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ORIGINAL ARTICLE

Delayed administration of adenoviral BMP-2 vector improves the formation of bone in osseous defects

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The direct, local, administration of adenovirus carrying human BMP-2 cDNA (Ad.BMP-2) heals critical-sized femoral bone defects in rabbit and rat models. However, the outcome is suboptimal and the technology needs to provide a more reliable and uniform outcome. To this end, we investigated whether the timing of Ad.BMP-2 administration influenced the formation of mineralized tissue within the defect. Critical-sized defects were created in the femora of 28 Sprague–Dawley rats. Animals were injected intralesionally with a single, percutaneous injection of Ad.BMP-2 (4×10^8 plaque-forming units) either intraoperatively (day 0) or 24 h (day 1), 5 days or 10 days after surgery. The femora were evaluated 8 weeks after surgery by X-ray, microcomputed tomography, dual-energy X-ray absorptiometry and biomechanical testing. The incidence of radiological union was markedly increased when administration of Ad.BMP-2 was delayed until days 5 and 10, at which point 86% of the defects healed. These time points also provided greater bone mineral content within the defect site and improved the average mechanical strength of the healed bone. Thus, delaying the injection of Ad.BMP-2 until 5 or 10 days after surgery enables a greater percentage of critical-sized, segmental defects to achieve radiological union, producing a repair tissue with enhanced mineralization and greater mechanical strength.
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Introduction

Gene therapy has many potential applications in orthopedic practice, including the enhancement of bone healing.[1,2] A growing literature describes the responsiveness of bone to transfer of osteogenic cDNAs encoding bone morphogenetic proteins (BMPs) -2, -4, -6, -7 and -9.[3–9] The properties of those BMPs have many potential clinical applications, such as to accelerate the healing of fractures, improve the repair of non-unions and segmental defects, enhance spinal fusion and provide better fixation for prosthetic implants.

Although most of the published data come from experiments utilizing *ex vivo* gene transfer to promote osteogenesis, *in vivo* gene delivery with simple adenovirus vectors provides an inexpensive, straightforward, alternative strategy for promoting bone healing.[3,10] The direct, local injection of adenovirus carrying human BMP-2 cDNA (Ad.BMP-2) heals critical-sized segmental defects in rabbits and rats.[10,11] This vector also accelerates the healing of tibial fractures in osteoporotic sheep,[12,13] and Ad.BMP-6 accelerates the healing of tibial fractures in rabbits.[3]

Despite these promising data, there is a need to ensure a more reliable and robust outcome. For instance, in a recent, detailed study in a rat femoral model, we found that 25% of critical-sized defects failed to heal in response to Ad.BMP-2 and cartilaginous areas persisted in many of the healed bones. Moreover, at 8 weeks after surgery, the mechanical strength of the healed bone was only 25% that of normal.[10] As part of a program to optimize the technique, this study focuses on whether the timing of Ad.BMP-2 administration affects the efficiency of bone formation within the defect.

There are several reasons to believe that delayed injection of Ad.BMP-2 may enhance healing to a greater extent than injection at the time of injury. First, Bertone *et al*.[14] observed better healing of a rabbit ulnar defect when administration of Ad.BMP-6 was delayed from 3 h to 7 days.

At the earlier time point, there was considerable heterotopic bone formation in the limb, presumably as a result of elution of the vector from the injection site.[3,14] Thus, it may be better to wait until a stable hematoma has formed. Along these lines, it may be more efficacious to delay the injection until the periosteum has become activated and osteoprogenitor cells have migrated into the defect. Moreover, studies by Ito *et al*.[15] have demonstrated in a murine model that expression of the

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coxsackievirus and adenovirus receptor (CAR), the receptor used by adenovirus, peaks 5 days after fracture. The purpose of this study was to investigate the effect of the timing of Ad.BMP-2 administration on bone formation within critical size bone defects in rats. We delivered Ad.BMP-2 0, 1, 5 or 10 days after surgery to rat femoral defects and evaluated bone formation by X-ray, micro-computed tomography (μCT), dual-energy X-ray absorptiometry (DXA) and biomechanical testing.

