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Time for treating bone fracture using rhBMP-2: A randomised placebo controlled mouse fracture trial

Mark Murnaghan, Lisa McILmurray, Moses T. Mushipe, Gang Li *

The Department of Trauma and Orthopaedic Surgery, School of Medicine, Queen's University of Belfast, Musgrave Park Hospital, Stockman's Lane, Belfast BT9 7JB, Northern Ireland, UK

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Abstract

Although the mechanisms of osteoinduction by bone morphogenic proteins (BMPs) are increasingly understood, the most appropriate time to administer BMPs exogenously is yet to be clarified. The purpose of this study was to investigate when BMP may be administered to a fracture arena to maximise the enhancement of healing. Forty mice with externally fixed left femoral fractures were randomised into four groups: Group I, the control group was given a placebo of 30 μl saline at day 0; Groups II, III and IV were given 30 μl saline plus 2.5 μg rhBMP-2, at post-operative days 0, 4 or 8, respectively. Sequential radiographs were taken at days 0, 8, 16. On day 22 the mice were sacrificed and both femora were harvested for biomechanical assessment in 3-point bending and histological evaluation. Radiographic analysis indicated that healing of fractures in Groups II and III was significantly greater ($p < 0.05$) than those in Groups I and IV, at both 16 and 22 days post-fracture. The highest median bone mineral content at the fracture site was evidenced in Group III and II. Furthermore, Group III also had the highest relative ultimate load values, followed by Groups II, IV and I. Greater percentage peak loads were observed between Group I and both Groups II and III ($p < 0.05$). Histological examination confirmed that at 22 days post-fracture, only fractures in Groups II and III had united with woven bone, and Groups I and IV still had considerable amounts of fibrous tissue and cartilage at the fracture gap. Data presented herein indicates that there is a time after fracture when rhBMP administration is most effective, and this may be at the time of surgery as well as in the early fracture healing phases.

Keywords: Fracture repair; Mouse; rhBMP-2; Biomechanical test; Radiography

Introduction

Approximately 5–20% of all tibial fractures fail to heal properly resulting in delayed or impaired healing. New treatment options used in conjunction with traditional treatment methods may enhance fracture healing. Of the various growth factors and cytokines involved in bone fracture repair processes, bone morphogenetic proteins (BMPs) have been proven to be key modulators. BMPs are potent cell signaling proteins that involve in many aspects of embryonic development, among the BMPs family, BMP-2 has been shown extensively to have the ability to improve or accelerate fracture healing [4,11,15,16,19,20]. In most studies however, BMP-2 administration has been on the day of surgery [4,6,9] and relatively high doses, particularly in large animals and primates [6,12,21] are required to exert any clinically significant effect. The aim of this study was therefore to investigate when rhBMP-2 may be administered to a fracture arena in order to improve fracture repair optimistically.

* Corresponding author. Tel.: +44 2890 902830; fax: +44 2890 902825.
E-mail address: g.li@qub.ac.uk (G. Li).

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Materials and methods

Animal model of fracture repair

All animal experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act (1986) at the Queen's University Belfast Biomedical Research facility. Three months old male Carworth Farms Lane-Petter (CFLP) mice were used, with age range from 12 to 14 weeks (mean 12.22 ± 0.58 weeks) and body weight mean 43.06 ± 5.91 g. A model of mouse open femur osteotomy was used as previously described [3]. Briefly, general anaesthesia was induced using a gaseous mixture of 3% isofluorane in a 50:50 mixture of N_2O_2:O_2 at 2 l/min in a sealed chamber. Once asleep animals were transferred to the operating table with gases delivered using a Hunt mask. In brief, via an antero-lateral approach the left femoral shaft was exposed, four holes with an angle of 7° subtended to the vertical were drilled via a specifically designed jig through both cortices using a 0.55 mm drill-bit. Pins (0.51 mm) were inserted into the holes and a metal crossbar was loosely placed over the pins and held 10 mm from the bone surface. A low energy transverse osteotomy was made between the two central drill holes. The geometry of the drilling jig and crossbar ensure a 0.7 mm discrepancy between the distance of the central two pinholes in the crossbar and in the femur. Thus as the crossbar is reduced onto the bone leaving a standard bone bar distance of 4 mm, compression is produced across the fracture gap. The tops of the pins were then cut and secured using a small drop of quick drying glue (Super-glue, Loctite) and the wound was closed.

Randomisation and injection of rhBMP-2

Prior to surgery, animals were randomly allocated to one of the four treatment groups, each containing 10 mice. Animals in Group I were injected with 30 μl of normal saline following surgery (day 0). Animals in Groups II, III and IV were all injected with 2.5 μg rhBMP-2 (Pepro-Tech EC Ltd, London, UK) which was reconstituted according to manufacturer's instructions and made up to 30 μl using normal saline, at days 0, 4 and 8 post-fracture, respectively. Injection was performed using a 25 gauge needle from a postero-lateral approach, guided by radiograph to ensure the solution was injected into the fracture gap.

Radiography analysis

At 8, 16 and 22 days after surgery, all animals were anaesthetised and placed inside a high-resolution digital radiography system (Faxitron MX-20 with DC-2 option, Faxitron X-ray Corporation, IL, USA) for radiographic analysis using an exposure of 24 kV for 3 s. To control the plane of radiography a specifically made X-ray jig was attached to the crossbar via two portals and the animal positioned centrally using laser crosshairs for guidance. The X-ray jig also contains a step aluminium phantom to enable standardisation and comparison of images across time points and between animals. Digital radiographs were saved in TIFF format and analysed by comparing the changes in pixel density across the fracture gap using the UTHSC-SA ImageTool program (ftp://maxrad6.uthscsa.edu). Changes in pixel density correspond with changes in bone mineralised tissue. The intra- and inter-observer variability was measured and the pixel density analysis was highly reproducible. In the early period of repair the fracture gap is filled with relatively less dense inflammatory tissue and thus the pixel density is low, however as repair progresses, hard callus is formed resulting in a rise in pixel values. The pixel density of the fracture gap is compared to two adjacent areas of uninjured bone. Initially there is less bone at the fracture gap than in the adjacent areas however with healing, callus is laid down and the pixel density at the fracture becomes relatively greater that the uninjured area adjacent to it. Thus values are represented as 'relative bone mineral content'.

Mechanical testing

After excision of both femora, muscle and soft tissues were removed with care taken not to disrupt the structural integrity of the fracture site. The external fixator and the pins were removed by cutting through the pins using a diamond cutting-disc and a haemostat was used to prevent pin-spinning, and the pin remnants were removed by gentle anti-clockwise rotation. Both femora were stored in containers humidified by saline-moistened gauze in the base before biomechanical testing at room temperature, 22 °C, and all tests were performed within 12 h post-excision. From each group, four or five specimen pairs were tested to failure by 3-point bending (100 N load cell, Lloyd Instruments Ltd, UK). Each specimen was placed on two lower supports that were 9 mm apart and force was applied at 5 mm/min at the mid-diaphysis on the anterior surface such that the posterior surface was in tension. Ultimate load and stiffness were determined from the load–displacement curves and the biomechanical properties of the healing fractures were expressed as percentages of the contra-lateral intact bone properties.

Histology examination

On the day of sacrifice, half of the animals were selected for histological evaluation. The healing left femur was disarticulated and amputated through the knee. Specimens were coded, fixed in 10% buffered formalin for 5 days and decalcified at 4 °C over a period of 3 weeks in 8% EDTA (pH 7.2–7.4). In order to preserve the fracture morphology the external fixators were removed at completion of the decalcification process. Decalcified samples were processed through graded alcohols, xylene and embedded longitudinally (on their coronal plane) in paraffin wax. Seven micrometer sections were cut and stained with Alcian blue/Sirius red and examined under light microscopy. In brief, following de-paraffin, re-hydration, nuclear staining with Weigert's hematoxylin, sections were stained with Alcian blue 8GX (0.1% in 1% acetic acid) and Sirius red F3B (1% in saturated picric acid). Alcian blue stains the proteoglycan-rich cartilage matrix (blue), while Sirius red stains the type I collagen fibril (red). The amounts of periosteal callus, fibrous tissues and cartilage in the fracture gap were compared qualitatively.

Statistical analysis

All quantitative data were transferred to statistical spreadsheets and analysed using a commercially available statistical program SPSS (Version 11, Chicago, IL, USA). Due to the small sample sizes non-parametric methods (LSD tests) were employed. Differences due to treatment and healing time were considered significant at $p < 0.05$ for all tests. Data were expressed on box plots. Post hoc, a power analysis was performed to determine the number of animals that would have been required to demonstrate true differences in measured biomechanical data (power = 80%, 1 tail and alpha = 0.05).

Results

Aetiology

There were no significant differences between animals in all groups, with regard to age, weight at surgery or weight-change during experiments. During surgery two animals died; one following a vascular incident, while the second suffered an intra-operative pin fracture and was sacrificed. These two animals were immediately replaced with littermates and thus each experimental group began with 10 animals.

Radiographic assessments

Qualitative assessment of serial radiographs shown in Fig. 1, suggested that at days 0 and 8, there were no apparent differences in the amount of periosteal callus

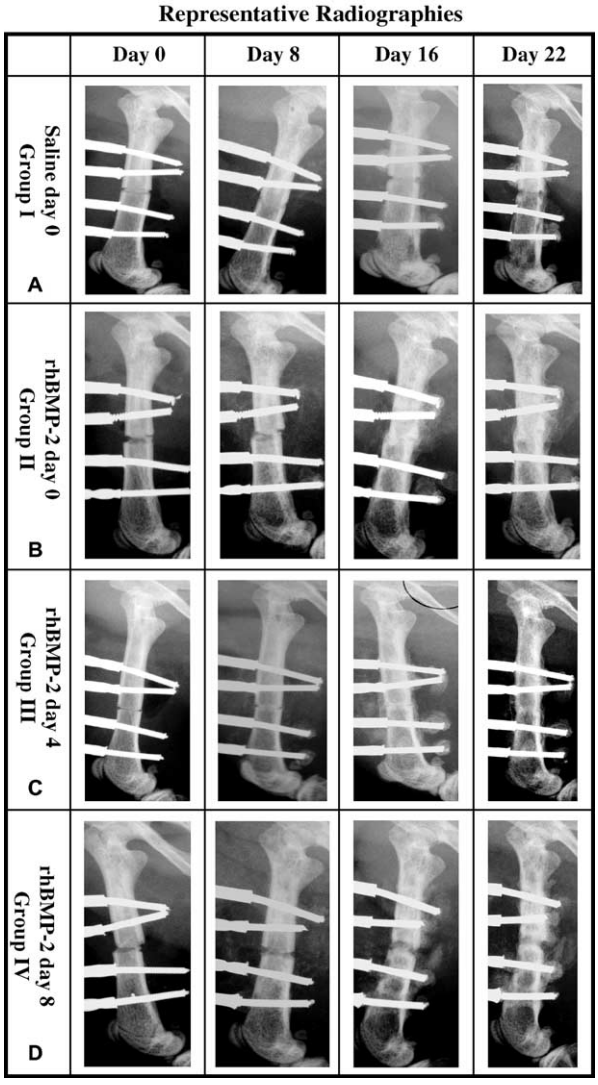

Fig. 1. Representative serial radiographies of animals from four experimental groups. A. Group I, saline injection at day 0; B. Group II, rhBMP-2 injection at day 0; C. Group III, rhBMP-2 injection at day 4; D. Group IV, rhBMP-2 injection at day 8. At day 0 and day 8 post-fracture, there were no significant differences seen on radiographies among the groups; at day 16 and 22 post-fracture, the fractures were fully united in Groups II and III, but the fracture gaps in Groups I and IV were still visible.

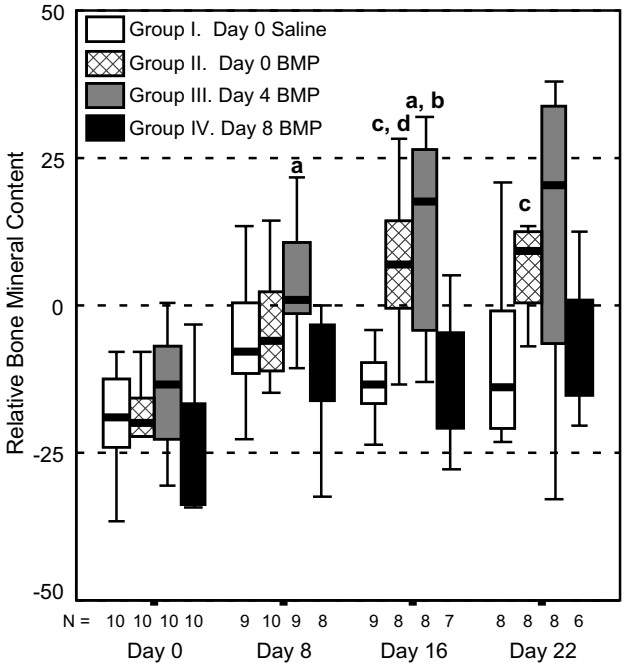

Fig. 2. Change of relative bone mineral content (BMC) at the fracture site during fracture healing. There were no significant differences between groups on day 0. All groups except Group I showed a gradual increase in BMC at the fracture site throughout the experiment. The BMC in Group IV did not differ significantly from Group I at any time point. The highest median BMC was seen in Group III at day 8, 16 and 22 post-fracture. At day 16 both Groups II and III had significantly greater BMC at the fracture sites than both those in Groups IV and I respectively. At day 22 only Group II was significantly different to Group I. a–d, $p < 0.05$; a: Group III vs Group IV; b: Group III vs Group I; c: Group II vs Group I; d: Group II vs Group IV. Number of animals in each group was denoted. Medians and range of variances were plotted.

formation in all the groups. At day 16 post-fracture there was no difference between Groups I and IV (Fig. 1, day 16), the amounts of periosteal callus were greater in Groups II and III compared to Groups I and IV. At day 22, fractures in Groups II and III were completely united as opposed to Groups I and IV which still displayed visible fracture gaps (Fig. 1, day 22).

The relative BMC of the fracture sites are shown in Fig. 2. There were no significant differences between groups on the day of surgery (day 0). As healing progressed, all groups except Group I exhibited a gradual increase in BMC at the fracture site throughout the experiment and the BMC in Groups II and III were greater than Group IV. The BMC in Group IV did not differ significantly from Group I at any time point. The highest median BMC was seen in Group III (day 4 rhBMP-2 injection) at day 8, 16 and 22 post-fracture, which had also showed the earliest rise in BMC at the fracture site. At day 16 both Group II (day 0 rhBMP-2 injection) and Group III had significantly greater BMC at the fracture sites than both those in Groups IV (day 8 rhBMP-2 injection) and I (day 0 saline injection) respectively. At day 22 only Group II was significantly different to Group I, although Group III had the highest median BMC, it also had a wider range and did not reach statistical significance.

Mechanical testing

Results of the biomechanical tests are shown in Fig. 3A and B. The former showed that there were no significant differences in percentage ultimate loads between the control Group I and Group IV. Groups III and II

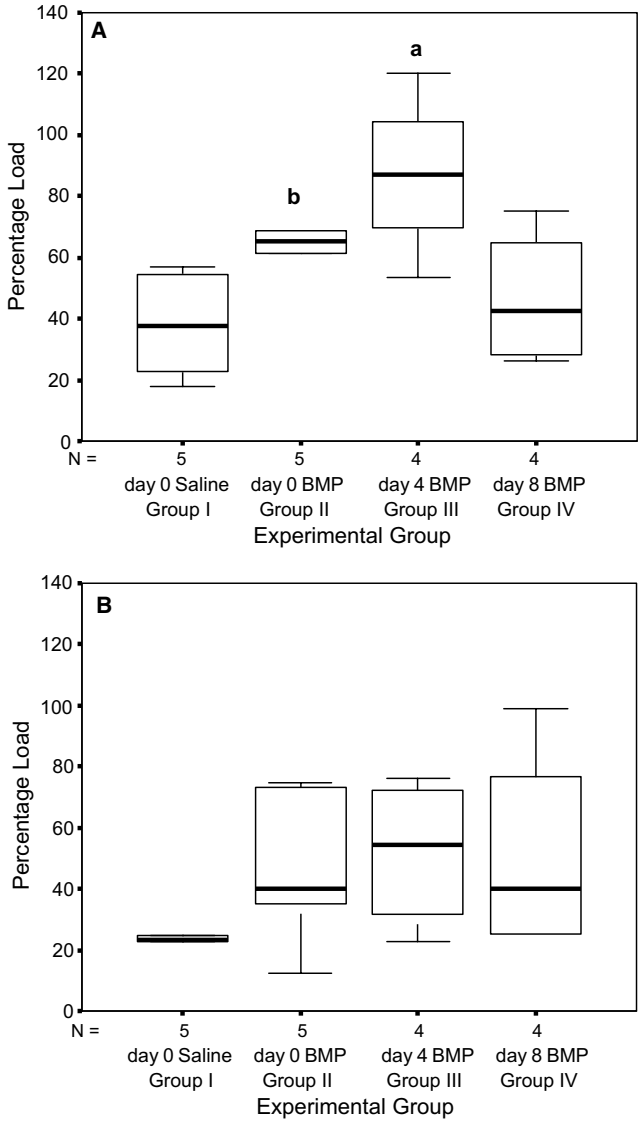

Fig. 3. Results of the biomechanical testing on specimens collected at day 22 post-fracture, all values were shown as a percentage of the values of the contra-lateral intact limbs. A. Animals in Groups II and III had greater percentage load of the contra-lateral intact limbs, which was statistically greater than Groups I and IV. B. There was a trend of increase in percentage stiffness in Groups II and III, who had the higher medians among the other groups, but it did not research statistical significance. a and b, $p < 0.05$; a: Group III vs Group I; b: Group II vs Group I. Number of animals in each group was denoted. Medians and range of variances were plotted.

exhibited the greater percentage loads indicating strength of the healing bone getting closer to the strength of contra-lateral intact bone. Compared to Group I, both Groups II and III had greater ultimate load properties ($p < 0.05$). Although stiffness data showed a similar trend to that obtained from the relative loads (Fig. 3B), there were no significant differences observed between the groups. Post hoc power analysis of the biomechanical data showed the number of animals required for detection of acceptable difference (Table 1).

Table 1
Number of animals required to demonstrate biomechanical property differences between Groups II and III

Expected difference, ε	20	25	30	35	40	45	50
Peak load	10	6					
Stiffness	100	40	22	14	10	8	6

Assuming $\mu_1 - \mu_2 \neq 0$, power = 80, alpha = 0.05 and tail = 1.

Histology examinations

On day 22 post-fracture, the amount of periosteal callus was greater in Groups II and III (no difference between Groups II and III), followed by Groups IV and I (Fig. 4A–D). The fracture gap consisted predominately of fibrous tissues and cartilage in Group I (Fig. 4A1), and cartilage was frequently see in the fracture gaps in Group IV (Fig. 4D1). Whereas in Groups II and III, periosteal and endosteal callus (woven bone) were mostly evident and no or little fibrous tissues or cartilage were present in the fracture gaps (Fig. 4B1 and C1). The number of animals with completed union (both cortices united with periosteal callus) at day 22 was: 2 out of 5 in Group I; 4 out of 5 in Group II; 5 out of 5 in Group III and 2 out of 5 in Group IV.

Discussion

The animal model used in the current study has been fully characterised previously [3,10] and the externally fixed fracture model is a fair representative of clinical fracture repair with no or little interference to endosteal callus formation. This study was aimed at assessing whether an alteration in timing of delivery of rhBMP-2 could improve the time to union, so that all animals were terminated at day 22 post-fracture as previous studies have shown that fracture callus in untreated animals begins to remodel between 25 and 32 days post-fracture in this model [10].

The study has demonstrated that a single percutaneous injection of rhBMP-2 to the fracture site in mice during the early stage of fracture repair enhances fracture healing. Radiographic analysis and biomechanical testing both demonstrated that the administration of rhBMP-2 at day 0 or day 4 post-fracture in this mouse model resulted in the augmentation of periosteal callus formation, bone mineral content and superior biomechanical properties when compared to later (day 8) administration of rhBMP-2 or and the saline administration without rhBMP. The observed results are in agreement with a previous study in a non-human primate fibular osteotomy healing model which showed that a single percutaneous injection of rhBMP-2 at 3 h or 7 days after surgery both induced acceleration of osteotomy healing [17,18]. Although there were con-

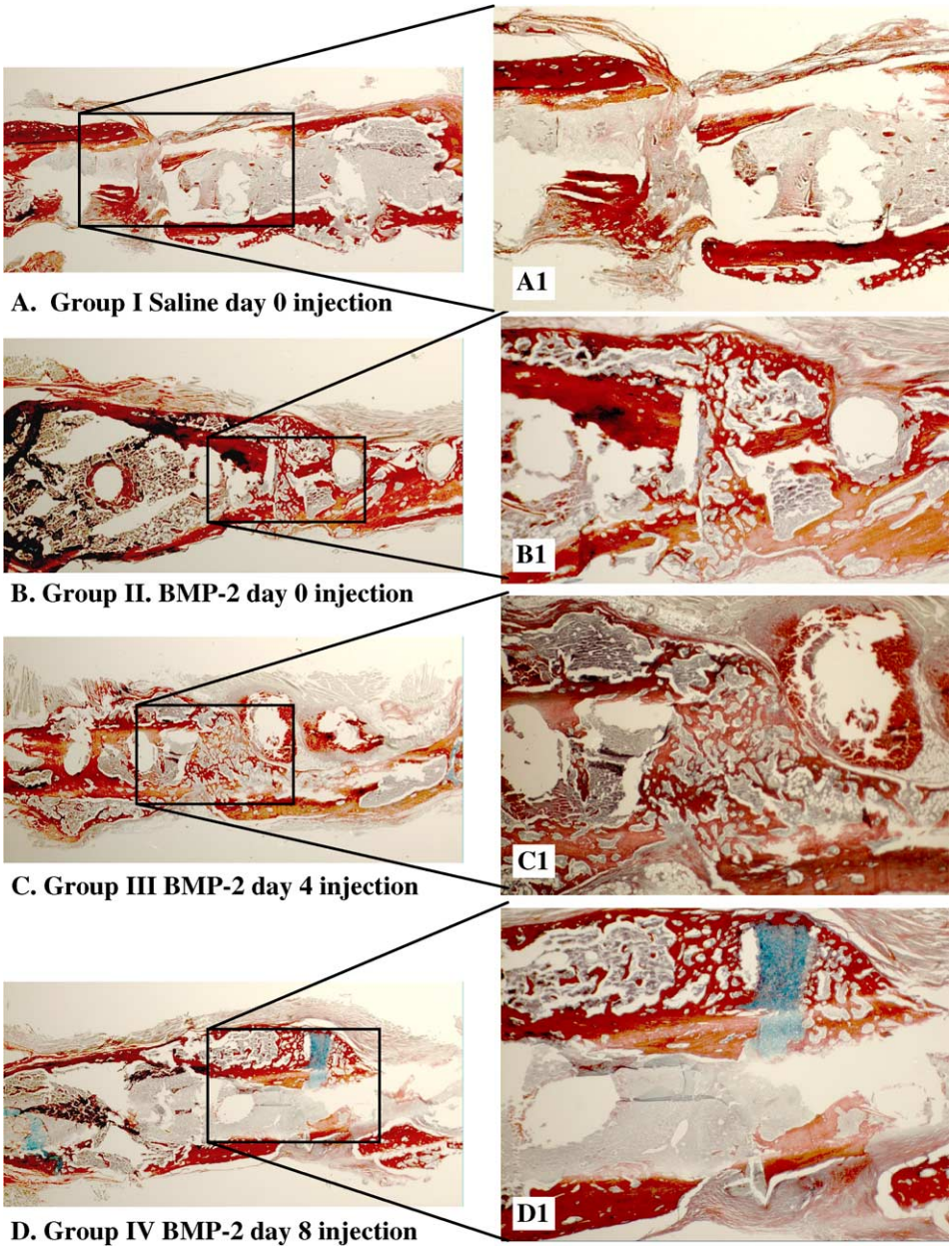

Fig. 4. Representatives of histological appearances from the experimental groups at day 22 post-fracture. A. Group I, saline injection at day 0; B. Group II, rhBMP-2 injection at day 0; C. Group III, rhBMP-2 injection at day 4; D. Group IV, rhBMP-2 injection at day 8. The amounts of periosteal callus were greater in Groups III and II (similar between the two groups), followed by Groups IV and I. Fibrous tissues and cartilage in the fracture gaps were still visible in Group I (A, A1) and cartilage islands were found in Group IV (D, D1). Whereas in Groups II (B, B1) and III (C, C1), fractures were mainly united with periosteal and endosteal callus (woven bone) and no or little fibrous tissues and cartilage remains were seen. A1–D1 was enlarged images from the boxed areas in A–D. Alcian blue/Sirius red staining, A–D: ×15 and A1–D1: ×40 magnifications.

cerns that early BMP-2 administration (at day 0) may not be the best to augment bone formation [5,22,23,25], we did not find significant difference in the amount of bone formation and fracture healing mechanical property between the groups that received rhBMP-2 at day 0 and day 4 post-fracture in the present study. Power analysis (the probability of detecting a difference) between Groups II and III showed that 6 and 40 animals would be needed for a significant detectable difference of 25% in the peak load and stiffness data, respectively. The peak load thus proved to be the more sensitive biomechanical parameter. In the present study we have used four or five animals for biomechanical testing in each group, thus the number has insufficient statistical power to detect a substantial difference between Group II and Group III.

As well as timing, dosage and carriers for rhBMP have to be considered carefully to gain the most effect of the treatment. Several studies have highlighted the importance of dosage dependence and carriers of

rhBMP on osseous union in different animal models [4,11,15,16,19,20]. Evidently, the most appropriate carrier for a given BMP may also depend on the specific clinical situation as well as the skeletal site to be treated [14]. Biodistribution studies indicated that approximately 70% of the administrated dose of rhBMP-2 was initially retained at the fracture site following implantation of rhBMP-2/ACS (absorbable collagen sponge), and that approximately 40% and 10% remained at 1 and 2 weeks after implantation, respectively [1]. Injection of rhBMP-2 is an appealing method of delivery, it allows a precise dosage of the protein and repeated deliveries are also possible, several studies demonstrated that a single percutaneous injection of rhBMP-2 to a fracture site does enhance fracture healing [4,9,17,18]. In the present mouse study we administrated 2.5 μg rhBMP-2 by a single percutaneous injection to the fracture site, the dose used is based on reported optimal dosages in humans [6]. To determine the dosage to give the mice, we used a dose calculator to convert the human dosage with the average mouse body weight of 40 g (dose calculator at http://www.fda.gov/cder/cancer/animalframe.htm). The resultant dose (10 μg) was further reduced by 4 times, to 2.5 μg rhBMP-2 per mouse. Overall, the lower the species, the less amount rhBMP-2 was needed for bone induction. It is reported that the working doses of rhBMP ranged from 80 to 300 μg in rats [4,15]; 40 to 860 μg in rabbits [1,9,11,12,20,21]; 500 to 750 μg in monkeys [17,18]; and 6 to 12 mg in human [6]. The exact level of endogenous BMP-2 produced at fracture sites is unknown, however it is assumed the likely physiological concentrations are in *pico*- or *nano*-gram levels based on previous in vitro human cell culture studies [14]. In the present study a total of 2.5 μg of rhBMP-2 promotes fracture healing and this is the first report to date on the effective dose of rhBMP-2 used in a mouse fracture study.

BMP-induced bone formation in vivo is clearly a complex multistage process involving the activities of multiple locally produced growth factors and systemically available hormones [14]. BMP-2 has been shown to induce differentiation of osteoprogenitor cells into osteoblastic cells [7,24]; BMP-2 has chemotactic effects on mesenchymal cells, osteoblastic cells [13] and endothelial cells [8], suggesting the enhancement of bone formation by rhBMP-2 may be related to an increase in recruitment of bone-forming cells and enhancement of neovascularization. In a mouse tibial fracture model, BMP-2 was found to be the earliest cytokine induced during fracture repair. This early release is followed by a sustained increase during soft and hard callus formation phases, whereas BMPs-3, 4, 7 and 8 were restricted during the phases of most active osteogenesis, e.g. hard callus formation [2]. BMP-2 may play a role in mediating the early inflammation cascades as well as early bone formation processes during fracture healing.

The early administration of exogenous rhBMP-2 at fracture site may not be desirable as excessive exogenous BMP-2 may interfere with the natural biological cascades of inflammatory cytokines and other growth factors, and may halt endogenous BMPs production. Recently, BMP-2 has been shown to act as an anti-proliferative and apoptosis-inducing agent for a variety of cell lines by activating signaling cascades that involve several cyclin-dependent kinase inhibitors causing cell cycle arrest [5,22,23,25]. BMPs have also been associated with anti-proliferative effects in vascular tissue by inhibition of platelet-derived growth factor (PDGF)-stimulated human aortic smooth muscle cell proliferation [23]. Cell proliferation was predominant at early stages of fracture healing, peaked at day 8; declined in later stages of fracture healing (days 16–24), when apoptosis became the dominant cell activity [10]. If BMP-2 does have anti-proliferative and apoptosis-inducing ability in some cell types (endothelial, epithelial, muscle, cancer) as reported, then administration of rhBMP-2 too early at the fracture site (day 0) may halt cell proliferation of inflammatory and angiogenic cells, producing negative effects on healing. From previous studies on rhBMP-2 administration that being given on the day of surgery, it has been noted that an exceptionally high dose of rhBMP-2 was needed per treatment (range 80–860 μg in rat and rabbit and 6–12 mg in human) [1,4,6,9,11,12,17,18,20,21] in order to induce a positive effect on fracture healing.

In conclusion, this study suggests that in the mouse fracture model employed, a single percutaneous injection of 2.5 μg of rhBMP-2 on day 0 or 4 after fracture has a positive effect on fracture healing. Data from the radiographic analysis, biomechanical testing and histological examinations all imply that the most effective timing of delivery of rhBMPs may be at the time of surgery (day 0) or/and in the early fracture healing phase (day 4 post-fracture) in the mouse model. It must be remembered that day 4 in the mouse is likely to equate to 7 to 10 days post-fracture in larger animals or humans.

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