Tissue Engineering and Regenerative Medicine, Vol. 8, No. 2, pp 158-163 (2011)

| Original Article |

The Effects of rhBMP-2 Injection at Distraction Osteogenesis of Rats' Tibia

Hae-Ryong Song[1, 2†], Sung Eun Kim[2†], Hyo-Geun Kim[1, 2], Young-Pil Yun[2], Ji-Hoon Bae[2], and Hak Jun Kim[2*]

[1] Division of Brain Korea 21 Program for Biomedical Science, and [2]Department of Orthopeadic Surgery, Korea University, College of Medicine, Guro Hospital, 80 Guro-Dong, Guro-Gu, Seoul, 152-703 Korea

(Received: October 4th, 2010; Accepted: January 30th, 2011)

Abstract：Distraction osteogenesis is the popular method of bone-lengthening procedure. Delayed consolidation of bone after distraction osteogenesis makes complications related to external fixator and refractures after removal of external frame. We hypothesized that only rhBMP-2(recombinant human bone morphogenetic protein-2) could accelerate the long bone healing. 18 Sprague-Dawley rats were divided into two groups after 5 mm lengthening by distraction osteogenesis. The control group was none-injected group and the experimental group was injected 0.5 cc of 0.05 mg/ml rhBMP-2. We evaluated the 6 samples respectively at different time point (2, 4, 8 weeks) using simple radiographs, micro-CT and histological stains. According to the simple radiographs, bone consolidation of percentage pixel count was higher in the experimental group at 2, 4, 8 week, and statistically significant higher than the control group at 8 weeks ($p < 0.05$). Percentage bone Volume, trabecular thickness in the experimental group was higher than the control group by evaluation with micro-CT. According to the histological examinations, neovascularization and new stromal cells were observed in the control group at 2 week- speciemen but intramembranous ossification was seen at 2 week- specimens in the experimental group. Intramembranous and enchondral ossification was seen in the experimental group but not seen in the control group at the 4 week-specimens. Trabeculae thickness in the experimental group was thicker than the control group at 8-week specimens. Only local injection of rhBMP-2 at the distraction site could accelerate the bone healing during distraction osteogenesis.

Key words: *distraction osteogenesis, rhBMP-2, rat*

1. Introduction

Distraction osteogenesis using external fixator is traditional new bone forming method for bony defect, and limb length discrepancy.[1-6] Long application of external fixator until bony consolidation can make pin site infection, refracture after removal of the fixator and nerve problem.[7, 8] There had been many reports that methods facilitated the consolidation of distraction osteogenesis. Ultrasound, injection of calcium sulfate, growth factor, mesenchymal stem cells have been.[9-15]

Epidermal growth factor (EGF) and bone morphogenetic protein-2 (BMP-2) are clinically used for regeneration of soft tissue and bone formation respectively.[16-18] There had been many reports about using BMP-2 with collagen, polymer coated gelatin sponge and chitosan hydrogel at distraction osteogenesis in long bone.[19-22] But there was little report about only BMP-2 injection at the distraction osteogenesis site of long bone.

The authors planned to study the early consolidation of distraction osteogenesis after injecting only recombinant human bone morphogenetic protein-2 (rhBMP-2) in rats' tibia model.

2. Materials and Methods

2.1 Experimental Animal Model

Thirty-six skeletal mature Sprague Dawley rats (male, 6 weeks, body weight 300-400 g) were anesthetized by intraperitoneal injection of Tiletamine/Zolazepam (0.025 mL/100 g, Zoletil® 50, Virbac Laboratories, France), Xylazine (0.025 mL/100 g, Rompun® 2%, Bayer Healthcare Korea, Korea). The right leg of rat was shaved and prepared for sterile isolation. A 2-cm skin incision was made over the medial aspect of right proximal tibia. The perisoteum and soft tissue carefully

†These authors contributed equally to this work
*Tel: +82-2-2626-3090; Fax: +82-2-2626-1164
e-mail: hjunkimos@gmail.com (Hak Jun Kim)

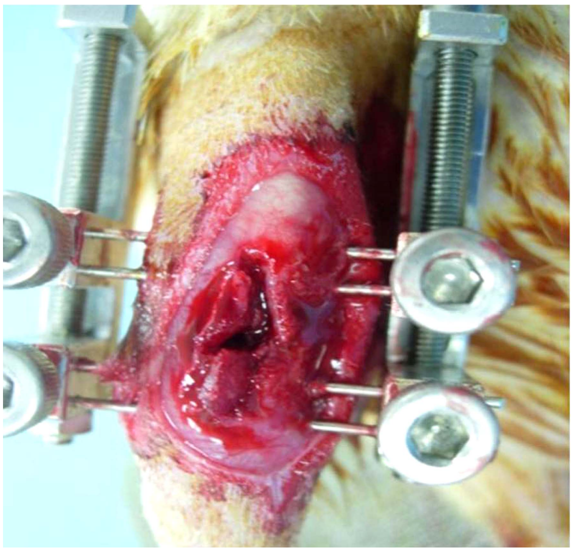

Figure 1. Photograph of surgical procedure.

retracted, and the tibia was exposed. Four 0.9 mm k-wires (Zimmer®, Warsaw, IN) were used to drill both cortices of the tibia. K-wires were clamped bilaterally with author own-designed external fixator (U&I, Kyunggido, Korea). The tibia was osteotomized between second and third k-wire. The osteotomized gap was compressed using the external fixator (Fig 1). Subcutenaous tissue and skin was sutured. The animals were allowed free movement in cages after recovery from anesthesia. After 7 days, the lengthening of 36 tibiae was initiated at a rate of 0.25 mm per 12 hours for 10 days. The rats were divided in to two groups. Group A (control group, 18 rats) didn't received any injection after the final distraction: Group B (experimental group, 18 rats) received an injection of 0.5 mL E-coli derived rhBMP-2 (0.05 mg/mL, Cowellmedi Co. Busan, Korea) into the distraction site under the fluoroscopic control. The 6 rats of each group were sacrificed 2, 4, 8 weeks after injection. The specimens from these rats were evaluated using gross findings, radiograph and micro-computed tomography, histologic analysis.

2.2 Radiographic and Micro-CT Evaluation

Radiographs were obtained whole specimens at 0, 2, 4 and 8 weeks using Xscan® (Xavis, Sungnam, Korea). Callus formation and bone consolidation at the distraction site were evaluated and also percent pixel count (% pixel count) was calculated using ImageJ® (CDC, Atlanta, GA) program.

The two specimens of each group were evaluated using micro-computed tomography (micro-CT, Skyscan 1173 (Skyscan, Belgium), Xenos, Suwon, Korea). Percentage bone volume (%BV), trabecular thickness (TT) and trabecular separation (TS) of distraction site were obtained from microCT in 2, 4, 8 week-specimen.

2.3 Hematoxylin and Eosin (H&E) Staining

The tibiae were harvested and fixed in 10% formaldehyde solution over several days. Routine decalcifying was done in 8% hydrochlroric acid/formic acid solution. Blocks were cut using a microtome and H&E staining was performed for hisotologic analysis. The specimens treated with the haematoxylin and rinsed with Distilled Water (DW). The Acid alcohol (0.3%) treatment was differentiated and then rinsed with DW. After rinse, the Eosin was treated for 2 min, and observed in light microscope for image analysis. Qualitative evaluation of osteogenesis was performed by examining the formation of new cortex and bone marrow at the distraction site.

2.4 Von Kossa Staining

The tibiae were harvested and fixed in 10% formaldehyde solution over several days. Routine decalcifying was done in 8% hydrochlroric acid/formic acid solution. Blocks were cut using a microtome and Von Kossa staining was performed for hisotologic analysis. The specimens were treated a 2% silver nitrate solution, and were exposed to sunlight for 1 h after which was rinsed with DW. Sodium thiosulfate (5%) was treated for 5 min, the samples were then rinsed in DW, and dried for image analysis.

Qualitative evaluation of osteogenesis was performed by examining the formation of new cortex and bone marrow at the distraction site.

2.5 Statistical Analysis

Percentage pixel count, percentage bone volume, trabecular thickness and trabecular separation were compared using Kruskal-Wallis test. $p < 0.05$ indicated a significant difference.

All animal experiment were performed under the guideline of Korea University IACUC (Institutional animal care and use committee).

3. Results

3.1 Gross Findings

Callus was visible at distraction site at 2-week specimen; callus of control group was more brittle than experimental group. There were no gross differences between control and experimental group at 4, 8 week specimens (Fig 2).

3.2 Radiographic Evaluation

Mean distraction length of control and experimental group

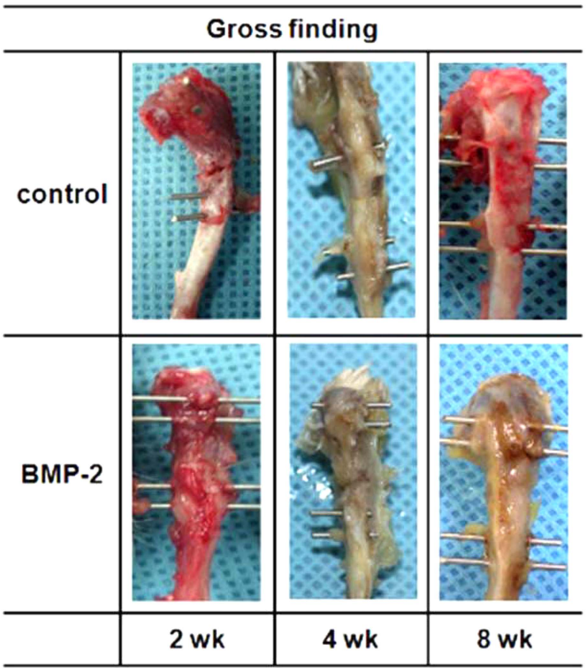

Figure 2. Gross finding examination of the control group and BMP-2 group at 2, 4, and weeks.

were 42.2±19.4 mm and 48.8±9.8 mm respectively. Mean % pixel count of control group was 77.9±8.4% at 2 weeks specimen and experimental group was 82.3±14.9%. The pixel count of experimental group was higher than control group, but there was no statistical significance ($p = 0.149$). At 4 weeks specimen, pixel count of control group was 82.3±14.9% and the experimental group was 101.2±8.4% that was statistically significant ($p = 0.033$). Also at the 8 week specimen, the pixel count of control group was 84.0±9.1 % and experimental group was 102.5±11.2 %($p = 0.04$) (Fig 3).

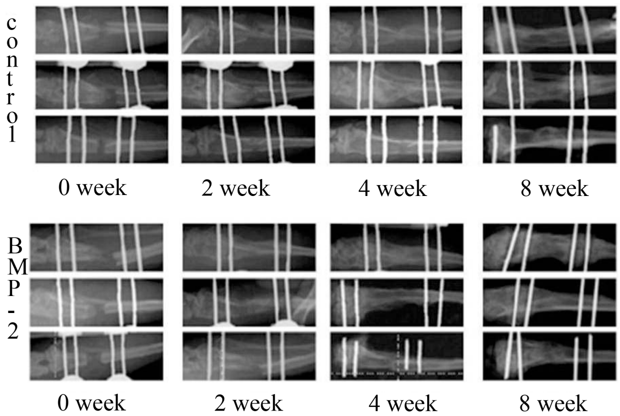

Figure 3. Representative 2D radiographs of both control and treated (BMP-2 injected) tibia at 2, 4, and 8 weeks after surgery.

