In vivo performance of a BP-linked hyaluronan-based hydrogel as carrier of bone morphogenetic protein-2

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Aims
Growth factors such as bone morphogenetic proteins (BMPs) [1] are often delivered during surgery by means of a collagen carrier, but rapidly diffuse away from the site of surgery [2]. Our aim was to develop a hydrogel with controlled release of BMP-2. This could be of great importance in the clinic where the highly controlled release could prevent side effects such as ectopic bone or immune response against the BMP-2 protein. Furthermore, bisphosphonates (BP) are used to prevent osteoporosis [3]. It would be interesting to investigate if they would have an effect on fracture healing. The aim of this study was to investigate the in vivo performance of hyaluronic acid (HA) hydrogel carrier of BMP-2 with and without BP compared to its analog lacking BP groups (control).

Method

Surgical Procedures
The surgeries were performed following a previously published protocol [4]. 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 hydrogel including BP [5] was used to fill the defect in one of the legs with the control in the other leg, with our without 6 µg of BMP-2. The wound was closed by 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 sacrificed after 30 days in a CO₂ 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).

Table 1: Hydrogel groups investigated.

<table>
<thead>
<tr>
<th>HA</th>
<th>HA-BP</th>
</tr>
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<tbody>
<tr>
<td>No BMP-2</td>
<td>CTRL (N=4)</td>
</tr>
<tr>
<td></td>
<td>BP (N=4)</td>
</tr>
<tr>
<td>BMP-2</td>
<td>CTRL-BMP (N=4)</td>
</tr>
<tr>
<td></td>
<td>BP-BMP (N=4)</td>
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**Micro-computed tomography**

All femora were scanned using a SkyScan 1176 (Bruker, Kontich, Belgium). The femur was placed in a plastic tube and then placed within the animal bed inside the micro-CT. Rat femora were scanned using the following protocol: 65 kVp; 385 µA; 1 mm aluminum filter; exposure time 987 ms; frame averaging 10; rotation 180°; rotation step 0.30°; field of view 20x20 mm²; and an isotropic pixel size of 8.67 µm. Reconstruction of cross sections was done using software package NRecon (SkyScan, Bruker, Kontich, Belgium).

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 unpaired t-test was used (p < 0.05) to determine statistical significance.

**Results**

No significant difference was found between the CTRL and BP groups. However, BMP-2 gave a significance difference in bone volume; CTRL compared to CTRL-BMP p=0.01, and BP compared to BP-BMP differed with p=0.01, see Fig. 1. These results correspond well with what can be seen in the images (see Fig. 3 and Fig. 4). In the groups without BMP-2 the defects were still open and only minor bone formation can be seen. Conversely, the femoral defects treated with the hydrogel containing BMP-2 were almost closed.

![Figure 1. Bone volume in for hyaluronan hydrogels; CTRL, CTRL-BMP, BP and BP-BM, (p<0.05, Student’s unpaired t-test, n=4)](image-url)
Figure 2: Rat femoral defect treated with hydrogels with and without BP and BMP after 4 weeks.
Conclusion
A procedure to distinguish newly formed bone from mature bone was identified. However, it is still in need of optimization, since the small differences in density make an automatic separation difficult.
Nevertheless, these preliminary results confirm that BMP-2 have a positive effect on bone regeneration [6]. Furthermore, BP hydrogels were found to have no effect on bone formation and could be a promising candidate as carrier of BMP-2.

References: