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αBSM failed as a carrier of rhBMP-2 to enhance bone consolidation in a sheep model of distraction osteogenesis

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Purpose: One of the problems associated with callus distraction is a long time period needed for consolidation of the newly formed bone. The goal of this study was to determine whether percutaneous injections of rhBMP 2 in αBSM would enhance bone consolidation. *Methods:* A unilateral tibial osteotomy combined with external stabilization was performed in 20 adult sheep. After a latency of four days, distraction was conducted for 20 days. Sheep were divided into three groups: group 1 received rhBMP-2/αBSM injections at day 23 and 30, group 2 buffer/αBSM injections at day 23 and 30 and group 3 did not receive any injection. The radiographs and *in-vivo* torsional stiffness measurements were obtained weekly during the following 50 days. Post-mortem bone densitometry (DXA) and mechanical testing were performed. *Results: In-vivo* stiffness assessments, DXA values and the maximum torsional moment of the sheep tibia treated with two rhBMP-2 injections were not significantly greater than those of both control groups. *Conclusions:* Presented application of rhBMP-2 in αBSM failed to enhance bone consolidation in distraction osteogenesis.

Key words: *rhBMP-2, growth factors, αBSM, bone consolidation, biomechanical testing, DXA*

1. Introduction

The process of generating viable osseous tissue by the gradual separation of osteotomized bone edges is called distraction osteogenesis. Associated with this procedure is a long time period needed for consolidation of the newly formed bone. Several investigators addressed this problem and tried to enhance callus maturation by mechanical or physical interventions, such as mechanical loading [9]. Furthermore, biological interventions were suggested. The application of growth factors expressed during distraction osteogenesis was supposed to enhance bone consolidation. Transforming growth factor β (TGF-β), IGF-1 and basic fibroblast growth factor (bFGF) were detected in osteocytes and osteoblasts obtained from the distraction zone in sheep and human [14]. Bernstein et al. asked whether bone healing in distraction osteogenesis can be accelerated by local application of IGF-1 and TGF-β1 [4]. They analysed the effect of locally applied IGF-1 and TGF-β1 from IGF-1/TGF-β1-enriched polylactide membranes on fracture healing in a sheep model of delayed callus formation and detected an accelerated bone healing for the application of growth factors. The authors concluded that locally applied TGF-β1 improves the mineral density of distraction gap and load to failure (energy absorbed during testing). In animal models, mRNAs of BMP-2 and BMP-4 were expressed throughout the entire distraction phase [5]. The effects for rhBMP have been shown in other contexts. One study showed that local administration of recombinant human bone morphogenetic protein-2 (rhBMP-2) in a rabbit distraction zone enhances bone

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mineral content (BMC) by almost 100% compared to untreated limbs [17]. A similar effect was found by administration of a single injection of BMP-7 to tibial rat distraction zones [20]. The effect of the injection of rhBMP-2 was demonstrated in a radiological, biomechanical and histological study on monkeys [27] and rabbits [21]. However, this effect could not be reproduced in a different model [10], whereas local delivery of adenovirus containing the gene for rhBMP-2 showed positive effects on mandibular callus formation [2]. Moreover, rhBMP-2 has been shown to consistently promote regeneration of skull, mandibular, and long-bone defects as well as fusion of vertebrae [3], [8].

The effects of BMP-2 largely depended on the carrier material used [25], [27]. Recently, Begam et al. concluded in their review that future clinical investigations are needed to define dose, scaffold and route of administration [3]. The usage of a biomimetic endothermically setting apatitic calcium phosphate bone substitute material (αBSM) showed enhanced bone formation and strength in a canine model of tibial osteotomy [6] and a rabbit ulna model [17]. Its injectability and ability to harden at body temperature in the presence of physiologic saline, and other buffering agents makes it an attractive clinical bone substitute and carrier for therapeutic agents in orthopaedic and dental applications.

Based on these successful enhancing methods we hypothesized that αBSM would be a valuable injection vehicle for rhBMP-2 administration in distraction osteogenesis. The goal of this study was to determine whether a series of two percutaneous injections of rhBMP-2 in αBSM carrier would enhance bone consolidation following tibial distraction in a sheep model. Specifically, we asked whether treatment with rhBMP-2/αBSM would lead to enhancement of biomechanical properties of the callus tissue throughout the consolidation period, and at the estimated completion of consolidation where the mechanical properties of the callus were approximately 50–70% of those of the intact contralateral limb.

2. Materials and methods

For this study a distraction osteogenesis model in adult sheep was chosen. Therefore, a unilateral, middiaphyseal tibial osteotomy stabilized with an external half-ring fixator (Smith+Nephew, Memphis, TN, USA) was performed in 20 adult, female sheep. All animal experiments were conducted with permission of local animal protection authorities according to US and German animal welfare laws (IRB Nr. 509.6-42502-00/355). The sheep were not restricted in loading the treated limb after surgery. To ensure minimum variability between the different animals regarding their surgical procedures, the osteotomy including cut of the periosteum was performed by an oscillating saw. Following a latency period of four days, a distraction of 1.25 mm per day was performed for twenty days. The sheep were randomly distributed into two groups: one group including six sheep received rhBMP-2/αBSM injections at day 23 and 30 after the primary surgery while the other group of six sheep received buffer/αBSM at the same days. The injection was made directly into the distraction zone using an 18 gauge injection needle. Fluoroscopy was used to ensure correct placement of the needle. A third group of eight sheep served as an additional control group and did not receive any injections.

rhBMP-2 and buffer formulations

For group 1 rhBMP-2 (4 mg) was reconstituted with 1.8 ml water for injection, resulting in the appropriate concentration of 2.2 mg/ml. For group 2 a buffer (5.0 mM glutamic acid, 5.0 mM NaCl, 2.5% glycine, 0.5% sucrose, and 0.01% polysorbate 80) was taken instead of rhBMP-2. Under aseptic conditions 1 ml of each solution was mixed with self-hardening αBSM. Injections were performed into three spots of the distraction at 25%, 50% and 75% of longitudinal distance under fluoroscopy. At day 23 and 30 sheep were randomly assigned to receive either an injection of rhBMP-2/αBSM (Pharmacia/Upjohn), or an injection of buffer/αBSM, which served as a carrier control. A third group (group 3) was treated with distraction osteogenesis without any injections, serving as control group.

Radiography

Antero-posterior radiographs of the distracted limb were taken before surgery, immediately after surgery and weekly thereafter in order to gain a qualitative follow-up of consolidation of the distraction gap.

In vivo stiffness measurements

During the 50 days of consolidation *in-vivo* torsional stiffness measurements of the distracted tibia were obtained weekly. For this measurement, a special grip, instrumented with a longitudinal variable differential transformer and a load cell, was con-

nected to the distal double ring of the external fixator. Using a stepper motor, the distal segment of the tibia was twisted against the proximal segment at a constant speed of 0.5 mm per second [28]. Applied moments and resulting angular displacements were monitored. *In-vivo* stiffness [Nm/°] was calculated from the initial linear portions of the moment-angle curves.

At the end of the distraction period (day 74) the sheep were euthanized and both tibiae removed and stored frozen.

Bone density measurements

Bone density parameters were obtained using dual-energy X-ray absorptiometry (DXA, QDR2000+, Hologic, Bedford, MA, USA). Rectangular regions of interest were placed into the distraction zones and bone mineral content (BMC [g]) and bone mineral density (BMD [g/cm^2]) of the distraction zone were determined. In addition, a similar-sized region in the mid-diaphysis of the contralateral, intact limb was scanned using DXA.

Biomechanical testing

The tibiae were then prepared for biomechanical testing by embedding them in polymethylmethacrylate (PMMA) 10 mm proximal and distal to the distraction zone. The intact tibiae were also embedded leaving a 45 mm gap in the mid-diaphyseal region. The embedded tibiae were mounted in a servo-hydraulic materials testing machine (MTS Systems Corporation, Eden Prairie, MN, USA). Different types of stiffness (compressive, torsional and bending stiffness in antero--posterior and medio-lateral orientation) were determined in randomized chronology. Therefore, the specimens were loaded non-destructively. The linear portion of the slope of the load-deformition curve was used to calculate the respective stiffnesses. Finally, the tibiae were tested to failure in torsion at a rate of five degrees per minute using displacement control to record the maximum torsional moment.

Statistical analysis

Descriptive statistics were computed for all outcome variables. The effect of rhBMP-2 treatment on *in-vivo* stiffness measurements was assessed using a multivariate ANOVA in combination with a Tuckey post-hoc test. Results from bone densitometry and biomechanical testing were evaluated using a Mann–Whitney-*U*-test. In all cases, differences were considered significant at $p < 0.05$.

3. Results

The findings of this study show that an injection of rhBMP-2 with αBSM carrier in the consolidation phase (day 23 and 30) were not significantly different from control injections of buffer/*α*BSM or the sheep receiving no injections.

In vivo stiffness measurements

The torsional *in-vivo* stiffness of the three analyzed groups reveals an increase in stiffness during consolidation. However, no significant differences of the *in-vivo* stiffness of tibiae of group 1 and 2 were calculated by multivariate ANOVA (Fig. 1). Furthermore, there was no significant treatment effect compared to the control group which received no injection. Thus, there was no significant overall treatment effect.

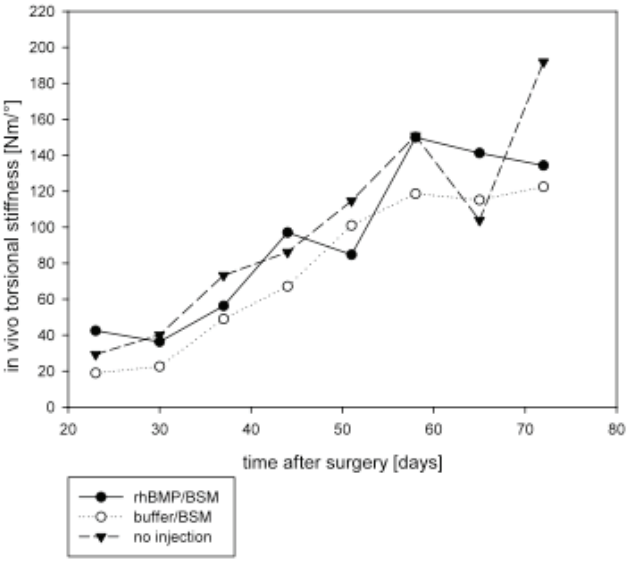

Fig. 1. Follow-up of in-vivo torsional stiffness during consolidation after distraction osteogenesis

Bone density measurements

The postmortem DXA analysis showed no significant differences between the treatment groups regarding the parameter BMC and BMD (Figs. 2a and 2b).

Biomechanical testing

Maximum torsional moment of the tibiae treated with rhBMP2/*α*BSM injection at day 23 and day 30 was 44.9 Nm ± 22.7 (51.8% of contralateral intact limb), while the group treated with buffer/*α*BSM injection on the same days revealed a maximum torsional moment of 42.1 Nm ± 25.4 (48.5% of contralateral intact

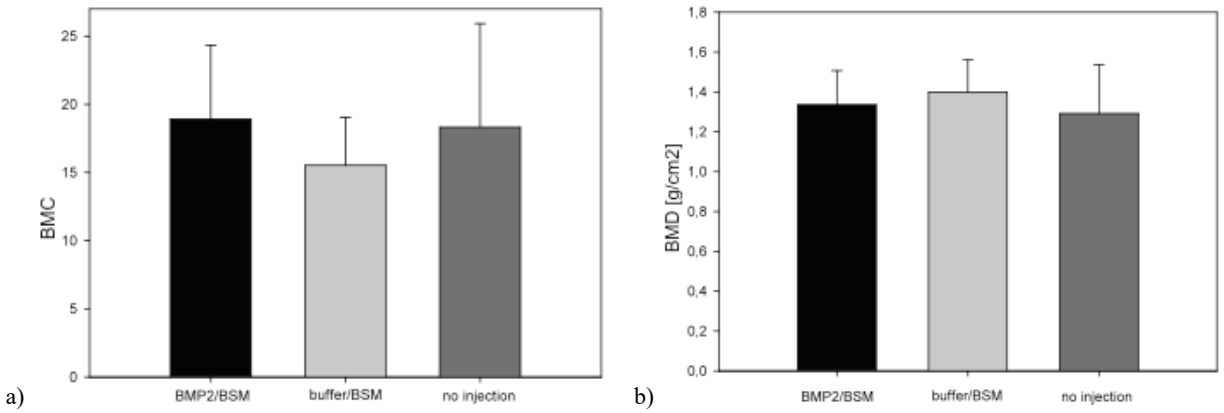

Fig. 2a, b: Comparison of DXA analysis post-mortem (BMC and BMD) including standard deviation

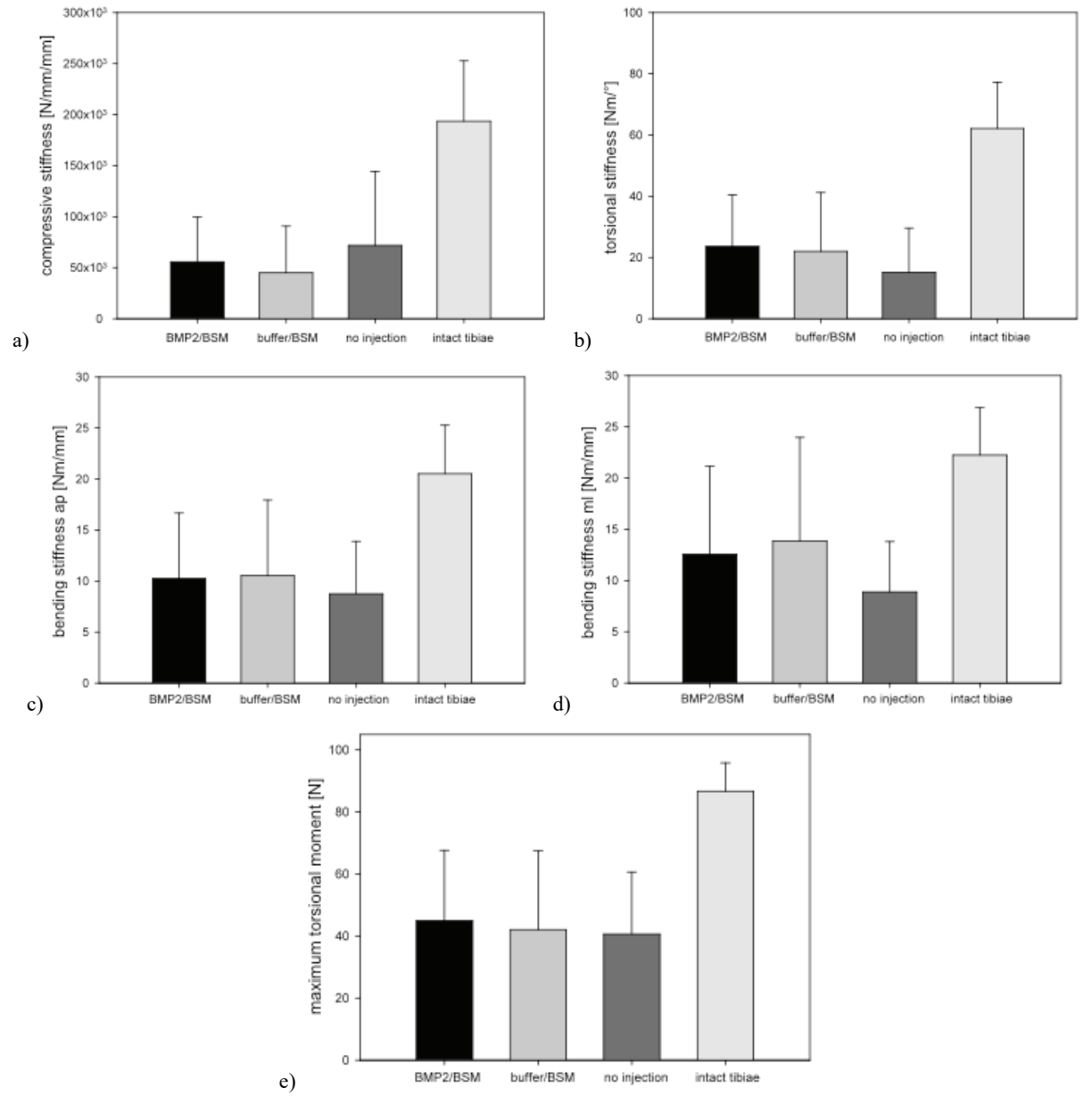

Fig. 3a–e: Comparison of post-mortem biomechanical testing including standard deviation (compressive, torsional, bending stiffness in antero-posterior and medio-lateral orientation and maximum torsional moment). The data are present as absolute values. For comparison the values of intact tibiae were presented

limb). The maximum torsional moment of the control group receiving no injection had a mean value of 40.7 Nm ± 20.0 (46.9% of contralateral intact limb). There was no statistical significant difference between the groups following a two-tailed Mann–Whitney--*U*-Test.

Also for the different types of stiffness no significant differences between these groups of treatment were determined, so that no significant overall treatment effect could be determined by post-mortem biomechanical testing. (Figs. 3a–e).

4. Discussion

This study concentrated on a problem whether a sequence of two injections of rhBMP-2 in αBSM administered at the end of the distraction period during distraction osteogenesis would be able to enhance bone consolidation. The results showed moderate effects by qualitative radiographic evaluation. Qualitative evaluation of bone formation from standard *in-vivo* radiographs, routinely performed in the clinical environment, showed increased mineralization in the distraction zone and more through bridging of the distraction gap in those animals treated with rhBMP-2. However, qualitative analysis by density measurements via DXA, *in-vivo* torsional stiffness measurements and biomechanical testing after 74 days were not able to show significant differences between the different groups of treatment. Overall, the results exhibit only moderate advantages of the rhBMP2-treatment with the dose and carrier used, and application time point chosen.

Unlike in our study, in previous works BMP's have shown promise for enhancing distraction osteogenesis. BMP's are cytokines that stimulate the transformation of undifferentiated mesenchymal stem cells into chondroblasts and osteoblasts [30]. Previous studies determined BMP's as important regulators of skeletal development and healing [3], [13]. After fracture, BMP's are thought to diffuse from resorbing bone matrix and promote osteogenesis by activating osteoprogenitor cells to develop into osteoblasts, which results in stimulation of new bone matrix formation [19]. As far back as 1990 it was published that within the group of growth factors only BMP's have shown the ability to singularly induce *de-novo* bone formation *in vitro* and *in vivo* [24]. More than 20 types of BMP's have been identified [8]. Recombinant replication techniques allowed to produce sufficient quantities of human BMP's in order to test their ability to enhance skeletal repair and regeneration.