Results

Radiographic evaluation

None of the animals treated with Ad. BMP-2 intraoperatively showed united bone 8 weeks after surgery. In group 2, treated 1 day after surgery, 3 of 7 animals (43%) showed united bones. In group 3, treated 5 days after surgery, 4 animals of 7 animals (57%) showed united bones. In group 4, treated 10 days after surgery, 6 of 7 animals (86%) showed bone union. χ^2 Analysis (13.3, $P=0.04$) of percentage of bony union indicated that there was a statistically significant improvement in the incidence of bony union when administration of the vector was delayed. Fisher's exact tests indicated that union rates were significantly higher with treatment group 4 (10 days) compared with group 1 (0 days; $P=0.001$) and with group 3 (5 days) compared with group 1 (0 days; $P=0.026$). In addition, there was a trend toward a difference between group 2 (1 day) and group 1 (0 days; $P=0.07$; Figure 1).

A corresponding increase in radiologic scores also revealed that better osseous filling occurs with later treatment. The overall analysis of variance (ANOVA) *F*-test indicated significant group differences ($F=6.93$, $P=0.002$) and *post hoc* Bonferroni comparisons revealed significant differences for 0 day versus 5 days ($P<0.05$) and 0 day versus 10 days ($P<0.001$). No other group differences reached significance (Figures 2 and 3).

μCT

The μCT evaluation of representative femora 8 weeks after treatment of animals in groups 1–4 confirmed osseous union of different degrees (Figure 4a–d). The μCT images of the femora of group 1 (0 day) showed the least bone formation (Figure 4a), whereas those of group 3 (5 days) and group 4 (10 days) showed the most bone formation (Figure 4c). The axial sections of the same specimen revealed gaps of unmineralized tissue within many of the treated defects (Figure 5b and d).

DXA

The BMC of group 3 (5 days) (0.137 ± 0.033 g, $P=0.02$) and group 4 (10 days) (0.131 ± 0.035 g, $P=0.009$) were significantly greater than that of group 1 (0 day) (0.090 ± 0.016 g; Figure 6). No other differences between the treatment groups reached statistical significance.

Mechanical testing

Torsional testing was used to compare the mechanical properties of the Ad.BMP-2-treated femora of the four groups to intact, contralateral femora 8 weeks after surgery (Figure 7a and b). The Ad.BMP-2-treated limbs of group 1 (0 day) achieved approximately 10% of the strength (0.033 ± 0.005 Nm) and stiffness (0.191 ± 0.061 Nm/rad) of the contralateral, intact femora (strength 0.300 ± 0.080 Nm, stiffness 2.000 ± 0.500 Nm/rad). Strength and stiffness of group 2 (1 day) (strength 0.080 ± 0.048 Nm, stiffness 0.531 ± 0.576 Nm/rad) and group 4 (10 days) (strength 0.074 ± 0.039 Nm, stiffness 0.684 ± 0.738 Nm/rad) reached both about 25% of the strength and about 25% (group 2 (1 day)) and 33% (group 4 (10 days)) of the stiffness of the intact, contralateral femora. The highest values for strength

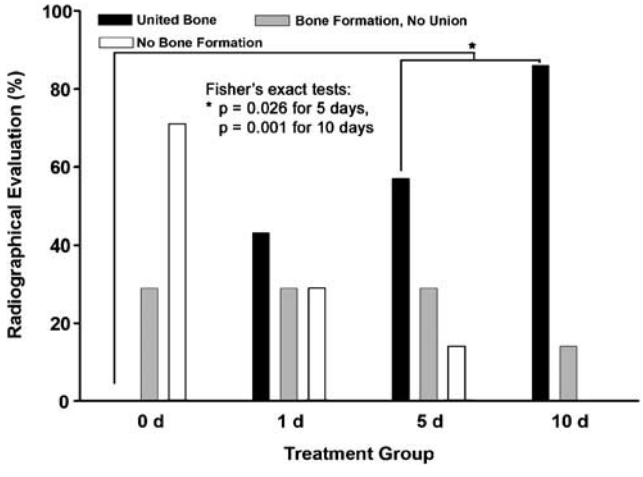

Figure 1 Radiographic evaluation at 8 weeks after surgery. The digital radiographs were examined by two blinded reviewers who assigned rats to one of three categories: bone ends were united (yes/no); bone formation within the defect without union (yes/no); no noticeable bone formation within the defect. Values given are percentage of animals per group for each category. * Denotes statistical significant differences between 0 day versus 5 ($P=0.026$) days and 0 days versus 10 days ($P=0.001$).