Table 1. The results of % pixel count using ImageJ® program

	2 week	4 week	8 week
Control	77.9±8.4	80.8±2.3	84.0±9.1
Experimental	82.3±14.9	101.2±8.4	102.5±11.2
p-value	-	$p < 0.05$	$P < 0.05$

The pixel count was increased according to the time but there was statistically significant increasing only in the experimental group (Table 1). There was significant difference at 4 and 8 week compared with 2 week in experimental group, respectively ($p < 0.05$). However, there was no significant difference between 4 and 8 week experimental group because of already generated sufficiently new bone at 4 week.

3.3 MicroCT Results

The percent bone volume were 23.8, 37.9, 36.1 % in control group and 33.8, 40.3, 47.6 % in experimental group respectively at 2, 4, 8 week (Fig 4). The percent bone volume increased according to the time in both group. The percent bone volumes of experimental group were higher than control group at different time point. Trabecular thickness were 0.4, 0.78, 0.67 mm in control group and 0.59, 0.63, 0.78 mm in experimental group respectively at 2, 4, 8 week. Trabecular separation were 1.89, 2.14, 2.34 mm in control group and 1.79, 1.89, 2.24 mm in experimental group respectively at 2, 4, 8 week.

3.4 Histological Results

At 2 weeks specimen, fibrous tissues and vessels were seen

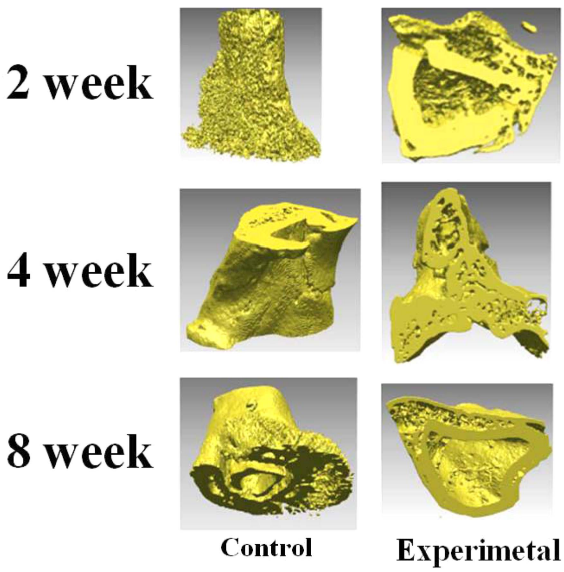

Figure 4. Representative microCT images of both control and treated (BMP-2 injected) tibia.

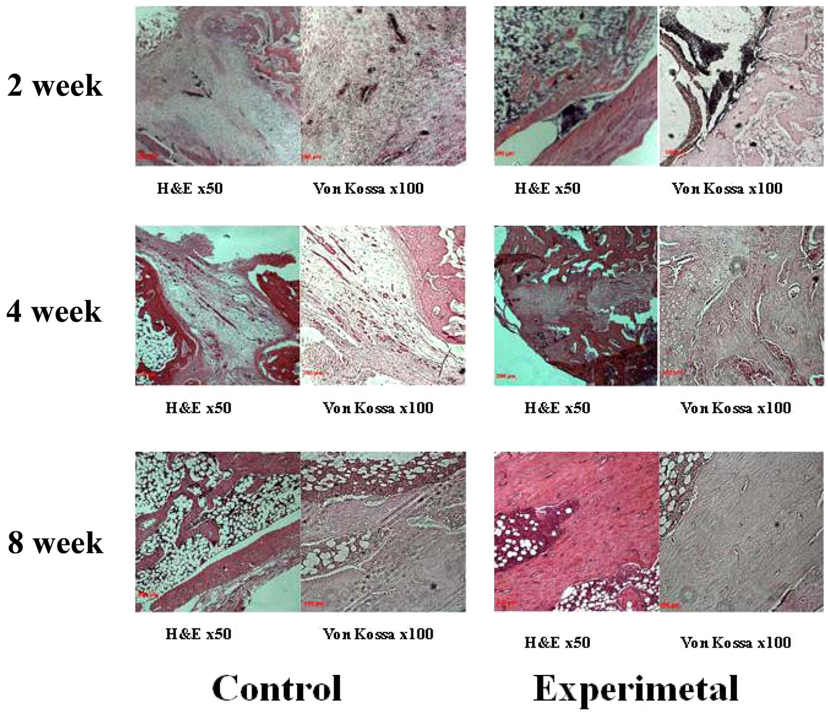

Figure 5. Histological results of the control group and experimental group at different time points with H&E stain and Von Kossa stain.

in the control group, but new bone formations were seen in the experimental group at H&E stain and Von-Kossa stain.

