4°C. The resulting tissue was processed in 0.5% (v/v) HCl solution for 30 min to denature the collagen, washed with deionized water and neutralized with 0.5% (v/v) NaOH solution. This collagen matrix was then subjected to mechanical stretching to reach desired dimensions, structure and alignment of the fibers. The tissue was immersed in a solution of 1% (v/v) HCl to ensure complete denaturation for 1 day. A dry CM was obtained by briefly treating it with acetone and air drying. At the end of the process, a CM with a thickness range of 200–400 μm was obtained. The structure of the CM was visualized using micro-CT imaging (Zeiss, Xrdia 520 Versa, Voxel size: 6 μm) with contrast enhancement using 10% potassium iodine solution for 10 min. The CM was further characterized using scanning electron microscopy (SEM) (Leica S260, Cambridge, U.K.) by sputter coating the CM with platinum [20]. The membrane contains one rough side with randomly distributed collagen bundles that form a rough/porous structure (Fig. 1A, C, E), which enable cell attachment and settlement. The membrane further contains a smooth side (Fig. 1A, B, D) consisting of aligned collagen bundles forming a knitted structure (Fig. 1F). The application of the CM in bone as well as its carrier properties in delivering rhBMP-2 and ZA has not been tested before.

The second biomaterial is a supermacroporous cryogel consisting of crosslinked gelatin-CaS-HA (Gel-CaS-HA) prepared via cryogelation [11,22]. Preparation, characterization and carrier properties of this biomaterial have been described elsewhere [11]. Briefly, the material is fabricated by mixing the polymeric (gelatin) and inorganic components (CaS/HAp) to form a slurry, after which a crosslinker (glutaraldehyde) is added and the polymerization occurs at sub-zero temperatures (−20 °C) [11]. After an incubation period of 12 h under cryo conditions, the crosslinked matrix is thawed at room temperature followed by re-freezing and freeze-drying. This process produces a spongy scaffold with a porous structure ranging from a few microns to approximately 100 μm, as shown earlier with SEM [11]. The delivery of rhBMP-2 and ZA via the Gel-CaS-HA scaffold has been described earlier in an extra-osseous, muscle pouch model [11] but local delivery of bone active molecules in a bone defect has not been performed to date.

2.2. Biomaterials used in the study

In this study we evaluated two biomaterials. The first biomaterial consists of a collagen membrane (CM) (Porcine Collagen Type I) developed at the University of Western Australia [20,21]. A detailed description of the fabrication process of the collagen membrane is provided elsewhere [20]. Briefly, porcine connective tissue rich in collagen type I was cleaned of the fat, followed by denaturing the non-collagenous proteins using a mixture of 1% (v/v) sodium dodecyl sulphate and 0.2% (v/v) LiCl overnight at 4°C. The resulting tissue was processed in 0.5% (v/v) HCl solution for 30 min to denature the collagen, washed with deionized water and neutralized with 0.5% (v/v) NaOH solution. This collagen matrix was then subjected to mechanical stretching to reach desired dimensions, structure and alignment of the fibers. The tissue was immersed in a solution of 1% (v/v) HCl to ensure complete denaturation for 1 day. A dry CM was obtained by briefly treating it with acetone and air drying. At the end of the process, a CM with a thickness range of 200–400 μm was obtained. The structure of the CM was visualized using micro-CT imaging (Zeiss, Xrdia 520 Versa, Voxel size: 6 μm) with contrast enhancement using 10% potassium iodine solution for 10 min. The CM was further characterized using scanning electron microscopy (SEM) (Leica S260, Cambridge, U.K.) by sputter coating the CM with platinum [20]. The membrane contains one rough side with randomly distributed collagen bundles that form a rough/porous structure (Fig. 1A, C, E), which enable cell attachment and settlement. The membrane further contains a smooth side (Fig. 1A, B, D) consisting of aligned collagen bundles forming a knitted structure (Fig. 1F). The application of the CM in bone as well as its carrier properties in delivering rhBMP-2 and ZA has not been tested before.

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2. Materials and methods

rhBMP-2 from the Infuse® Bone Graft kit (Medtronic, Ireland), ZA (0.8 mg/mL) (Novartis, Switzerland), pentobarbital sodium (60 mg/mL), diazepam (5 mg/mL) ketamine hydrochloride (50 mg/mL) (Intervet), xylazine hydrochloride (20 mg/mL) (Bayer) and buprenorphine (0.3 mg/mL) (Scherer Plough) were purchased from the pharmacy (Apoteket AB, Sweden). Collagen Membrane (CM) was kindly provided by Ortho Cell Australia. Male Sprague-Dawley rats were purchased from Taconic (Denmark).

2.1. Study plan

The study was divided in following sections based on the aims of the study: 1) Analyze the feasibility of a collagen membrane in delivering rhBMP-2 and ZA in an ectopic muscle pouch model, 2) Evaluate the carrier properties of a supermacroporous biomaterial in delivering locally rhBMP-2 and ZA in a metaphyseal bone defect with an aim to guide cancellous bone regeneration. 3) Evaluate the potential of the collagen membrane delivering low dose rhBMP-2 to guide cortical bone defect healing in the same defect model.

2.3. Sample preparation, animal models and surgical procedures

2.3.1. Ectopic abdominal muscle pouch model

Pre-sterilized circular pieces of CM were cut with a biopsy punch (diameter 4 mm). The animals were divided into following groups: 1. CM with saline, 2. CM containing 10 μg rhBMP-2 (CM + rhBMP-2 (10 μg)) and 3. CM containing 10 μg rhBMP-2 and 10 μg ZA (CM + rhBMP-2 (10 μg) + ZA (10 μg)). In the CM + rhBMP-2 group, a total of 60 μg rhBMP-2 was reconstituted in 75 μL saline to a concentration of 0.8 mg/mL. From the stock solution, 12.5 μL of the saline containing 10 μg rhBMP-2 was pipetted on each piece of the membrane. In group 3 with rhBMP-2 and ZA, a total of 60 μg rhBMP-2 was solubilized in 75 μL of ZA (concentration of 0.8 mg/mL supplied as a reconstituted solution in saline) to a concentration of 0.8 μg/mL. A total of 12.5 μL of this solution containing 10 μg rhBMP-2 and 10 μg ZA was pipetted on each membrane. Scaffolds were incubated with the rhBMP-2 and rhBMP-2 + ZA solution for at least 30 min at room temperature to allow for homogenous soaking of the material prior to implantation. Samples belonging to the only CM group (group 1) were incubated with 12.5 μL of saline. The volume of the liquid pipetted on
the membranes in all groups was just enough to cover the membranes without overflowing.

Ten male Sprague-Dawley rats with average weight of 351 ± 9 g were used. Animals were anaesthetized using a cocktail of pentobarbital sodium and diazepam administered via the intra peritoneal route. A midline abdominal incision approximately 1.5 cm long was made and a muscle pocket (approximately 5 mm wide) in the rectus abdominis on each side of the midline separated by a minimum distance of 1.5 cm was created using scalpels. Five animals received CM alone in the left pocket and CM + rhBMP-2 (10 μg) in the right pocket. Likewise, five more animals were implanted with CM alone in the left pocket and CM + rhBMP-2 (10 μg) + ZA (10 μg) in the right pocket. All membranes were implanted in a flat fashion within the muscle pouch. The muscle and skin wounds were closed using non-resorbable sutures and animals had free access to food pellets and water immediately post-operation through the duration of the experiment. Animal sacrifice was performed using CO₂ asphyxiation 4-weeks post implantation. Harvested specimens were cleaned of surrounding muscle tissue, wrapped in saline soaked gauze and stored in 5mL Eppendorf tubes followed by radiography and micro-CT imaging on the same day.

2.3.2. Tibia defect model

Gel-CaS-HA scaffolds were cut into cylinders measuring 4 mm in diameter and 3 mm in height. This was done by cutting the scaffold into cylinders of 3 mm height using a sterile surgical blade following which a biopsy punch with a diameter of 4 mm was used to obtain a cylinder with 4 mm diameter and 3 mm height. Scaffold sterilization was performed by incubating the scaffolds in 70% EtOH overnight followed by two quick changes of 99.5% EtOH for 20 min each. Scaffolds were then air dried in a laminar hood. Pre-sterilized circular pieces of CM measuring 6 mm in diameter were cut using a biopsy punch. Immobilization of the Gel-CaS-HA and CM materials with bone active molecules was performed as per the dosages specified in Table 1. In G1, the defect was left untreated. In G2, the Gel-CaS-HA scaffold was incubated with 20μL saline for at least 30 min before implantation. In G3, 100μg ZA contained in 125μL saline (original concentration 0.8mg/mL) was mixed with 75μL saline (final concentration 0.5mg/mL) and 20μL of this solution containing 10μg ZA was pipetted on each of the Gel-CaS-HA scaffolds. In G4, 50μg rhBMP-2 was reconstituted in 125μL of ZA solution containing 100μg ZA following which 20μL of the mixture containing 5μg rhBMP-2 and 10μg ZA were pipetted on each scaffold. In groups G5 and G6, similar procedure followed during preparation of groups G3 and G4 were repeated, respectively with the only difference that a CM incubated with 10μL saline was applied as an endosteal cover. In G7, the Gel-CaS-HA scaffold was prepared by following the same steps described during the preparation of scaffolds in G3. Additionally, the CM pieces were incubated with rhBMP-2 solution. This solution was prepared by solubilizing 20μg rhBMP-2 in 100μL saline (concentration 0.2mg/mL). Each CM was incubated with 10μL of this solution containing 2μg rhBMP-2. In G8, 30 μg rhBMP-2 was suspended in 125μL ZA solution (pre-solubilized in saline) and further diluted with 75μL saline (final rhBMP-2 concentration 0.15mg/mL and final ZA concentration 0.5mg/mL). From this solution, 20μL containing 3μg rhBMP-2 and 10μg ZA were pipetted on each Gel-CaS-HA scaffold. To combine the CM with rhBMP-2 in G8, the same steps described for functionalizing the CM in G7 were followed. No overflow of the