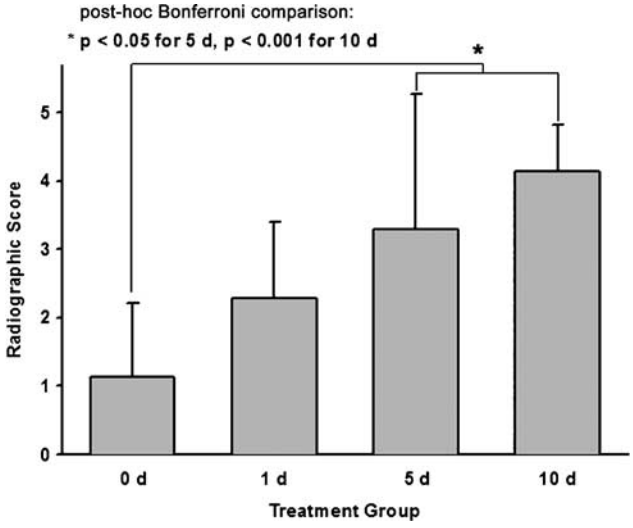

Figure 2 The radiographs were scored according to a 6-point scale, where 0 points indicate no bone formation, 1 point: less than 25% of the defect area filled with bone, 2 points: 25–50% bone filling, 3 points: 51–75% bone filling, 4 points: 76–99% bone filling and 5 points: indication 100% filling of the defect by bone. Values given are means$\pm$s.d. * Denotes statistical significant differences between 0 day versus 5 ($P<0.05$) days and 0 day versus 10 days ($P<0.001$).

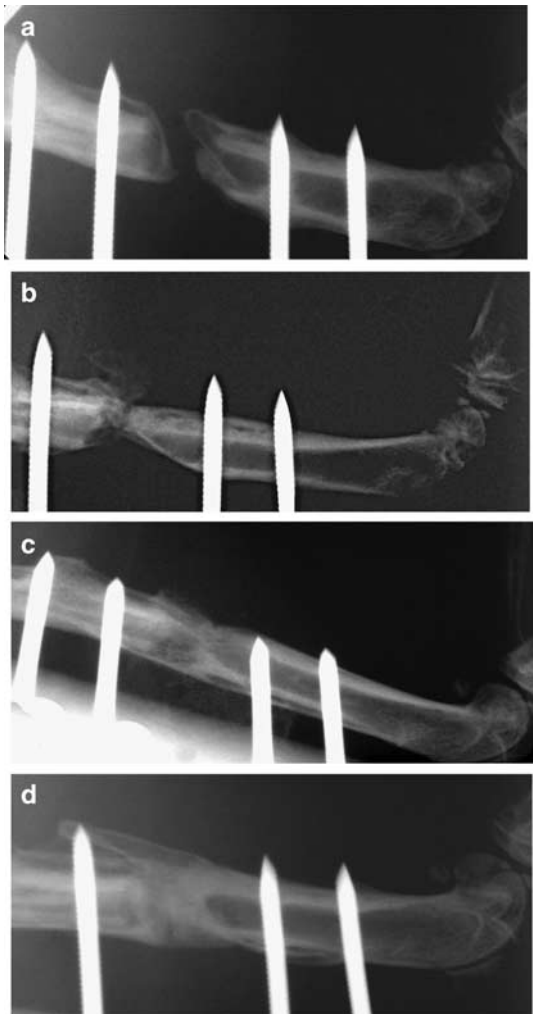

Figure 3 Representative radiographic images of segmental bone defects after direct injection of Ad.BMP-2. Defects treated with Ad.BMP-2 intraoperatively (**a**), or 1 day (**b**), 5 days (**c**) or 10 days (**d**) after surgery displayed different amounts of bone formation within the defect 8 weeks after surgery.

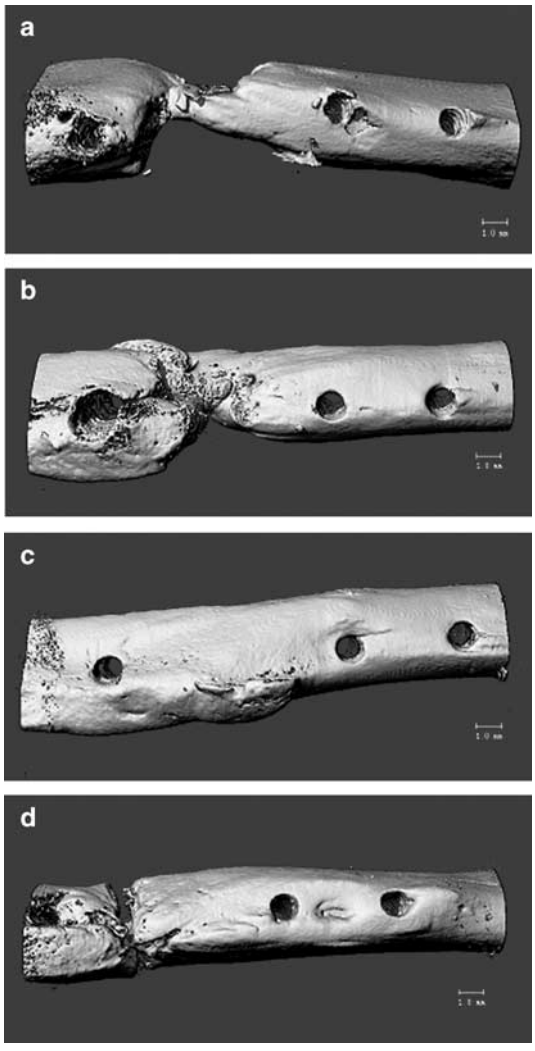

Figure 4 Representative μCT images of femoral defect sites treated with Ad.BMP-2 intraoperatively (**a**) or 1 day (**b**), 5 days (**c**) or 10 days (**d**) after surgery displayed different amounts of bone formation within the defect 8 weeks after surgery.

and stiffness were found in group 3 (5 days). These femora achieved more than 40% of the strength (0.132 ± 0.151 Nm) and more than 50% of the stiffness (1.007 ± 1.462 Nm/rad) of the intact, contralateral femora. Although the mean strength and stiffness of the healed bones increased with delayed administration of Ad.BMP-2, the individual variability was too high to allow statistical significance.

Discussion

Previous studies in rabbits have provided contrasting data on the effect of delaying administration of an osteogenic gene with adenovirus. Baltzer *et al*.[11] reported healing of critical-sized segmental defects in rabbit femora by early injection of Ad.BMP-2, whereas Bertone *et al*.[3,14] found only heterotopic bone formation when Ad.BMP-6 vector was injected 3 h after ulna osteotomy. In the former study, the surrounding musculature was tightly sutured to form a tight chamber from which virus could not escape.[16] Very high transgene expression was noted in the muscle around the defect, and this may have compensated for the lack of a stable hematoma. In the ulna osteotomy model, there was no equivalent attempt to retain virus, leading to tracking of vector into the limb.

The study described here confirms that the simple expedient of delaying the application of Ad.BMP-2 improves consequent bone formation in our rat model. Osseous union according to radiological findings was achieved in 86% of animals when the vector was injected 10 days after surgery, compared with 0% of animals when vector was injected immediately after surgery and 43% of animals treated after 1 day. These radiological findings were largely consistent with data obtained with μCT, DXA and mechanical testing. Although the results are strongly in favor of a delayed injection of Ad.BMP-2 into the defect site, it is not clear whether a 5 or a 10-day delay is better in this model. The radiological evaluation (Figures 1 and 2) favors the later time point, but DXA-based BMC showed similar results for 5 and 10 days, and mechanical data trend toward the 5-day delay. Further

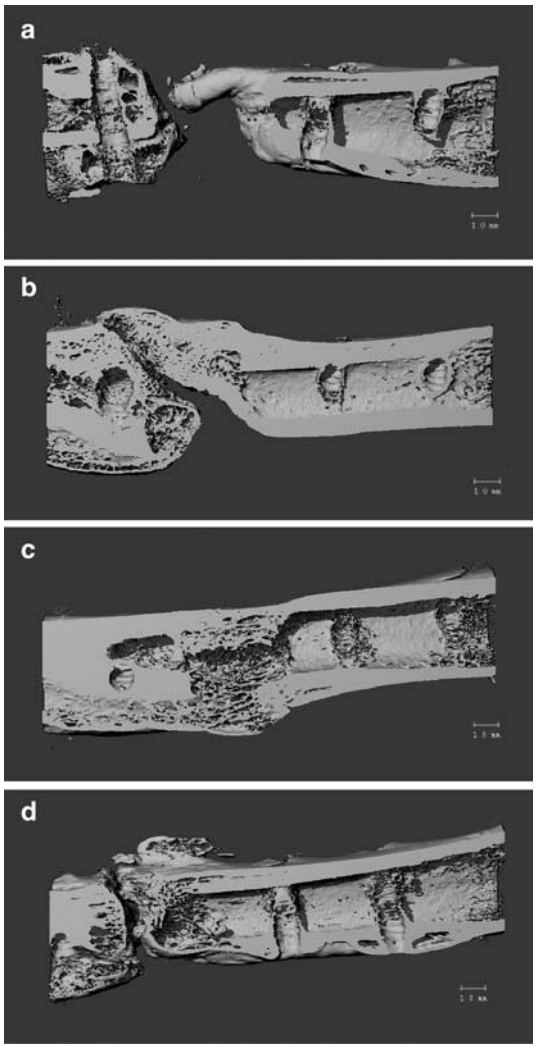

Figure 5 Representative axial, longitudinal sections of μCT images of femoral defect sites treated with Ad.BMP-2 intraoperatively (**a**) or 1 day (**b**), 5 days (**c**) or 10 days (**d**) after surgery displayed different amounts of bone formation and revealed areas of undecalcified tissue within the defect 8 weeks after surgery.

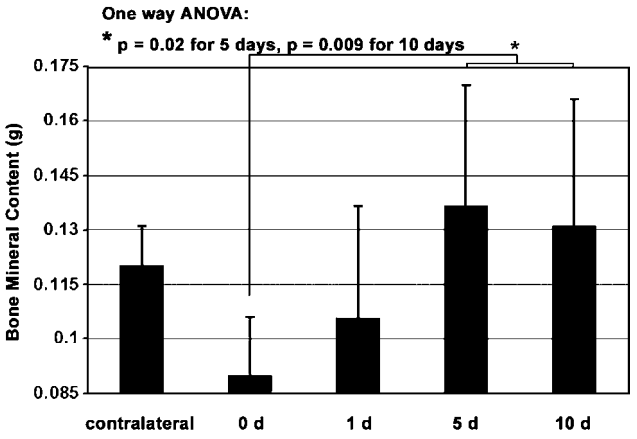

Figure 6 DXA evaluation of the BMC of contralateral femora and defects treated with Ad.BMP-2 for groups 1–4 and corresponding areas of the contralateral, intact femora. Values given are means $\pm$ s.d. * Denotes statistical significant differences between 0 day versus 5 ($P = 0.02$) days and 0 day versus 10 days ($P = 0.009$).

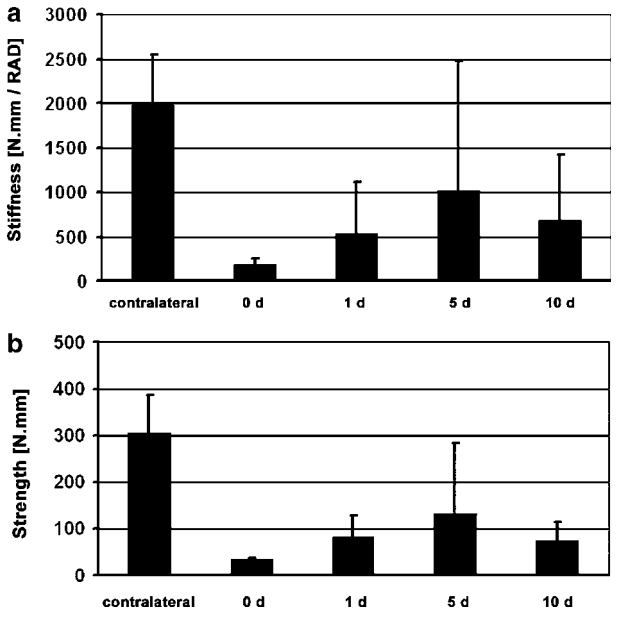

Figure 7 (**a**) Stiffness of Ad.BMP-2-treated femora 8 weeks after surgery in comparison to the contralateral, intact femora. Values given are means $\pm$ s.d. (**b**) Strength of Ad.BMP-2-treated femora 8 weeks after surgery in comparison to the contralateral, intact femora. Values given are means $\pm$ s.d.

studies using larger numbers of rats are needed to clarify if a 5 or 10-day delay is more beneficial for the formation of bone.

Although delayed gene transfer increased the bone mineral content (BMC) of the lesions, there was not a corresponding, statistically significant increase in mechanical strength. This is partly explained by high interanimal variability, and also by the μCT data of the axial sections of the defects. Although clearly more mineralized tissue was seen in the defects of the 5 and 10-day groups, there was often interspersed unmineralized tissue visible which interfered with increase in mechanical stability. Even the best results achieved only 40–50% of the mechanical strength of the intact bone, and further optimization is needed to address this.

There are several reasons why the osteogenic response to the local administration of Ad.BMP-2 may be stronger at later delivery times. For one, studies in mice show that expression of the adenovirus receptor, CAR, in bone is increased by fracture, with peak message abundance occurring at day 5,[15] a kinetic that agrees very well with our findings. Additional events in the defect site may also contribute to the greater responsiveness at later time points. Seeherman *et al*.[17] found that delayed treatment with rhBMP-2/calcium phosphate matrix accelerated osteotomy site healing in nonhuman primates. This result was in part explained by an increased cellular infiltrate at the osteotomy site after 7 days. We can assume that after 5 and 10 days, the defect site will be populated by cells which are both susceptible to *in vivo* transduction with adenovirus vectors and which can also mount an osteogenic response to BMP-2.

Although delayed application of Ad.BMP-2 was advantageous, the technique requires further refining as osseous union is still not reliably achieved in all animals. There are several other factors deserving attention. This includes the quality of the adenoviral preparations. A high ratio of infectious particles to non-infectious particles is desirable as there is a possible influence of the immune system triggered by a viral load. Also, the mechanical properties of the external fixator can have a significant influence on the healing and remodeling of the defect. Finally, the dose of vector may be an important variable.

Materials and methods

Study design

A 5 mm, critical-sized mid-femoral defect was created in the right hind limb of each of 28 male Sprague–Dawley rats (weight 400–425 g) and stabilized by an external fixator. The rats were assigned to one of four groups. All groups (seven rats each) received the human BMP-2 cDNA in an adenoviral vector (Ad.BMP-2). The four groups received AdBMP-2 intraoperatively (group 1) or one (group 2), five (group 3) or 10 (group 4) days after surgery. Adenoviral suspension (40 μl) (4×10^8 plaque-forming units (PFU)), appropriately diluted in phosphate-buffered solution (PBS), was injected percutaneously into the defect. The presence of new bone formation was monitored by X-ray at 0, 4 and 8 weeks. All animals were killed 8 weeks after surgery; femora were harvested and bone formation was evaluated by DXA, μCT and mechanical testing.

