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Research Paper
Bone Regeneration

Endocultivation: the influence of delayed *vs.* simultaneous application of BMP-2 onto individually formed hydroxyapatite matrices for heterotopic bone induction

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Abstract. When bone morphogenetic protein (BMP) is delivered to matrices *in vivo* may affect tissue engineered bone constructs for jaw reconstruction after cancer surgery. This study compared the effects of BMP application at different times after matrix implantation for heterotopic bone induction in a rat model. Hydroxyapatite blocks were implanted unilaterally onto the surface of the latissimus dorsi muscle. A second block was implanted onto the contralateral muscle after 1, 2 or 4 weeks and 200 μg rhBMP-2 was injected into the blocks on both sides. Bone formation and density inside the blocks was analysed by CT and histology. 8 weeks after BMP application increases in bone density within the scaffolds were most pronounced in the simultaneous application group (179 HU). Less pronounced increases were observed for the 1 (65 HU), 2 (58 HU) and 4 (31 HU; $p < 0.0001$) week delay group. Homogeneous bone induction started from the central channel of the blocks. Capillaries and larger vessels were seen in all constructs, samples receiving delayed BMP treatment demonstrated significantly greater neovascularization. Delayed application of BMP was less effective for heterotopic bone formation than simultaneous application. A central channel allows homogeneous bone induction directly from the centre of the blocks.

Key words: endocultivation; heterotopic bone; bone induction; bmp; rat; delay; simultaneous; stem cells; bone density; vascularization; reconstruction; bone defect; intramuscular; extrasceletal tissue engineering; bone engineering.

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The *in vivo* tissue engineering of heterotopic bone by intramuscular endocultivation, where the patients serve as their own bioreactor, has yielded customized vascularized bone grafts that have already been used in humans for jaw reconstruction after ablative tumour surgery.[1–3] Midface reconstructions are difficult because of the complex anatomy.[4,5] Three dimensional (3D) planned reconstructions are being studied[6–8] based on computed tomography (CT) or magnetic resonance imaging (MRI) data.[9] The origin of materials used ranges from hydroxyapatite or derivates based on polylactic acid (PLA) or polylactic-co-glycolic acid (PLGA).[4]

This cultivated bone is mostly cancellous with no cortical layer. Excess bone formation is often observed outside the matrices.[2] It remains a challenge to achieve thorough and homogeneous bone induction throughout the entire graft.[3]In a pilot study, the authors administered BMP-2 for bone induction 4 weeks after the implantation of the scaffold (delayed application).[10] They used bovine hydroxyapatite (HAP) blocks but did not observe any improvement in bone induction with a delayed application. One reason was that excessive soft tissue ingrowth may have prevented the penetration of the BMP deep within the scaffolds. Earlier studies tested the use of a PLA membrane to avoid soft tissue ingrowth into HAP scaffolds placed onto the mandible. This technique enhanced homogenous bone formation.[11–13]

The results of the first study led the authors to modify the matrices[14] and examine the effect of delays shorter than 4 weeks (this study). The special design of the matrices included a central channel to allow BMP injection directly into the centre of the blocks and a porous structure.[14]

The novel aspect of this study was the administration of cytokines for bone induction and cell differentiation at several different time intervals. Usually, the scaffold and cytokines are implanted simultaneously at the same operation in animal models.[2,15] At the time of implantation no vascularization or osteoblasts are present in the centre of the scaffolds. The cellular machinery for bone formation has to migrate from the surface of the scaffold to the centre. The authors reasoned that a delay in the application of cytokines might allow undifferentiated mesenchymal cells to penetrate into the scaffold, thereby enhancing the number of stem cells within the scaffold at the time of application of the cytokines (BMP). The authors reasoned that should this occur, vascularization within the scaffold would be promoted and there would be an increased likelihood of homogenous osteogenesis within the scaffold, with consequently higher bone density.

The aim of this study was to evaluate heterotopic bone formation on a novel HAP scaffold produced using computer aided design (CAD) in a rat model. In particular, the influence of soft tissue ingrowth with delayed application of BMP-2 at different intervals was compared to the simultaneous placement of the matrix together with the BMP-2.

Materials and methods

24 female Lewis rats (4 months old, about 200 g each) were used for this study. The study protocol was in accordance with the German Animal Welfare Act (Animal Experiment Permit V-742-72241.121-14 (39-5/04)). All animals received food and water *ad libitum.* Every surgical procedure and each CT examination was performed under general anaesthesia with ketamine (10%, 1 μl/g body weight, intraperitoneal) and xylazine (2%, 0.6 μl/g body weight). The implanted HAP blocks were specially designed and produced as previously published.[16] A spray-dried granulate HA19 (BioCer Entwicklungs-GmbH, Bayreuth, Germany) was chosen as the raw material for 3D printing of HAP scaffolds.[17]

Each animal was implanted with a HAP block on the left side. After different delay periods allowing for soft tissue ingrowth in the first block, a second block was inserted on the right side and BMP was applied to both blocks (Fig. 1). This allowed the authors to examine simultaneous and delayed application of BMP and its effect on heterotopic bone formation *in vivo* in the same animal. The animals were separated into three groups (8 animals each): the first group had a delay period of 1 week, the second group a period of 2 weeks and the third group a period of 4 weeks. These groups are referred to as the '1 week delay', '2 week delay' and '4 week delay' groups. The delay period reflects the time given to allow for soft tissue ingrowth into the first block. The right side (simultaneous application) served as a control, where no time for soft tissue ingrowth was given.

The surgical procedure and the amount of rhBMP-2 solution applied are reported in earlier publications.[14] The skin was shaved and disinfected before surgery. Over a median incision on the thoracic spine, the latissimus dorsi muscle on the left side was displayed and a pouch above the muscle was created. A special CAD HAP block (about 2 cm × 1 cm × 1 cm) with one side rounded off and a central channel (Fig. 2) was implanted. The pouch and the skin were sutured separately.

In the second operation, a similar HAP block was inserted through the same approach on the right side. Additionally, 1 ml of rhBMP (containing

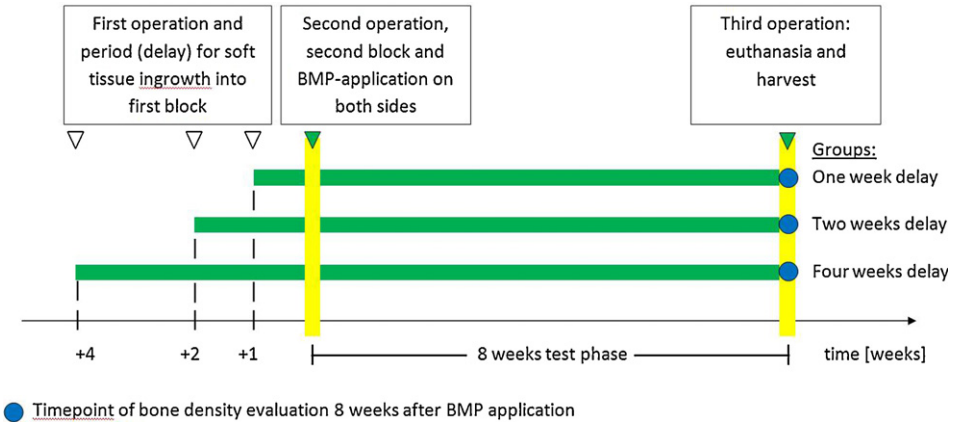

Fig. 1. Flowchart showing the experimental set up. Note the different intervals for soft tissue ingrowth and similar intervals for evaluation of the effects of BMP.