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment and Doses</th>
<th>Sample Size (n)</th>
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<tbody>
<tr>
<td>G1</td>
<td>Empty</td>
<td>10</td>
</tr>
<tr>
<td>G2</td>
<td>Gel-CaS-HA</td>
<td>10</td>
</tr>
<tr>
<td>G3</td>
<td>Gel-CaS-HA + ZA (10 μg)</td>
<td>10</td>
</tr>
<tr>
<td>G4</td>
<td>Gel-CaS-HA + ZA (10 μg) + rhBMP-2 (5 μg)</td>
<td>10</td>
</tr>
<tr>
<td>G5</td>
<td>Gel-CaS-HA + ZA (10 μg)</td>
<td>10</td>
</tr>
<tr>
<td>G6</td>
<td>Gel-CaS-HA + ZA (10 μg) + rhBMP-2 (5 μg)</td>
<td>12</td>
</tr>
<tr>
<td>G7</td>
<td>Gel-CaS-HA + ZA (10 μg)</td>
<td>10</td>
</tr>
<tr>
<td>G8</td>
<td>Gel-CaS-HA + ZA (10 μg) + rhBMP-2 (5 μg)</td>
<td>10</td>
</tr>
</tbody>
</table>

Gel-CaS-HA denotes gelatin-calcium sulphate-hydroxyapatite biomaterial, CM denotes collagen membrane, ZA denotes zoledronic acid and rhBMP-2 denotes recombinant human bone morphogenic protein-2. The first 4 groups (G1-G4) were not covered with a CM endosteally.
bioactive molecule containing solution occurred during the immobilization process. After the addition of bioactive molecules, both Gel-CaS-HA and CM were incubated with the additives at room temperature for at least 30 min before implantation. Total number of experimental groups with sample size/group is also mentioned in Table 1.

A total of 82 male Sprague-Dawley rats with an average weight of 510 ± 16 g were used for the tibia defect model. Animals were anesthetized using a cocktail of ketamine (100 mg/kg) and xylazine (11 mg/kg) by intraperitoneal administration. The surgical procedure was similar to what has already been described by Horstmann et al. [12]. Briefly, the right knee was shaved and sterilized using chlorohexidine EtOH. A skin incision measuring approximately 1 cm was made medially at the proximal tibia starting at the knee joint. Small layer of muscle was scraped using scalpels after which the periosteum was rigorously scraped in both proximal and distal directions to expose the flat surface of the tibia. Drilling was performed near the insert of the medial collateral ligament. Using a handheld drilling burr (Ø 4.5 mm), the cortical bone and the underlying cancellous bone was drilled until the posterior cortex was reached. This gave a circular cortical defect of 4.5 mm in diameter extending 3 mm downwards into the cancellous bone. The wound was cleaned using sterile gauze and either left empty or filled with Gel-CaS-HA based on the groups described in Table 1 and the whole experiment is schematically presented in Fig. 2.

The diameter of the Gel-CaS-HA (4 mm) was intentionally kept smaller than the defect diameter (4.5 mm) in order to allow for the swelling of the biomaterial and to ensure the biomaterial would fit in the defect without any micro-structural damage that would affect the porous structure of the material. In the groups involving CM, once the cancellous defect was filled with Gel-CaS-HA and bioactive molecules (Table 1, G5-G8), a pre-cut piece of CM with (G7, G8) or without rhBMP-2 (G5, G6) was placed on the defect with smooth side facing the Gel-CaS-HA in the marrow cavity and rough side facing the muscle. The CM was carefully packed under the endosteum in a circular fashion using a blunt, flat end elevator ensuring that the Gel-CaS-HA did not protrude outwards into the cortical defect (Fig. 2). At this stage, the muscle wound was closed using a single resorbable suture and the skin incision was closed using single mattress sutures. After 8-weeks of healing time, the animals were sacrificed using CO2 asphyxiation.

2.4. X-ray and micro-CT

The radiographs (top view) of the CM specimens harvested from the muscle pouch after 4-weeks of implantation were obtained using the scout view of a micro-CT scanner (nanoScan, Mediso Medical Imaging System, Budapest, Hungary). Subsequently, samples were scanned in the same micro-CT instrument (circular scan, voltage 65 kV, exposure time 1300 ms, 720 projections, maximum zoom). Images were reconstructed using a RAMLAK filter with 100% cut-off (isotropic voxel size 10 μm). In the tibia defect model, the right proximal tibiae were harvested at 8-weeks post defect creation and subjected to micro-CT imaging with the same settings as above, but with 480 projections. The images were analyzed to quantify the extent of bone formation in the defects as described below.

2.5. Region of interest (ROI) selection and image analysis

2.5.1. Ectopic abdominal muscle pouch model

DICOM images were converted to bitmap (BMP) images using imageJ (National Institute of Health, U.S.A) followed by importing the images to CTAn (v.1.9.1.0 Skyscan, Belgium). Due to the ectopic location of the CM in the muscle pouch, the entire volume of the harvested specimen above a predefined grayscale threshold (90–255) was considered as newly mineralized tissue for quantification. The threshold was defined based on visual inspection. Bone volume (BV) was used as an outcome variable.

2.5.2. Tibia defect model

Images from all samples were aligned in Dataviewer (v. 1.4, Skyscan, Belgium) and imported to CTAn (v.1.9.1.0 Skyscan, Belgium) for further analysis. Three separate regions of interest (ROIs) were defined for the analysis of new bone formation in the tibia defect model. ROI1 (Defect ROI) consisted of a conically shaped ROI in coronal view, starting with 4.5 mm diameter at the bottom of the old cortex, extending down into the defect for 2 mm with the smallest diameter at the bottom being 1.5 mm (Fig. 3, top row). Due to the triangular anatomy of the tibia, ROI 1 was dynamic (conical with larger diameter at the top compared to the bottom) with varying diameters at the top and bottom to avoid including old cortical bone in the analysis and only study bone formed within the defect.

