Transport Disc Distraction Osteogenesis with Recombinant Human Bone Morphogenic Protein-2 for Large Calvarial Defect Reconstruction

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Transport Disc Distraction Osteogenesis with Recombinant Human Bone Morphogenic Protein-2 for Large Calvarial Defect Reconstruction

Directed by Professor Yong Oock Kim

The Doctoral Dissertation
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Seung Yong Song

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The Graduate School
Yonsei University

Decemeber 2013

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Seung Yong Song

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Transport Disc Distraction Osteogenesis with Recombinant Human Bone Morphogenic Protein-2 for Large Calvarial Defect Reconstruction

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Large calvarial defect can be developed by trauma, surgery or congenital anomalies. This condition can be dangerous to patients because brain is more susceptible to external impact than normal. To cover the calvarial defect, autologous bone or biomaterials was used conventionally, but complications including donor morbidity and infection were also reported. On the other hand, attempts using transport disc distraction osteogenesis (TDDO) to treat a calvarial defect has been made. But, till now, consistency and reliability of this method are lacking. In this study, we used an internal distractor, which was more stable than an external distractor, combined with humoral factor, recombinant human bone morphogenic protein-2 (rhBMP-2), to achieve sufficient bone formation using TDDO in the calvarial defect. BMP-2 is known to enhance consolidation, shorten the period of distraction, and eventually accelerate bone regeneration. Author compared the effect of mechanical factor (TDDO) and the combination of mechanical (TDDO) and humoral (rhBMP-2) factor.

Fourteen mongrel dogs were divided into two groups, one was control (n = 6) and the other was experimental group (n = 8). The specially designed internal

distraction device was applied. It had two guide rails which enhance stability of device and allowed drug delivery through cannulated rotator rod. After 5-day latency period, distraction was initiated. The distraction rate was 2 mm/day. It took 13 days for completion of whole defect length distraction. rhBMP-2 of 10 μg/day was injected during whole period of distraction. Consolidation period was 3 months. Area of osteogenesis was measured by three dimensional computed tomographic images, strength was assessed by compression strength measurements and histologic evaluation was conducted.

Two dogs of the experimental group were excluded from this study due to infections. Therefore, 6 animals in the control group and 6 animals in the experimental group were compared. In the comparison of area of regeneration, the control group treated with only TDDO without rhBMP-2 showed 68.04 ± 18.07% of bone regeneration compared with original defect. On the contrary, the experimental group treated with TDDO and rhBMP-2 showed 94.64 ± 5.29% of bone regeneration. Average of area of osteogenesis was higher in rhBMP-2 treated group with statistical significance. ($p < 0.01$) Regenerated bone of the control group showed strength of 23.85 ± 6.19 N/mm^2 in compression test and regenerated bone of the experimental group showed strength of 53.75 ± 18.66 N/mm^2 in the same test. Comparison was performed with the ratio of regenerated bone strength to normal bone strength of each group. Regenerated bone of the experimental group showed increased strength with statistical significance. ($p < 0.05$) Histology of regenerated bone in the experimental group was more similar with normal cancellous bone compared to that of the control group. Osteoblastic rimming was more prominent in the regenerated bone of the experimental group and the number of osteoblasts was also increased compared to that of normal bone or the control group with statistical significance. ($p < 0.01$)

TDDO with internal distraction device of delivering rhBMP-2 can enhance bone regeneration of large calvarial defect in dog model. Regenerated bone nearly covered large calvarial defect and strength of regenerated bone was similar with

that of normal bone. These results indicate that the clinical human application of TDDO combined with rhBMP-2 is possible.

Key words: distraction osteogenesis, transport disc distraction osteogenesis, transport distraction osteogenesis, calvarial defect, calvarial reconstruction, recombinant human bone morphogenic protein-2

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I. INTRODUCTION

Large calvarial defect can be developed by trauma, surgery or congenital anomalies.[1,2] In humans, it cannot be healed spontaneously older than 2 years of age.[3] This condition is not only aesthetically unacceptable but also dangerous to patient because brain is more susceptible to external impact than normal.

Conventionally, autologous bone or biomaterials were used for the reconstruction of calvarial defect. But, bone harvesting procedure needs longer operation time and can cause donor morbidities. On the contrary, biomaterials including polyemthyl metacrylate, porous polyethylene, hydroxyapetite and titanium are abundant and easier to use than autogenous bone graft, but postoperative infection and exposure of implant have been reported in considerable number of cases.[1,2]

This report is an animal experiment of new surgical modalities, so called transport disc distraction osteogenesis (TDDO), that can treat the large calvarial bone defect in regenerative concept, prior to human application.

DO was first described by Alexander Codivilla at 1905.[4] This technique creates bone *de novo* between corticotomy surfaces undergoing gradual distraction.[5] Its clinical application was refined by Ilizarov. He used external fixators after osteotomy for endochondral bone formation in the long bone.[6] Application of DO

on membranous bone was reported by Snyder et al. on mandible of dog at 1973 at first, and later on humans.[7,8]

Based on these results, transport disc distraction osteogenesis (TDDO, also called bifocal DO) was introduced in the long bone.[9] This technique used small bone fragment (transport disc) compared to defect size and it was moved periodically by distraction for osteogenesis.[10] In 2002, first study of TDDO on calvarial defect in rabbit model was reported by Bouletreau et al.[5]

However, TDDO on calvarial defect is not popular, since the report of only one human application in 2010.[2] A few animal studies were conducted since the first report with animal model.[1,11-17] This seemed to be because no methodology showed consistent and satisfactory results till now.