At 4 weeks specimen, newly developed cartilaginous tissues were observed in the distraction area of control group, but large amount of new bones and cartilaginous tissue in experimental group.

At 8 week specimen, woven bones and cancellous bones were seen in the distraction area of control group, but thick cortices and cancellous bone in experimental group (Fig 5).

4. Discussion

Distraction osteogenesis is unique technique to make intramembranous and enchondral ossification simultaneously which can be occurred by new vessel, collagen and fibroblast from surrounding soft tissues.[6, 23] Distraction osteogenesis had been used for bony defect and retardation of bony development. [4-6, 24] But, long term application of external fixator would make infection of pin site, contracture of joint and fracture of lengthened site.[8, 25, 26] To overcome the disadvantages, ultrasound, electrical stimulation and intake of vitamin D was advocated for patient in clinical situations.[27, 28] Some experimental trial were reported to improve the consolation which bone mesenchymal cell bone substitutes, platelet rich plasma and growth factors were introduced into the distraction site.[9, 12, 14, 29]

Recently, to shorten the treatment time and accelerate bone formation in distraction osteogenesis, various growth factors such as BMP-2, BMP-7 and[9] Y. Mizumoto, T. Moseley, M. Drews, V.N. Cooper 3rd and A.H. Reddi, Acceleration of regenerate ossification during distraction osteogenesis with recombinant human bone morphogenetic protein-7, *J Bone Joint Surg Am* (2003), pp. 124–130 85-A Suppl 3. View Record in Scopus Cited By in Scopus (32) basic fibroblast growth factor (bFGF) have been studied in different distraction models for enhancing bone consolidation.[12, 37, 38] Surprisingly, the application of bone-relevant growth factors in long-bone distraction osteogenesis has not been generally studied but has great potential for new discoveries with clinical relevance and significance. Although the application of BMP-2, BMP-7 and bFGF resulted in accelerated bone formation in distraction models, these factors lack specificity for osteoblasts and often require superphysiological doses for different applications, which have resulted in various adverse effects, including ectopic bone formation and local severe inflammatory reaction.[39-44] To improve bone formation, great efforts have been made to find and evaluate novel bone-relevant growth factors. Here, BMP-2 have been known as potent bone forming growth factor and lately used for spinal fusion with collagen.[30] There were also studies about BMP-2 in distraction osteogenesis[11, 19, 20, 22, 31, 32], but there little report that only E-coli derived rhBMP-2 was injected into the distraction site exogenously without carrier. Carriers such as collagen and gelatin had the ability of tissue regeneration themselves, so the pure effect of BMP-2 could not be assessed in previous reports. We could identify the new bone formation of E-coli derived rhBMP-2 from this study. We could observe the early consolidation at the distraction site after simple injection of rhBMP-2.

There were many experimental animal models of distraction osteogenesis. Intramembranous ossification could be observed at distraction site using dog and rabbit model.[33-35] But intramebranous and enchondral ossification could be seen at rat's distraction model which could be occurred in human distraction osteogenesis. Early ossification could be seen at rat's distraction osteogenesis model.[36] In this study, we could seen intramembranous and encondral ossification simulateously.

The limitation of this study was that injected rhBMP-2 could not be traced. The location of rhBMP-2 could not be seen at radiographs. The methods which trace the injected rhBMP-2 should be developed.

5. Conclusion

Only E-coli derived rhBMP-2 can accelerate the consolidation of distraction osteogenesis. Injection of rhBMP-2 at the distraction site to treat the bony defect or retardation of bone formation could accelerate the early consolidation in clinical situation.

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