Vector production

Serotype 5, E1, E3 deleted, first-generation adenoviral vector Ad.BMP-2, was constructed by *cre-lox* recombination as described earlier.[18] The transgenes were cloned into the E1 domain, with expression driven by the human cytomegalovirus early promoter. Recombinant adenoviruses were propagated in 293/Cre8 cells. High titer preparations were generated by amplification in 293 cells, purification on cesium chloride (CsCl) gradients and dialysis against 10 mM Tris-HCl, pH 7.8, 150 mM NaCl, 10 mM $MgCl_2$, 4% sucrose buffer.[19] Viral titers were estimated as 10^{12}–10^{13} particles/ml by optical density and 10^{10}–10^{11} PFU by standard plaque assay.

Defect model

An established, critical size, femoral defect rat model,[20] was used in this study, according to the method described earlier.[10] Briefly, following anesthesia the diaphysis of the femur was exposed. Then an external fixator was affixed to the femur. A 5 mm osteotomy was then made using a sterile, round dental burr attached to a dental hand piece. The wound was then closed in layers. Virus was administered under general anesthesia either immediately after the surgery, or 24 h, 5 or 10 days after surgery. Viral suspension (40 μl) (4×10^8 PFU), appropriately diluted in PBS was drawn into an airtight 50 μl Hamilton syringe and administered by a single injection. Animals were killed 56 days after treatment. All operative procedures were approved by the Institutional Standing Committee on Animals. Both femora of each animal were harvested and immediately frozen for biomechanical testing, DXA and μCT evaluation ($n = 7$/group).

Radiographic evaluation

Bone formation was monitored by radiography at 4 and 8 weeks using a digital dental X-ray unit (Heliodent DS, Sirona, Germany). Under general anesthesia, the rats were placed in a ventral position and the hind limbs were laterally rotated. The digital radiographs were examined by two blinded reviewers who assigned rats to one of three categories: bone ends were united (yes/no); bone formation within the defect without union (yes/no); no noticeable bone formation within the defect. The radiographs were also scored by the same reviewers according to a 6-point scale, where 0 point indicate no bone formation, 1 point: less than 25% of the defect area filled with bone, 2 points: 25–50% bone filling, 3 points: 51–75% bone filling, 4 points: 76–99% bone filling and 5 points: indication 100% filling of the defect by bone.[21]

μCT

The segmental defect region was scanned using a desktop microtomographic imaging system (μCT40, Scanco Medical AG, Bassersdorf, Switzerland) equipped with a 10 mm focal spot microfocus X-ray tube. The entire defect region was scanned using a 34 μm isotropic voxel size, at 70 keV energy, 250 ms integration time and requiring approximately 600 μCT slices per specimen. Images were reconstructed, filtered and thresholded as described previously, with the threshold determined after pilot scans on several specimens.[22]

DXA

The mineral content of the defect region was assessed by DXA (PIXImus 2, GE-Lunar, Madison, WI, USA). Specimens were placed on a lucite block to simulate soft tissue during scanning. The scans were acquired using the small animal high-resolution mode. The total BMC was measured within a region of interest that included only the defect zone.[23] By assessing the total mineral content rather than the area bone mineral density, errors associated with positioning were minimized.

Mechanical testing

Following all non-invasive imaging, specimens were tested to failure in torsion. Both ends of each specimen were embedded in polymethylmethacrylate to provide an appropriate and reproducible gripping interface with the testing module. Specimens were tested to failure under deformation control at a constant deformation rate of 5 rad/min. Angular deformation and applied load data were acquired at 10 Hz. The applied load and angular displacement were used to compute the torsional stiffness and strength of the healing defect.

Statistical methods

The radiographic evaluation of the percentage of animals showing bone union was assessed by χ^2-analysis (χ^2-test = 13.2, $P = 0.04$) and Fisher's exact tests were conducted to compare two groups at a time. The radiographic scores were analyzed by overall ANOVA F-test ($F = 6.93$, $P = 0.002$) and post-hoc Bonferroni comparison. Statistical significance of DXA and biomechanical data was determined by using one way ANOVA ($P < 0.05$ was considered statistically significant).

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