![Fig. 2. Schematic of the tibia defect model emphasizing the surgical technique, defect dimensions and biomaterial based approach for guiding both cancellous and cortical regeneration.](image)

![Fig. 3. A schematic explaining the micro-CT based regions of interests (ROIs) used to evaluate the extent of bone formation in the tibia defect model.](image)
ROI2 (Cortical ROI), was a 4.5 mm diameter (coronal view) circle starting from the bottom of the old cortex and extending outwards to quantify the cortical bone/callus formed in the cortical defect (Fig. 3, middle row). The height of ROI2 was variable to include all newly mineralized tissue formed in the cortical/callus region.

ROI3 (6.5 mm full bone ROI) included the full extent of bone formation proximal (1 mm) and distal (1 mm) to the implanted scaffold (Fig. 3, bottom row). Besides, ROI3 included newly formed trabecular and cortical bone as well as the original bone. A square shaped ROI measuring 8 mm × 8 mm was drawn in the trans-axial view and the height of the ROI was chosen to be 3.25 mm proximal and 3.25 mm distal from the middle of the defect (total 6.5 mm).

Thresholding was set at 100–255 in all images, as determined by visual inspection. Bone volume (BV)/Tissue volume (TV) (%) was used as an outcome variable in ROI1, whereas Bone volume (BV) was used as an outcome variable for ROI2 and ROI3 due to variable tissue volume.

### 2.6. Assessment of cortical healing

An orthopedic surgeon (MT) assessed healing of the cortical defect and defects were classified as bridged or not bridged [12]. Assessment was performed based on the micro-CT images from approximately the middle of the defect in the sagittal and trans-axial view where the defect width was largest.

#### 2.7. Representative histological evaluation

Muscle pouch specimens and the tibia samples were fixed in a pH-neutral 4% formaldehyde solution for 24 h. Following fixation, samples were placed in a 10% (w/v) Ethylenediaminetetraacetic acid (EDTA) solution (room temperature) buffered to pH 7.3–7.4 for 2-weeks for the muscle pouch samples and 5 weeks for the tibia defect samples with regular replenishments of EDTA solution every 3rd day. Some samples (especially ZA containing specimens) from the tibia defect study were not decalcified by 5-weeks, due to which, a second decalcification step was added wherein the EDTA-solution was removed, samples washed in deionized water and then placed in a 5% (v/v) formic acid solution for 24 h. Once the decalcification was deemed complete by physically testing the samples (poking with a needle and bending), specimens were washed for 24 h in deionized water. Dehydration of the samples was performed using an increasing EtOH gradient ranging between 70 and 99%, followed by xylene treatment. Finally, samples were embedded in paraffin using routine procedures.

Paraffin-embedded tissue samples were sectioned to 5 μm thickness using a microtome (HM355S, Thermo Scientific, U.S.A). Sections were collected on super frost microscope slides (Thermo Scientific, U.S.A), allowed to attach to glass slides on a slide warmer for at least 1 h followed by incubation at room temperature for at least 24 h. Sections were then deparaffinized and rehydrated using standard procedures with a decreasing EtOH gradient, followed by staining with hematoxylin and eosin (H&E), dehydrated again, cleared in xylene and mounted. Additionally, in the tibia defect study, collagen matrix staining using Picrosirius red was performed per the manufacturer’s protocol (Abcam, U.K).

### 2.8. Statistical methods

Power calculations for estimation of group sizes were based on our previous studies, which included comparisons between multiple treatment groups [12,23]. Data is presented as mean ± standard deviation (SD) unless otherwise stated. Micro-CT data from the tibia defect model were first evaluated for normality using Shapiro–Wilk test on the residuals and the distribution of the residuals. Normally distributed data were tested using ANOVA with Tukey post-hoc or Games-Howell post-hoc test based on the homogeneity of variances (Levene’s test) [12]. When data were not normally distributed, Kruskal–Wallis multi sample test was used. Micro-CT data from the abdominal muscle pouch model comparing only two groups were tested using Mann-Whitney U test, where a non-parametric test was chosen due to the small sample size.

#### 2.9. Animal ethics statement

Both the abdominal muscle pouch model (permit number: M124-14) and the tibia defect model (permit number: M79-15) were approved by the Swedish board of agriculture. Animals had free access to regular food pellets and water throughout the duration of the experiments. Animals were housed two/cage with 12 h light/darkness cycles.

### 3. Results

#### 3.1. Ectopic abdominal muscle pouch model: establishing CM as a carrier

This experiment evaluated if a CM can be used to deliver rhBMP-2 and ZA and subsequently lead to bone formation in an ectopic muscle pouch model. Radiographs taken at the time of micro-CT scanning showed no radiolucency in the CM alone group (Fig. 4A, top). Identifying them in the muscle pouch after 4-weeks was also difficult. CM + rhBMP-2 group showed scattered radiolucency in different regions of the material and prominent radiolucency towards the edges (Fig. 4A, middle). CM + rhBMP-2 + ZA group exhibited highest radiolucency throughout
the volume of the specimens (Fig. 4A and bottom). Samples that exhibited radiographic bone formation were further scanned using a micro-CT scanner and CM + rhBMP-2+ZA (12.6 ± 5.1 mm³) group produced significantly higher bone volume (BV) compared to the specimens in the CM + rhBMP-2 (1.2 ± 0.7 mm³) group (p < 0.01) (Fig. 4B). Histologically, CM alone did not show any bone formation and the scaffold was infiltrated with fibrous-like tissue at the extremities (Fig. 4C). In the CM + rhBMP-2 group, prominent bone formation was observed on the edges. The specimens were predominantly filled with marrow like tissue and small amounts of bone towards the middle of the specimen (Fig. 4C). Co-delivery of rhBMP-2 and ZA with the CM exhibited significant bone formation both at the sides as well as towards the middle of the specimens (Fig. 4C). Remnants of the CM were observed in all tested groups.

