Premixed calcium phosphate cement as a carrier of bone morphogenetic protein.

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Aims
Bone defects caused by bone loss due to trauma or tumours are common, with grafting of transplanted tissue currently as the gold standard for bone replacement. However, there are many issues inherent with autograft, including donor site morbidity, supply of tissue and infection [1]. In order to overcome these drawbacks, a diverse array of synthetic biomaterials is under development [2]. Calcium phosphate cements (CPC) are a group of injectable calcium phosphates [3]. CPCs are particularly advantageous due to their low setting temperature and ease of administration by syringe, which allow for minimally invasive surgery. Their physiological setting temperature also makes them convenient carriers for drugs such as bone morphogenetic protein 2 (BMP-2) [4], which some surgeons use routinely during surgery for its ability to promote differentiation of mesenchymal stem cells to osteoblasts. Our aim was to develop an easy-to-handle alloplastic material with osteoinductive properties, accelerating bone ingrowth by stimulating osteogenic differentiation.

Method
Surgical Procedures
The surgeries were performed following a previously published protocol [5]. Shortly, rats were weighed and anaesthetized with 1-2.5 % isoflurane, 1.0 l min-1 oxygen and 0.8 l/min nitrous oxide and placed on a 37° C heat pad during surgery. One dose of 225 mg/kg antibiotics (Zinacef, GlaxoSmithKline AB, Sweden) was administered subcutaneously. The legs were shaved and washed with chlorhexidine (Fresenius Kabi, Uppsala Sweden). A 2.5 cm long skin incision was made to expose the femur. Cortical bone defect was drilled through the anterior cortical bone with a 1.9 mm low-speed drill (Dormer, France). The defect was placed distal and anterior of the third trochanter in a standardized way and with a size of about 6 x 2 mm.

A total amount of approximately 40 µl of premixed CPC [6] with and without 6 µg of BMP-2 was placed in the defect in the left leg and right leg and the wound was sutured with resorbable 4.0 suture (Polysorb, Tyco Healthcare, Gosport, UK) subcutaneously and intracutaneously. 0.05 mg/kg buprenorphine (Temgesic, Sheringer Plough, Brussel, Belgium) was administered subcutaneously daily for 2 days for analgesia. The rats were killed after 5 weeks in a CO\textsubscript{2} chamber. Incisions were made at the knee and the hip to collect the femora, which were preserved in 4% paraformaldehyde (Histolabs Products AB, Gothenburg, Sweden). A total of 8 femora treated only with CPC (control) and 7 femora treated with CPC+BMP-2 (BMP-2) were collected.

Micro-computed tomography
All femora were rehydrated in saline solution for 24 h before the micro-CT acquisition. The femur was placed vertically into a plastic cylinder filled with saline solution and positioned inside the micro-CT (model Skyscan 1072, Skyscan, Kontich, Belgium). Rat femora were acquired using the following protocol: 60 kVp, 163 µA, 1 mm aluminium filter, exposure time...
5.9 s, frame averaging 2, rotation 180°, rotation step 0.45°, field of view 20×20 mm² and an isotropic pixel size of 8.93 μm. Reconstruction of cross sections was done using software package NRecon (SkyScan, Bruker, Kontich, Belgium).

A circular region of interest (ROI) with a diameter of 1 mm was centered over the CPC still present within the bone cavity. The volume of interest (VOI) investigated was composed of a stack of 225 consecutive ROIs, resulting in a cylindrical VOI of 1 mm in diameter and 2 mm in height. Porosity was calculated for the selected VOI, using a global threshold.

To determine the amount of newly formed bone, an elliptic ROI was placed to include the whole cortical shell. The VOI investigated was composed of a stack of 400 consecutive ROIs, centered at the middle of the defect. The images were binarized to separate the newly formed bone from the mature bone and the background, using a global thresholding procedure. Thresholds were visually determined for all acquired dataset and thereafter the average value was applied to all specimens. Bone volume (BV) was calculated using CTAn (SkyScan, Bruker, Kontich, Belgium).

Three-dimensional reconstructions of the samples were obtained using CTvox (SkyScan, Bruker, Kontich, Belgium).

Data was analyzed using the GraphPad Prism software package (version 5.0c). Values are given as mean ± standard deviation (SD). Student’s un-paired t-test was used (p < 0.05) to determine statistical significance.

**Results**

The porosity of the CPC left within the defect of the two groups was not significantly different (p=0.53, see Fig. 1A). Conversely, a significantly higher volume of newly formed bone was found in the group with BMP-2 (p=0.03 see Fig. 1B).

Differences in bone regeneration are also clearly visual (see Fig. 2). In the group where BMP-2 had been added a bone bridge covering the defect can be seen, while in the control group such a bone bridge does not seem to be present.

![Figure 1: Porosity (A) and Bone Volume (B) of Control CPC (red) and CPC with BMP-2 (blue).](image-url)
Figure 2: (Left) Femoral defect treated with CPC; (Right) Femoral defect treated with CPC and BMP-2.

**Conclusion**

The similarity in X-ray absorption of bone tissue and CPC made it difficult to separate the two materials. Calcium phosphate cements degrade within the body and will therefore have a lower density at the surface, similar to the density of the newly formed bone. This led to a repeated error in all the bone volume data. To our knowledge, there is no existing method to separate bone from calcium phosphate. Further analysis will be done trying to address this problem. Nevertheless, it was possible to determine a difference between the two groups, a difference that was also clearly visible in the pictures. These results are promising for a new easy-to-handle alloplastic material with osteoinductive properties.

**References:**