The findings of the current study were thus not anticipated, as data from using rhBMP-2 in αBSM carrier in animal models of bone defects and osteotomies showed dramatically enhanced bone healing [6], [17], [26], [27]. In comparison with various carriers αBSM showed superior performance [26]. In bone, αBSM remodels itself into bone tissue and promotes bone healing. αBSM treatment has been shown in several animal models to be effective in promoting healing of surgically created critical size defects and restoring bone biomechanical strength to values equal to or greater than values achieved with autograft controls. The incorporation of rhBMP-2 with αBSM was shown to be effective in stimulating bone formation and accelerating restoration of the differentiated phenotype in an osteotomy model [15]. Edwards et al. performed bilateral tibial osteotomies and stabilized the limbs with external fixators [6]. Four hours after the surgery, one limb was treated with a single percutaneous injection of rhBMP-2/αBSM paste or an equal volume of αBSM alone. At four and eight weeks the scores for radiographic union, callus area, leg load bearing and biomechanically tested stiffness in bending and in torsion were significantly greater for the rhBMP-2/αBSM-treated limbs than they were for the αBSM-treated or untreated control limbs ($p < 0.05$). In another study in rabbits bilateral mid-ulnar osteotomies were injected with either 0.1 mg rhBMP-2/αBSM or buffer/αBSM [17]. Contralateral osteotomies served as untreated surgical controls. Radiographs demonstrated complete bridging of the BMP limbs at four weeks, whereas none of the BSM or untreated limbs were bridged. Post-mortem analysis showed 62% more mineralized callus (pQCT), 63% greater torsional stiffness and 103% greater strength for the BMP treated limbs. Seeherman et al. confirmed similar positive effects after injection of rhBMP-2 in αBSM using histological, radiological and biomechanical testing [26], [27]. Thus, the existing results vary regarding the effect of rhBMP-2 in αBSM. It probably depends on the animal model, the time after osteotomy, the dosis and the applied carrier.

Other studies evaluated carrier different to that investigated in the current study. Eguchi et al. used a composition of BMP-2 (0, 30, or 100 μg), β-tricalcium phosphate powder (βTCP, 100 mg/animal; particle size, <100 μm), and polyethylene glycol (PEG; 40 mg/animal) for percutaneous injections. Their data also suggest the potential application of BMP-2 in accelerating callus formation and in enabling rapid bone transporting [7]. Another study on the efficacy of rhBMP-2 was conducted by Nunotani [21]. The delivery system for rhBMP-2 used a polymer-coated

gelatin sponge (PGS). The group of rhBMP-2/PGS revealed the highest torsional stiffness an strength. The efficacy was also proven radiologically and histologically [21]. Xiong et al. investigated the feasibility of using novel hollow hydroxyapatite microspheres as an osteoconductive matrix and a carrier for controlled local delivery of BMP-2 [29]. Hollow hydroxyapatite microspheres (100 ± 25 μm) with a core (60 ± 18 μm) and a mesoporous shell (180 ± 42 m2/g surface area) were prepared by a glass conversion technique and loaded with recombinant human BMP-2 (1 μg/mg). There was a gentle burst release of BMP-2 from microspheres into the surrounding phosphate-buffered saline in vitro within the initial 48 hours, and continued at a low rate for over 40 days. These results indicate that BMP-2-loaded hollow hydroxyapatite microspheres could be a potential new option for bone graft substitutes in bone regeneration [29]. Lee et al. compared the bone regenerative effects of a rhBMP-2-loaded collagen-based biphasic calcium phosphate composite (BCPC) and rhBMP-2-loaded biphasic calcium phosphate (BCP) [16]. They determined a positive effect of BCP – as well as BCPC – loaded rhBMP-2 on bone regeneration. In addition, collagen/chitosan microspheres composite scaffold was proven as a promising carrier of BMP-2 for the treatment of segmental bone defects [11], while Li et al. suggested, according to their results, a rabbit model fibrin to be a good carrier for rh-BMP-2 [18].

There are several explanations regarding the differences between this study and the previous ones. First, the distraction zone reflects a very different morphological setting than a bone defect or osteotomy does. While only blood and debrided tissue is present in defects, a distraction zone is completely filled with collagenous callus mass ready to mineralize. Any injection of viscous material in the distraction zone produces high pressure and compromises the natural tissue. Pressure necrosis may be possible. Thus, any enhancing effects on consolidation could be counterbalanced by negative effects on the mineralisation of the natural tissue, resulting in no overall gain with the proposed treatment. This may be the reason for using rhBMP-2 in solvent with a very low viscosity, like described in previous studies [20]. Second, comparison between the previous studies and the current one are complicated because different animal models and different distraction regimens were used. The effect in large animals like sheep may be different compared to small animals like mice or rats. In three studies investigating the effect of BMP-2 or BMP-7 on distraction osteogenesis, differences are apparent between early and late administration of BMP during distraction [10], [20]. The late-administration procedure failed in exhibiting effects and responsible investigators suggested that these findings could be explained by the lack of BMP receptors they found in tissue of later stages while they found strong expression of BMP receptors in early tissue. Seeherman et al. revealed best results after delaying the treatment of rhBMP-2 for one week and concluded that this accelerated the healing process because of an increase in the number of responding cells and an increase in direct bone formation [26]. Therefore, the time point could be crucial in distraction osteogenesis and may explain the result of the current study. Thus, the found discrepancies of no significant differences in biomechanical data post-mortem and differences in X-ray appearance during the time course could be explained by the one late time point of sacrifice after 74 days with a possibility of different mechanical properties at earlier time points, where X-ray analysis showed a difference.

These results of this study provide evidence for possible future treatment in patients undergoing distraction osteogenesis because of congenital deformities and shortenings or bone defects due to trauma, tumor, or infection. The safety and efficacy of rhBMP-2 has been established in several animal models. These studies have shown that rhBMP-2 promotes healing of segmental defects [13], fracture and fracture nonunions [12] and spine fusions [3], [8]. In addition, partially purified human BMP has been shown to be safe and efficacious in enhancing healing in human femora and tibia [12].

Local injections carry the risk of infection. However, the risk of infection depends on the sterile properties of the injection procedure and the tissue in which the injection is placed. In contrast to the tissue environment observed after traumatic fractures where large amounts of necrotic tissue and interrupted blood flow provide an excellent basis for bacterial growth, the distraction zone has no necrotic particles and is a highly vascular region with nearly three times higher blood flow [1]. Therefore, there is only a minimum risk of local infection from the injections.

The time point of injection was chosen at the first days of the consolidation period in order not to promote preliminary bone consolidation with insufficient gap creation. This regimen was also chosen by Okazaki et al., who tested the effect of bFGF injections on bone consolidation [22]. The *in-vivo* stiffness measurements suggest that an earlier injection might be possible, while still achieving the full distraction distance. The possibility of administering the treatment injection near the start of the

distraction phase should be investigated in future studies.

In this study, the progress of bone consolidation was measured using *in-vivo* stiffness testing. This method has been previously validated [28] and used in other studies [23]. Although the accuracy may be affected by pin loosening during the end phase of an experiment, *in-vivo* torsional stiffness may be used as a predictor of the bone load bearing capacity up to approximately two-thirds of the healing process [28]. It is important to note that *in-vivo* stiffness is measured using very low forces and cannot be directly compared to stiffness measurements assessed during the destructive testing.

Another limitation of this study was the inability to measure weight-bearing in the operated and contralateral, non-operated limbs during consolidation, as differences in weight-bearing could affect the healing and ultimately the mechanical properties of the regenerate.

In conclusion, these data support the potential feasibility of rhBMP-2 for enhancing treatment in distraction osteogenesis. However, because of the poor magnitude of the effects, further investigation in dose, carrier and application time point important before testing in a clinical trial is to be considered.

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