3.2. Tibia defect model: Gel-CaS-HA groups for cancellous bone regeneration (G1-G4)

The three groups (G2-G4) were used to test the ability of Gel-CaS-HA alone or immobilized with ZA or rhBMP-2+ZA in regenerating cancellous bone in a tibia defect model and compare it with the empty defect (G1).

3.2.1. Micro-CT

In the defect ROI (ROI1), all Gel-CaS-HA scaffold treated groups (G2-G4), irrespective of the addition of ZA (G3) or rhBMP-2+ZA (G4), showed significantly higher BV/TV when compared to the empty group wherein the defect was drilled and left empty to heal (Fig. 5A). In the cortical ROI (ROI2) i.e. new bone formation in the cortical defect, groups G1-G4, G3 and G4 exhibited significantly higher BV compared to the empty group and G4 also had significantly higher BV compared to G2 (Fig. 5B). In ROI3 i.e. full 6.5 mm bone ROI, BV was significantly higher in the scaffold groups immobilized with ZA (G3) and rhBMP-2+ZA (G4) when compared to G1. G4 also had significantly higher BV than scaffold alone (G2). No significant differences were seen between G1 and G2 as well as between G3 and G2 (p = 0.06) (Fig. 5C).

3.2.2. Cortical bone healing

Representative images of the extent of cortical healing from each group is shown in Fig. 6. In the empty group, all cortices healed with a thin neo-cortex. Addition of Gel-CaS-HA in the medullary compartment impaired cortical healing and the scaffolds were protruding outwards into the cortical ends in several specimens.

3.2.3. Representative histology

In the empty group (G1), no signs of new bone were seen in the medullary cavity (Fig. 7). The defect was filled with marrow like tissue. On the cortical side, a thin neo-cortex was formed, which covered the entire width of the defect (Fig. 7).

In G2 (Gel-CaS-HA alone), new cancellous bone was seen in close proximity to the scaffold. Bone was also observed within the pores of the scaffold. Some new cortical bone covered the cortical defect, however a large portion of the cortical defect was not healed and was filled with fibrous tissue like structures. Remnants of the scaffold in the cancellous defect were also visible (Fig. 7, G2).

In G3, the medullary compartment was filled with large amounts of trabecular bone (yellow *) especially around the scaffold. Most of the new bone was seen in close proximity to the scaffold edges regenerating outwards from the scaffold. Large parts of the scaffold remained unresorbed. Similar to G2, the cortical defect in G3 also remained unbridged with only some cortical regeneration (Fig. 7, G3).

Similar to G3, the extent of cancellous bone regeneration in the medullary compartment was higher in G4 compared to G1 and G2. The marrow cavity was filled with new cancellous bone proximal and distal to the scaffold and remnants of scaffold were visible. Incomplete bridging of the cortex was seen in the representative histological images (Fig. 7, G4).

In terms of collagen matrix deposition, the empty control group (G1) showed no collagen deposition within the defect or regions surrounding the defect but the cortical defect was bridged and rich in...
collagen (Fig. 8). G2 exhibited slight amounts of collagen deposited in and around the scaffold. Both groups G3 and G4 had abundant collagen deposition in the regions that exhibited new bone formation in Fig. 7. All gel-CaS-HA groups (G2-G4) demonstrated impaired cortical healing (Fig. 8).

3.3. Tibia defect model: Gel-CaS-HA + CM groups for cortical bone regeneration (G5-G8)

Groups G5-G8 were used to evaluate whether a CM alone or immobilized with low dose rhBMP-2 could aid in restoring cortical bone regeneration, which was not seen in G2-G4.

3.3.1. Micro-CT

In the defect (ROI1), the BV/TV was significantly higher in all Gel-CaS-HA and CM treated groups (G5-G8) compared to the empty group (Fig. 5A). No differences between G2-G8 were seen. In the cortical ROI (ROI2), the BV was significantly higher in G6 and G7 when compared to G1 and G2 (Fig. 5B). In ROI3, the BV fraction was significantly higher in groups G5-G8 when compared to the empty group (Fig. 5C). G5, G6 and G8 also had significantly higher BV when compared to G2.

3.3.2. Cortical bone healing

Fig. 9, shows representative micro-CT slices from G5-G8 showing the extent of cortical healing in each group and the total number of cortices healed/treatment group. It was evident that in the groups where rhBMP-2 was added on the CM (G7 and G8), a greater number of cortices healed with a prominent bony callus bulging outwards compared to the empty control and the rest of the groups treated with only Gel-CaS-HA (G2-G4). Addition of only CM to the ZA treated Gel-CaS-HA (G5) appeared to have a radio dense mineral precipitation in the region where the membrane was originally placed but cortical bridging was not achieved in that group. In groups G6-G8, a dual response was seen on the cortex with radio dense mineral precipitation in the areas where the CM was originally applied as well as an outer cortical shell, which bridged the entire defect in several cases (Fig. 9).

3.3.3. Representative histology

The cancellous bone regeneration in G5-G8 treated groups was similar to G3 and G4. Large amount of new trabecular bone was seen growing around the scaffold (Fig. 10). Large parts of the scaffold remained un-resorbed.

In G5 and G6, the cortical defect was not completely bridged (Fig. 10) but cortical regeneration in close proximity to the damaged cortical ends was seen creeping towards the center of the defect. In the representative images in Fig. 10, G7 and G8 showed completely bridged cortical defects as indicated by the yellow # symbols. In all CM treated groups i.e. G5-G8, remnants of the CM covering the defect were not seen indicating a possible resorption of the membrane after 8-weeks.

All CM treated groups with Gel-CaS-HA and ZA or Gel-CaS-HA and ZA + rhBMP-2 indicated abundant collagen deposition in the
cancellous defect, predominantly in the outer regions of the scaffold and its surroundings (Fig. 11). Complete cortical bridging with uniform picrosirius red staining in the cortical region of representative images from groups G7 and G8 was observed.

4. Discussion

4.1. Selection of bioactive molecules, doses and experimental groups

Experimentally, rhBMP-2 till date has remained as the most potent osteoinductive molecule capable of inducing bone formation in various anatomical sites including non-osseous sites like in the muscle [24]. It has never proven superior to autograft in randomized clinical studies regarding the rate of healing. The only FDA approved device for the delivery of rhBMP-2 today is the Medtronic® absorbable collagen sponge (ACS). A comparison of the in-vivo release kinetics of rhBMP-2 from the ACS with other recently developed biomaterials including Gel-CaS-HA, showed more sustained release of the protein by the latter [11]. With improved biomaterials as carriers, we might be able to show an improved effect of BMP-2 over autografts, reducing the genuine shortage of autografts available at surgery [25]. This is paving way for the desired off-the-shelf tools in the form of biomaterial carriers that can efficiently deliver rhBMP-2.

While one of the reasons for the underperformance of rhBMP-2 can be attributed to its carrier, also the supraphysiological doses used clinically induce osteoclast formation [26] and thereby premature bone resorption leading to an overall reduced net bone formation. Several studies have shown that by combining BMP with systemic or local ZA treatment [9,11,23,27,28], it is possible to hinder the excessive osteoclast activity thereby maintaining an increased net bone turnover. In a previous study using the Gel-CaS-HA in an abdominal muscle pouch model, we have shown that co-delivery of rhBMP-2 and ZA via the Gel-CaS-HA can reduced the effective rhBMP-2 doses by 4 times. TRAP staining confirmed the hypothesis and indicated reduction in the osteoclast associated TRAP activity in the rhBMP-2+ZA group [11].

This study used two distinct biomaterials delivering bone active molecules to guide cancellous and cortical bone regeneration. The doses for rhBMP-2 and ZA in the abdominal muscle pouch model and the tibia defect model in this study were taken from previously published studies by our group [11,12]. These are in line with other reports in experimental bone healing models in rats, including the abdominal muscle pouch model and a critical sized femoral diaphysis defect model [29,30] with locally delivered rhBMP-2. Zara et al. reported local rhBMP-2 doses of 22.5 μg/animal or higher led to local inflammatory reaction and cyst formation in a femoral defect model [29]. Regarding local ZA delivery, Perdikouri and co-workers recently used a femoral condyle defect in rats and treated it with increasing concentration of local ZA delivered via a biphasic CaS-HA biomaterial [31]. They reported a reduction in bone mineral density in the defect area with an
increase in local ZA doses. Belfrage et al., reported similar findings in a bone chamber model in rats [32]. These results suggest that local ZA delivery at higher doses can have a negative impact on bone formation. Whether it is possible to further reduce the doses of rhBMP-2 and ZA is only speculative at the moment. Taken together, the literature suggests that too high local doses of rhBMP-2 and ZA can potentially have negative effects on bone formation and lowering the doses further should be pursued.

The experimental groups in the abdominal muscle pouch model were based on previous studies [11,27,28] and CM + ZA group was not included. Earlier data showed that local delivery of ZA in an ectopic model of bone formation in rats did not induce bone (unpublished findings). In the tibia defect model, a group with Gel-CaS-HA + rhBMP-2 alone was not included since rhBMP-2 induces osteoclastogenesis and always leads to less bone formation compared to rhBMP-2 + ZA [11,23,27,28]. Although, results from the muscle pouch study indicated that CM + rhBMP-2 + ZA regenerates significantly higher amount of bone compared to the CM + rhBMP-2 group, CM + rhBMP-2 groups (G7 and G8) were chosen instead for the tibia defect study. The tibia defect model used in this study was a follow up of an earlier study wherein it was noted that cortical healing was impaired when the cancellous cavity was filled with a CaS/HA biomaterial with bioactive molecules [12]. Based on the results from that study, it was also noted that out of all treatment groups, CaS/HA + ZA was the only group with no specimen showing complete cortical bridging. It was hypothesized that ZA might not have a similar anabolic effect on cortical bone as compared to the cancellous bone and thus only CM + rhBMP-2 groups were used for cortical bone regeneration in G7 and G8.

4.2. Collagen membrane in the muscle pouch

In the first part of the study, we used an established abdominal muscle pouch model to perform a feasibility analysis of the collagen membrane (CM) and its carrier properties in-vivo. The ectopic muscle model is an efficient model to study the osteoinductive properties of a carrier biomaterial due to its extraosseous location [24]. The muscle pouch results indicated that the combination of CM with rhBMP-2 + ZA regenerated significantly higher bone volume when compared to CM + rhBMP-2 group without a bisphosphonate. This could be
attributed to the excessive osteoclastogenesis by rhBMP-2 [11, 27, 28].
As expected, CM alone did not show any radiographic signs of bone formation at all. Osteoinduction of biomaterials has been shown to occur in other large animal models without bioactive molecules but chemical signals from calcium phosphates either embedded in the biomaterials or precipitated on the biomaterial surface in-vivo appears to be necessary along with other physico-chemical properties like porous structure and surface properties [13, 15, 16, 33].

4.3. Gel-CaS-HA scaffold for cancellous bone regeneration

Most previous animal models used to study fracture healing are diaphyseal models dealing with non-unions or critical defects. However, metaphyseal bone regeneration differs from diaphyseal. Focusing on other indications like subchondral fractures, aseptic prosthetic loosening and bone loss after debridement of infections or tumors has led to an increased interest in metaphyseal bone regeneration as a different entity to diaphyseal. The metaphyseal bone is highly vascularized and contains a rich stem cell source. The tibia defect model described in this study has previously been used to evaluate the bone forming potential of a ceramic biomaterial consisting of CaS/HA [12]. We have earlier reported that local delivery of ZA and ZA + rhBMP-2 using a microporous CaS/HA ceramic biomaterial induced significantly higher volume of mineralized tissue in the defect when compared to empty group, allograft group and CaS/HA material alone [12]. Though the defect dimensions in the earlier study and the present study are different (cortical Ø 3 mm vs. cortical Ø 4.5 mm), none of the defects created in the two studies appeared to be critical defects, since the majority of the cortices in the empty group healed in both studies. Almost no cancellous bone regeneration was seen in the empty group emphasizing the need of scaffolding for bone tissue regeneration. It should be noted that the cortical healing was impaired in both studies, irrespective of the type of biomaterial used or whether bioactive molecules were added. We speculate that the biomaterials protrude out through the cortical defect thereby hindering the damaged cortical edges from completely bridging within the time frame of the study.

The BV/TV in the defect ROI (ROI1) in G2-G4 was significantly higher than the empty group indicating that the addition of ZA or rhBMP-2 and ZA thus does not have an effect on the ingrowth of bone into the scaffold. This could be because most of the new bone formed due to the addition of ZA (G3), and rhBMP-2 + ZA (G4) occurred outwards from the scaffolds, which is measured in ROI3. The effect of the addition of ZA and rhBMP-2 + ZA was more prominent in ROI3 (which includes regions proximal and distal to the scaffold) as seen both via micro-CT and representative histology images. The addition of ZA alone (G3) induced significant bone formation around the scaffold, indicating a possible anabolic role of ZA, which challenges the notion of ZA only being anti-catabolic [34–36].

4.4. Gel-CaS-HA scaffold combined with collagen membrane for cortical bone regeneration

With impaired cortical healing using Gel-CaS-HA from the first part of the study (G2-G4), we sought to guide cortical tissue regeneration with the aid of a CM placed endosteally at the defect site. The hypothesis was to use the CM as a barrier to prevent the scaffold from protruding outwards into the cortical defect and simultaneously provide a template for stem cells to populate the cortical defect. The release of rhBMP-2 would further accelerate the differentiation process of stem cells into osteoblast like cells thereby bridging the cortical defect. In G5 and G6, the CM prevented the Gel-CaS-HA scaffolds from bulging out into the defect. In the area originally covered with the membrane, a radio dense mineral precipitate forming a white rim was seen in the micro-CT images. Since histology was done on decalcified specimens, it was not possible to characterize the type of mineral deposition, although it was evident from histology that the white rim seen in the micro-CT image was not cortical bone. We could not identify the collagen membrane after 8-weeks, which might imply that the membrane leads to mineral precipitation early on but gets resorbed at a later time. Addition of rhBMP-2 to the CM in G7 and G8 led to complete cortical bridging seen as a bulging callus covering the entire cortical defect in 50% and 70% of specimens, respectively. Callus formation and cortical bridging are important aspects of indirect bone healing necessary to achieve complete repair [3].

Noteworthy is that while micro-CT results from the cortical ROI (ROI2) indicated some mineralization in the membrane only groups (G5 and G6), these groups did not score equally high as G8 in the visual assessment of cortical healing. This is most likely because the grayscale values in the micro-CT images are too similar to differentiate between low-density mineral precipitations on the CM from viable cortical bone. Furthermore, the large spread of data in G8 could also have contributed to these results.

Significant radiological and histological bone formation was noticed in all bioactive molecule treated groups. The Gel-CaS-HA biomaterial provides a spatio-temporal release of rhBMP-2 and ZA in-vivo and releases approximately 65% of rhBMP-2 over a period of 4-weeks, thereby providing a continuous supply of the osteoinductive molecule during the initial weeks of bone repair [11]. In terms of ZA delivery, the biomaterial released nearly 40% ZA on day 1 post implantation with almost no further release of ZA. rhBMP-2 delivery is necessary for osteogenic differentiation of progenitor cells and the constant presence of ZA is necessary for preserving the new bone formation. This is verified by significant bone formation in all bone active molecule treated groups (G3-G8). Bone was predominantly seen around the scaffold rather than in the middle of the defect, which was still covered by the unresorbed Gel-CaS-HA scaffold. Approximately 50% of the Gel-CaS-HA scaffold degraded after two months as seen from the in-vitro degradation experiment earlier [11]. It can be inferred that due to the slow progression of bone into the pores of the scaffold, bone preferentially grew in the regions around the scaffold. Maybe the resorption rate of the Gel-CaS-HA scaffold could be modulated further to achieve even more cancellous bone regeneration within the original defect. It must however be noted that most biomaterials are pre-tested in rodent models of bone healing, which have a faster healing rate than humans [37], and a fast resorbing biomaterial in rodent models might not necessarily be optimal for human use.

4.5. Possible source of cells

The cells responsible for healing of bone originate from various sources including the marrow canal, periosteam, skeletal muscle and blood vessels [3, 38]. The cancellous bone regeneration especially in G3-G8 i.e. rhBMP-2 and ZA treated groups, could possibly be due to the BMP based recruitment and stimulation of stem cells from the medullary canal including the endosteum also shown by Yu et al., earlier [39]. ZA at low doses is also shown to induce osteogenic differentiation of mesenchymal stem cells (MSCs) [40]. Taken together, this justifies the source of cells for cancellous bone regeneration in this study.

Cortical healing is more complex and depends on several factors including mechanical stability of the bone and availability of healthy periosteam. Yu et al. reported that periosteal stem cells are a strong target for BMP-2 to form the fracture callus. Another study from Liu et al. suggested the role of myogenic cells from the Myo D lineage significantly (almost half the cells) contributes to fracture healing in an open fracture model with intentionally damaged periosteam [41]. In the present study, we scraped the periosteam both proximal and distal to the defect following which a hole was drilled into the bone. Many cortices did heal in the groups where CM with rhBMP-2 was used, and we speculate that the collagen membrane acted as a physical barrier for stem cells from the marrow cavity to migrate to the membrane and guide cortical healing. This is because the membrane is non-permeable to cells at least during the early phase before being degraded.
Furthermore, due to a physically damaged periosteum, we speculate that the cortical healing seen in G7 and G8, with rhBMP-2 delivered via the CM, is due to muscle derived stem cell population. Muscle cells possess BMP receptors and respond to BMP treatment [42]. In a compartmental (mechanically stable) defect in mice, cortical healing was impaired without the presence of MSCs from the marrow compartment and the defect was instead replaced by scar tissue [43]. This contradicts our findings and we speculate that cortical healing in G7 and G8 is muscle mediated and the defect used in our study is also somewhat mechanically stable. Lineage tracking studies are necessary to elucidate the cellular composition of both cancellous and cortical bone regenerated in this study.

4.6 Limitations

In the tibia defect model, we did not use a group wherein only CM was used to cover the defect without the presence of Gel-CaS-HA underneath. While the addition of this group could show the ability of the CM alone in healing of the defect, it was surgically difficult to ensure a firm endosteal placement of the membrane without a support from underneath. Also, this study was carried out using a specific collagen membrane and we did not compare the developed Gel-CaS-HA with the FDA approved absorbable collagen sponge in this model. However, their potential in locally delivering rhBMP-2 and inducing bone formation in the abdominal pouch model has been compared earlier [11].

5. Conclusion

In an ectopic muscle pouch model, we established that the dual delivery of rhBMP-2 and ZA via a collagen membrane (CM) regenerates higher bone volume in comparison to delivering rhBMP-2 alone. Secondly, a macroporous Gel-CaS-HA scaffold can be used to deliver ZA or ZA + rhBMP-2 for cancellous bone regeneration in a metaphyseal defect in the tibia. Addition of rhBMP-2 to the ZA in the scaffold does not provide an additive effect in cancellous bone regeneration. Addition of Gel-CaS-HA scaffold to the cancellous defect can impair cortical healing despite the addition of bone active molecules. In the empty group, all cortices healed but no cancellous bone regeneration was seen. It can thus be inferred that scaffolding and bone active molecule delivery is critical for cancellous bone formation. This holds true for cancellous bone regeneration but is not necessarily applicable for cortical bone regeneration. Protrusion of the Gel-CaS-HA scaffold through the cortical bone is suspected to interfere with the normal cortical bone repair process. The results indicated that a barrier in the form of a CM, delivering low dose rhBMP-2, at the endosteum and cortical bone interface at the defect site significantly enhances the cortical healing. Thus, we show a promising approach of combining a porous Gel-CaS-HA scaffold and a CM loaded with bone active molecules for guiding cancellous and cortical bone repair, respectively. This strategy could be translated into the clinical setting for improved treatment in patients with metaphyseal bone defects.

Author contributions

MT, LL, MHZ, AK, HI, DBR and DL designed the study. AK, IQ and DBR did the Gel-CaS-HA fabrication. MHZ was involved with the fabrication of CM. MT, DBR and DL performed animal surgeries. DL, DBR and HI performed micro-CT imaging, analysis pipeline and image analysis. DBR and DL contributed to histology. DBR wrote the first draft of the manuscript and all named authors contributed in revising the manuscript.

Disclosures

LL is a board member of Bone Support AB, Lund, Sweden. LL and MHZ are board members of Ortho Cell, Australia. All other authors have nothing to disclose.

Data availability

The data associated with this manuscript is available upon request to the corresponding author or the senior author.

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