One of the most remarkable animal studies in the application of TDDO for calvarial defect was conducted by Hong and followed by Yun.[14,16]

Hong et al. conducted an animal study of TDDO with an external type of distraction device without any supplementary maneuvers, such as growth factors. Nevertheless, they concluded that TDDO could induce considerable amount of bone with hardness comparable to normal bone. However, the volume of newly generated bone was not sufficient.[14]

Yun et al. conducted more advanced experiment of TDDO. They used internal type of distraction device different from previous study. It had advantages of increased mechanical stability and reduction of subcaute inflammatory reaction during DO, which resulted in more bone regeneration despite of short consolidation period. This model was more close to human application but still come short for being used as reliable modality.[16]

To obtain sufficient bone formation by TDDO in calvarial defect, two factors were considered to be important in this study. One is the stability of distractors. Hong et al. used external distractors and experienced loss of distractors in three of ten animals.[14] External distractor has an advantage of strong distraction force, easy handling of the distraction vector but a disadvantage of inevitable instability

because substantial portion of its structure is exposed to outside of skin especially in young children or animals.[18] These physical limitations can cause subcaute or chronic inflammation, which may disrupt bone regeneration during distraction period. Actually, animal study conducted by Yun et al. demonstrated increased bone regeneration using internal distractor compared to Hong's study without exposure or loss of devices.[16] However, even in their cases, non-functioning distractor was reported. After all, distraction device itself is an important factor for successful distraction.

The other is humoral factors. Among the various factor which can induce bone growth, bone morphogenic protein (BMP) can be considered primarily to facilitate the bone regeneration in DO.[19-24] BMP belongs to the transforming growth factor β superfamily. It promotes vascular invasion, bone formation, bone remodeling and bone marrow differentiation by directing cell differentiation of mesenchymal stem cells to osteoblasts and osteocytes, which in turn build and renew the extra cellular matrix.[25-27] Since this protein is known to have strong osteogenetic effect, it has been already used clinically in orthopaedic surgery or dental surgery using commercially available product.

Since first introduced by Urist et al., more than 20 subtypes of BMPs were reported. Among them, type 2 and 7 were more vigorously studied and clinically applied.[26,28,29] Originally, BMP was purified from animal but recombinant human BMP (rhBMP) was then released.[30-32] rhBMP has high purity, so its effect is known to be more predictable and less harzardous to human.[26] Most studies in recent years used rhBMP instead of purified BMP.

In this study, rhBMP-2 planned to be supplemented with TDDO. BMP-2 is known to enhance consolidation, shorten the period of distraction, and eventually accelerate bone regeneration.[19,22,23,33,34]

It may be the most desirable if rhBMP-2 is continuously released during the whole distraction period into distraction zone because BMP has short half life, 7 to 16 minutes *in vivo*.[26] In this study, rhBMP-2 was injected periodically with newly

designed drug-delivering distraction device.

In this study, author compared the effect of mechanical factor (TDDO) and the combination of mechanical (TDDO) and humoral (rhBMP-2) factors. This kind of study would be the first trial for the reconstruction of large cranial defect with TDDO. This study will provide feasibility and reliability for TDDO to apply clinically than previous studies.

II. MATERIALS AND METHODS

1. Experimental Animals and Groups

Fourteen mongrels (mongrel female dog, 5-month gestational age) were divided into two groups, one was control (n = 6) and the other was experimental group (n = 8). All experimental animals were maintained at the animal facility, Yonsei University College of Medicine, and all experimental procedures were performed after the permission of the Institutional Animal Care and Use Committee (2011-0032).

2. Operative Procedures

All animals were anesthetised by intravenous injection of midazolam (0.2 mg/kg; Bukwang Pharmaceutical Company Ltd, Ansan, Republic of Korea) and propofol (3 mg/kg, Pofol V; Dongkook Pharmaceutical Company Ltd, Jincheon, Republic of Korea); In succession, endotracheal intubation was performed with inhaling isoflurane (Foran; Choongwae Pharmaceutical Corporation, Hwaseong, Republic of Korea). Vital signs and oxygen saturation level were monitored similar with other operations. To reduce cerebral edema, the concentration of isoflurane was maintained at 2% to 3%, and the CO2 concentration was reduced by inducing the hyperventilation state. Animal was placed in the dorsal position and sterilized with povidone iodine. All surgical procedures were conducted in aseptic technique. About 8 cm incision was made in the midline of the scalp, and the calvarium was exposed by elevating the muscles and the periosteum bilaterally. Ostectomy was performed with burr for creation of calvarial defect. Defect size was 33 x 35 (width x length) mm and long axis was centered on the midline of the cranium. Transport disc (TD) size with 30 x 8 (width x length) mm was prepared. For easy movement of transport disc, its size was smaller than width of the defect. Total length of distraction was 26 mm. The periosteum of the TD was removed.

The internal bone distractor was specially designed for this experiment with guide rails, and was fixated using titanium screws at the border of remnant cranium. For the injection by once a day of rhBMP-2 during distraction period,

inlet was located at the rotating part of the distractor. It was sealed by silicone to prevent retrograde infection. Injected rhBMP-2 was flowed out to distraction zone. (Figure 1)

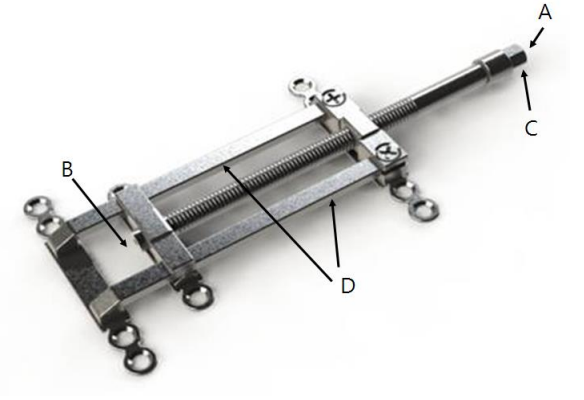


Figure 1. A newly designed drug-releasing internal distractor. A: inlet of vehicle, sealed with silicone to prevent retrograde infection. B: outlet of vehicle. C: rotator rod. D: guide rails

Microplates were used to fixate a transport disc, and it was connected to the distractor. Muscles and the skin were sutured by #3-0 Vicryl and #4-0 nylon sutures. (Figure 2)

After the surgery, the analgesic ketorolac (Tarasyn; Roche Pharmaceutical Company Ltd, Basel, Switzerland) and the antibiotic ceftriaxone (50 mg/kg; Donghwa Pharmaceutical Company Ltd, Ansan, Republic of Korea) were injected intramuscularly for 2 days.

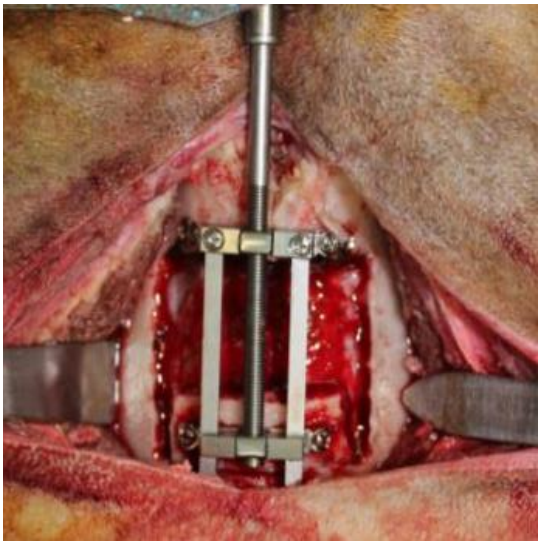


Figure 2. Intraoperative photograph. The newly designed drug-delivering internal distractor was applied in calvaria of mongrel dog. The size of calvarial defect and transport disc (TD) was 33 x 35 (width x length) mm and 30 x 8 (width x length) mm, respectively. Periosteum and dura mater were detached from TD.

3. Distraction

After 5-day latency period, distraction was initiated. The distraction rate was 2 mm/day. It took 13 days for completion of whole defect length distraction. Recombinant human BMP-2 (BioVision, Milpitas, CA, U.S.A.) was prepared at the concentration of 10 μg/ml with phosphate buffered saline and injected once with a volume of 1 ml/day with 24G syringe through cannulated rod. It was injected for 13 days, during whole time of distraction. Consolidation period was 3 months. (Figure 3)

4. Radiologic Studies

A plain X-ray of the skull was taken in all experimental animals 5 days before starting the distraction. Another X-ray was taken near the end of the bone distraction period to check the status of the distraction device. Computed tomography (CT) was performed (Somatom Sensation 64; Siemens Medical Systems, Erlangen, Germany) at the end of the study after euthanizing the experimental animal. (Figure 3)

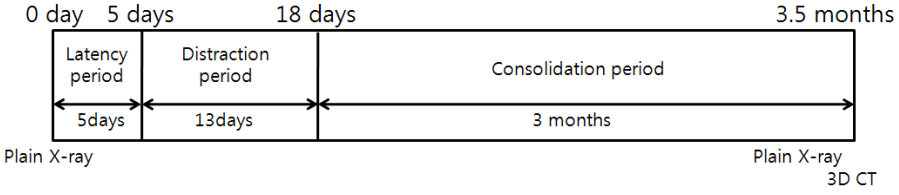


Figure 3. Schedule of experiments. This study was designed with 5 days of latency period, 13 days of distraction and 3 months of consolidation period. Radiologic studies included plain X-rays at postoperative day 0 and nearly end of the consolidation period and 3 dimensional computed tomography (3D CT) at the end of the study.

5. Area of Osteogenesis

Quantitative analysis of the area of regenerated bone was accomplished by measuring the area of the bone defect from CT images. The newly formed bone area was measured by subtracting the bone defect area that remained in the three-dimensional CT from the bone defect area created at the time of surgery (33 x 35 mm). We compared the calculated ratio (in percent) of the measured new bone formation area compared to the initial bone defect area.

6. Strength

After removal of distraction device, newly formed bone specimen was obtained from the TDDO site. Each specimen was harvested with a full-thickness of a bone. The strength of the harvested bone was estimated by compression strength

measurements. These measurements were done using an Instron 5848 microtester (Instron, Norwood, MA, U.S.A.), and the calculation was performed using the Poisson ratio for the effective module of ultrasound indentation. The strength value is presented in N/mm^2 (Pa). The strength of the experimental group and the control group was measured 3 times. Finally, the measured data were compared between the regenerated bone by TDDO and the normal calvarial bone by the strength ratio of regenerated bone to normal bone. Normal bone was obtained from ostectomized bone for creation of calvarial defect.

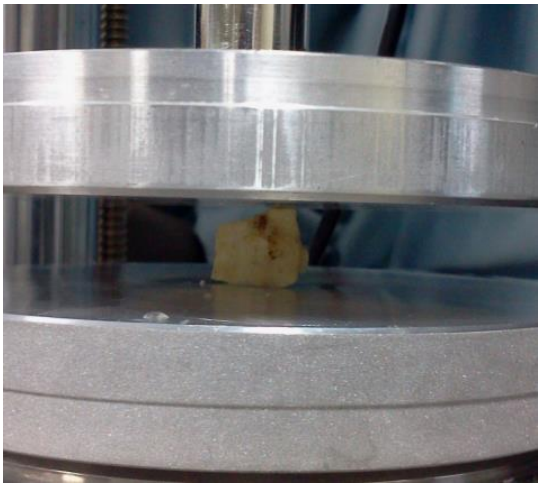


Figure 4. Compression test using Instron 5848 microtester. Strength was measured in each bone sample by 3 times.

7. Histologic Examination

The piece of extracted bone was fixed in 4% phosphate buffered formalin (pH 7.73) solution for 14 days. After the dehydration process, the samples were placed in an alcohol and acetone solution. Then, they were further fixed with a methyl-methacrylate solution (Merck, Hohenbrunn, Germany) to prevent calcium demineralization. Next, the bone pieces were cut into even smaller pieces with a

diamond saw at 30-μm intervals along the axis of distraction. The samples were then stained with hematoxylin and eosin, and a visual comparison was done under a microscope.

To compare the number of osteoblasts in the bone marrow of the normal, control and experimental group, manual counting was performed in ten randomly selected areas at high power field (x100).

8. Statistics

All data from our experimental results are represented as mean ± standard deviation (SD). To test the validity of our values, comparisons between groups were performed by the Student's *t* test. We regarded a value as statistically significant when the $p < 0.05$. All data analyses were performed using the Statistical Package for the Social Sciences (SPSS v. 15.0; SPSS Inc, Chicago, IL, U.S.A.).

III. RESULTS

During the experiment, two dogs of the experimental group were excluded from this study due to infections. After all, data of 6 dogs in the control group and 6 dogs in the experimental group were used for analysis of results. And, at the end of the experiment, bone specimen for strength measurement was inadequate in one animal. So, strength tests of the control group were performed in only five animals. Radiologic study was performed according to predetermined schedule, plain X-ray at immediate postoperatively and the end of distraction and 3 dimensional CT at the end of consolidation. (Figure 4, 5, 6)

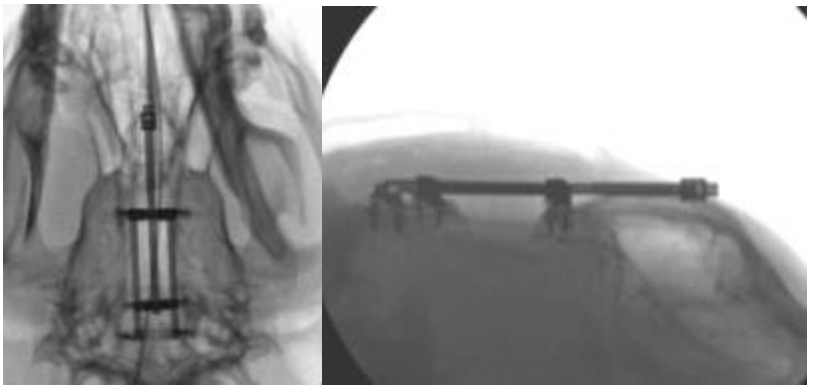


Figure 5. Immediate postoperative plain X-ray. Position of distraction device was checked by this study.

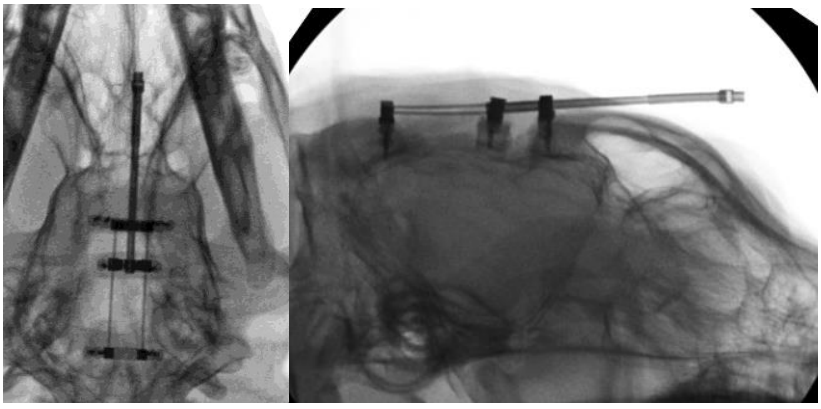


Figure 6. Plain X-ray at the end of distraction. Position change or deformation of distraction device was checked by this study.

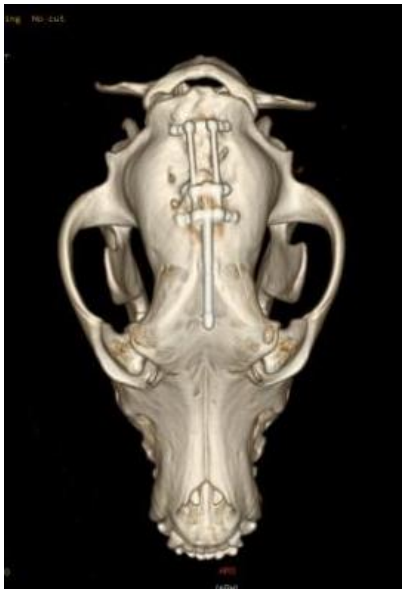


Figure 7. Three dimensional computed tomography at the end of the consolidation period

1. Area of Osteogenesis

The control group treated with only TDDO without rhBMP-2 showed 68.04 ± 18.07% of bone regeneration compared with original defect. On the contrary, the

experimental group treated with TDDO and rhBMP-2 showed 94.64 ± 5.29% of bone regeneration. (Figure 7, 8)

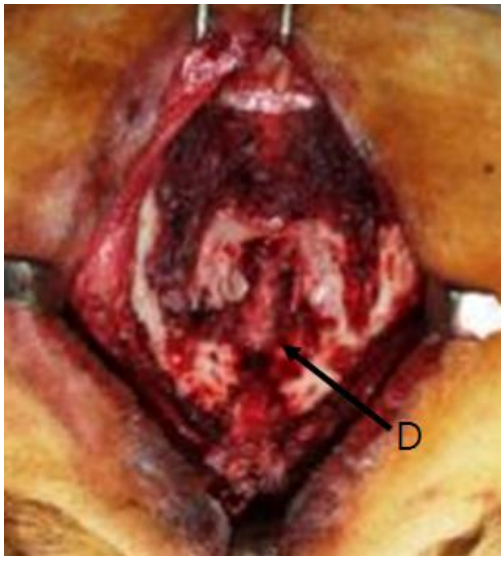


Figure 8. Photograph at the end of the consolidation period from the control group. The control group was consisted of the animals treated with only transport disc distraction osteogenesis for calvarial defect. Defect (D) was not fully covered by newly generated bone in the control group.

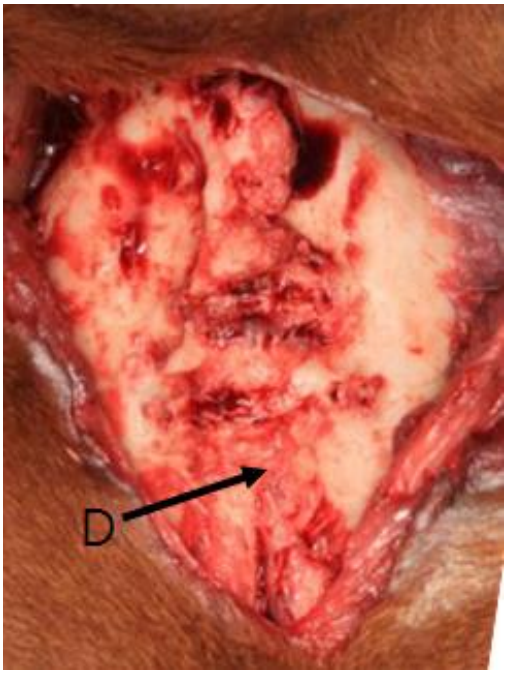


Figure 9. Photograph at the end of the consolidation period from the experimental group. The experimental group was consisted of the animals treated with transport disc distraction osteogenesis combined with recombinant human bone morphogenic protein-2 for calvarial defect. Defect (D) was nearly covered by newly generated bone in the experimental group.

Area of osteogenesis was higher in rhBMP-2 treated group with statistically significant difference. ($p < 0.01$) (Table 1, Figure 9)

Table 1. Area of regeneration. Area ratio of bone regeneration was the ratio of regenerated area to the original defect. It was higher in the experimental group but there was no statistical significance.

	Control (without rhBMP-2[1]) (%[2])	Experimental group (with rhBMP-2) (%)
1	95.21	93.77
2	68.87	99.03
3	72.47	98.79
4	54.00	94.29
5	49.33	84.83
6	68.38	97.12
Average±SD[3]	68.04 ± 18.07	94.64 ± 5.29**

[1]rhBMP-2: recombinant human bone morphogenic protein-2

[2]percent ratio of regenerated area to original defect size

[3]SD: standard deviation

**$p < 0.01$

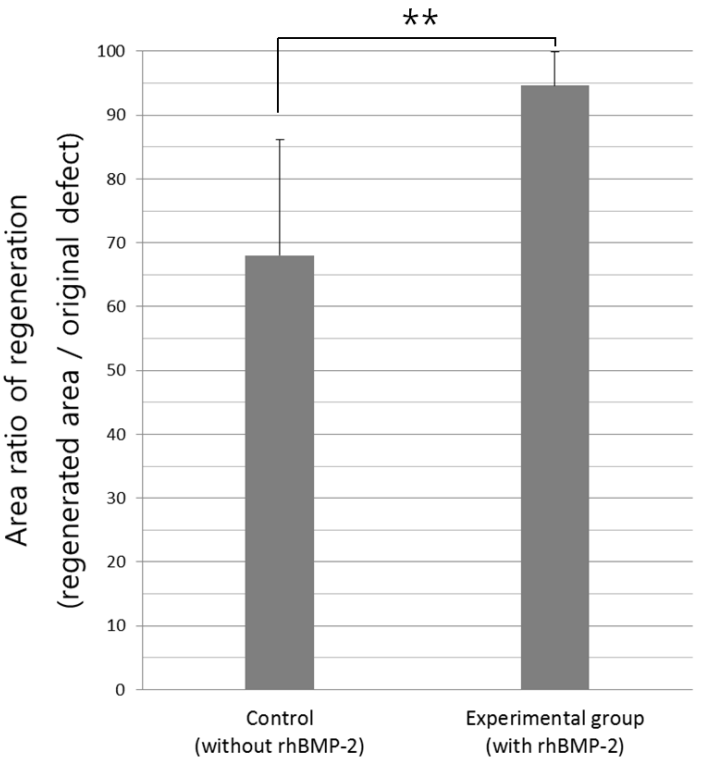


Figure 10. Comparison of area of regeneration with graph. Calvarial defect was treated with only transport disc distraction osteogenesis (TDDO) in the control group and TDDO with rhBMP-2 in the experimental group, respectively. The experimental group showed increased new bone formation with statistical significance. (p = 0.008) (rhBMP-2: recombinant human bone morphogenic protein-2)

2. Strength

Regenerated bone of the control group showed strength of 23.85 ± 6.19 N/mm^2 and that of the experimental group showed strength of 53.75 ± 18.66 N/mm^2 in compression test. We compared two groups with the ratio of regenerated bone strength to normal bone strength. Regenerated bone by TDDO combined with

rhBMP-2 showed increased strength with statistical significance. ($p < 0.05$) (Table 2, Figure 10)

Table 2. Comparison of strength. Compression test was performed and strength was measured. The experimental group showed significantly increased bone strength compared with the control.

	Control (without rhBMP-2[1])			Experimental group (with rhBMP-2)		
	Normal bone (N)	Regenerated bone (R)	Ratio (R/N)	Normal bone (N)	Regenerated bone (R)	Ratio (R/N)
Strength (N/mm^2)	25.28	15.26	0.60	60.87	29.06	0.48
	32.40	20.21	0.62	23.21	34.83	1.50
	27.75	Not measurable	Not measurable	46.70	75.74	1.62
	43.14	25.41	0.59	42.82	55.20	1.29
	54.95	31.00	0.56	76.18	70.31	0.92
	123.40	27.39	0.22	84.48	57.36	0.68
Average	51.15	23.85	0.52	55.71	53.75	1.08*

[1]rhBMP-2: recombinant human bone morphogenic protein-2

*$p < 0.05$

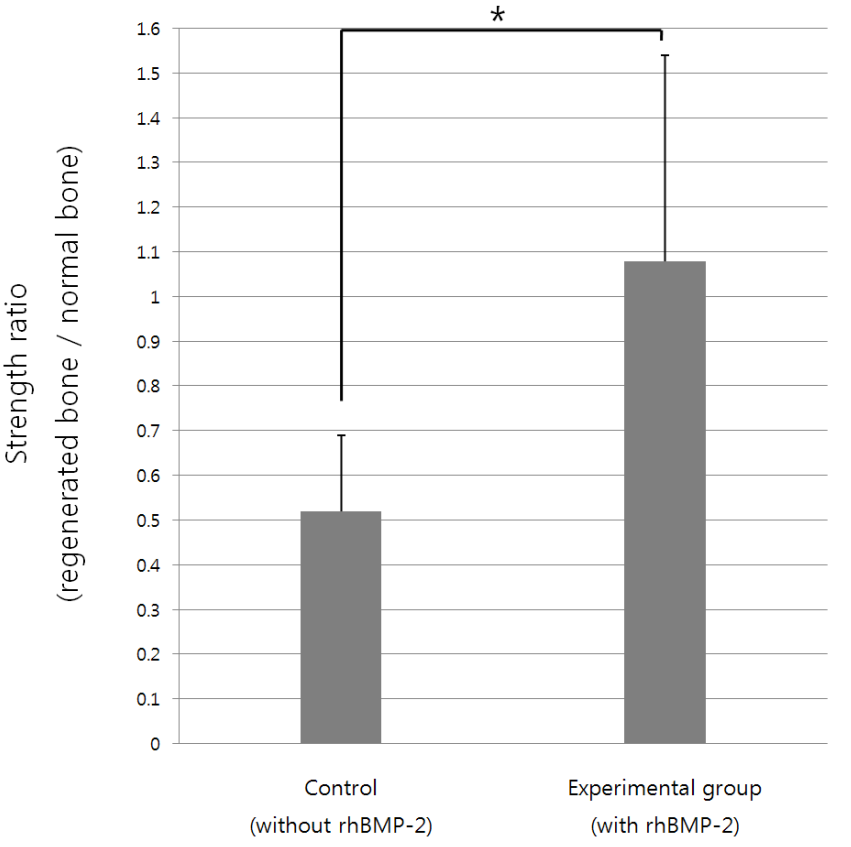


Figure 11. Comparison of strength with graph. Strength ratio was the ratio of regenerated bone to normal bone in each group. Regenerated bone from transport disc distraction osteogenesis with recombinant human bone morphogenic protein-2 showed significantly increased bone strength ratio compared to the control group. (*$p < 0.05$)

Curves of compression test in the control group showed lower slope although there were some variations in each sample. Some bony specimen of the control group did not have ultimate load and just showed increasing pattern of load according to the increase of compression extension. And curves of compression test in the experimental group showed higher slope and ultimate load although

there were also some variations in each sample (Figure 12, 13)

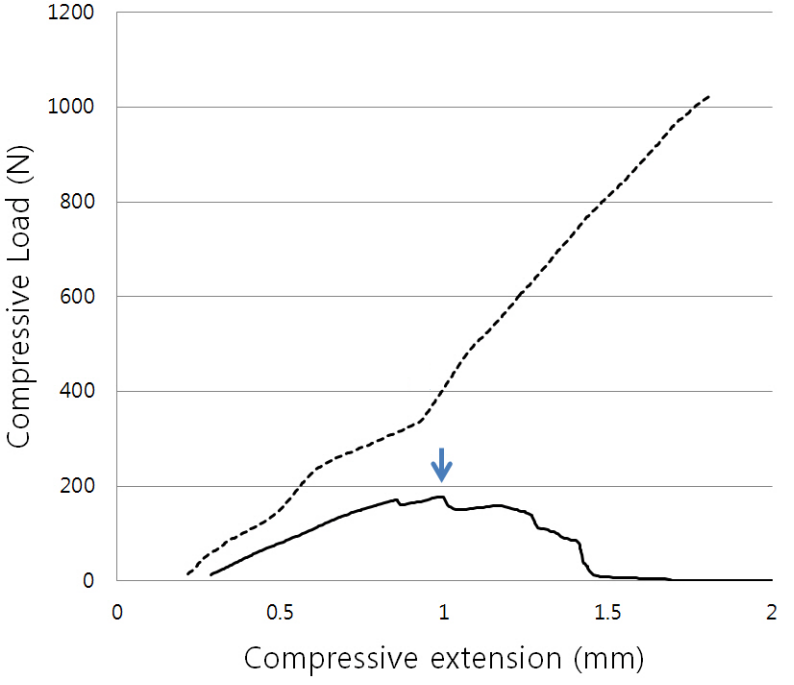


Figure 12. Compression test curves of one sample from the control group. Slope and ultimate load (arrow) were higher in the normal bone than the regenerated bone by distraction without rhBMP-2. (solid line: regenerated bone, dotted line: normal bone)

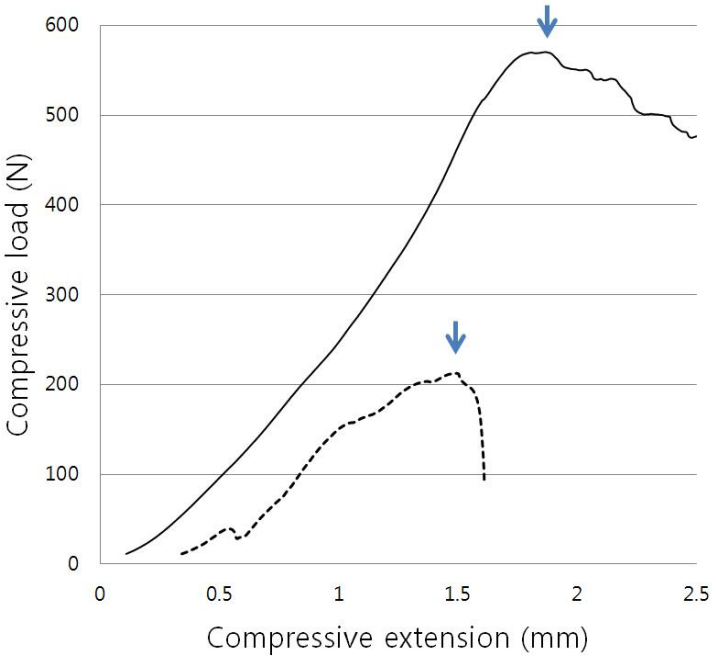
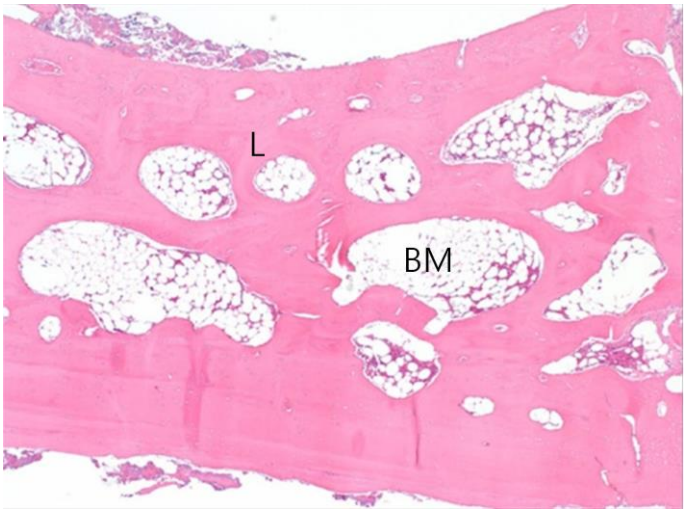


Figure 13. Compression test curves of one sample from the experimental group. Slope and ultimate load (arrow) were higher in the regenerated bone than the normal bone by distraction with rhBMP-2. (solid line: regenerated bone, dotted line: normal bone)

3. Histology

Histologic examination revealed that normal bone components such as bone marrow, lamella and osteocytes existed in the normal, control and experimental group. However, bony specimen of the control group showed reduced lamella portion compared to normal bone and its lamella was not matured compared to the normal bone. So, it can be considered as woven bone rather than mature bone. (Figure 13) The bony specimen of the experimental group had similar thickness and cortex portion with normal bone. These findings indicate that regenerated bone of the experimental group is more close to the normal cancellous bone. (Figure 14)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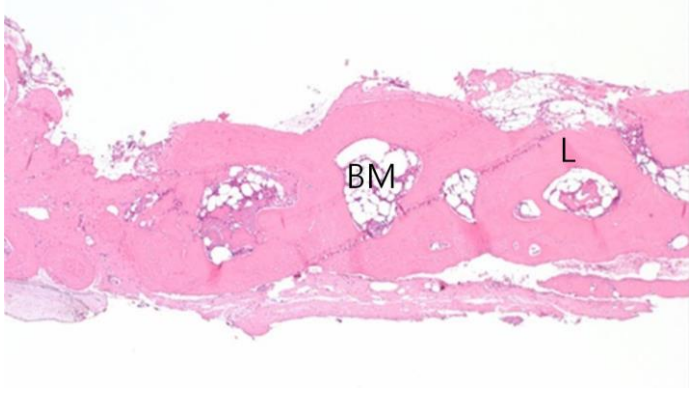
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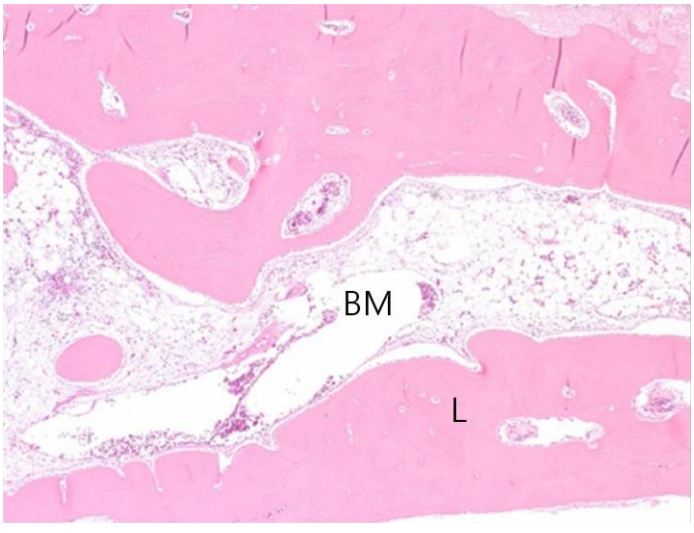
BM
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Figure 14. Microscopic findings of normal (upper), control (middle) and experimental (lower) group (x40). Normal bone components, such as bone marrow (BM), lamella (L) and osteocytes are observed in all groups. However, cortex of regenerated bone in the control group is relatively small and consists of woven bone. Regenerated bone of the experimental group has similar thickness with normal bone and the amount of cortex is also similar with normal bone. It is more close to the histology of normal cancellous bone.

In the bone marrow, osteoblasts rimming was observed in the control and the experimental group. (Figure 14) The number of osteoblasts was significantly higher in the experimental group compared to the control group. ($p < 0.01$) On the other hands, the control group also had significantly increased osteoblasts compared to normal bone. ($p < 0.01$) (Figure 15)

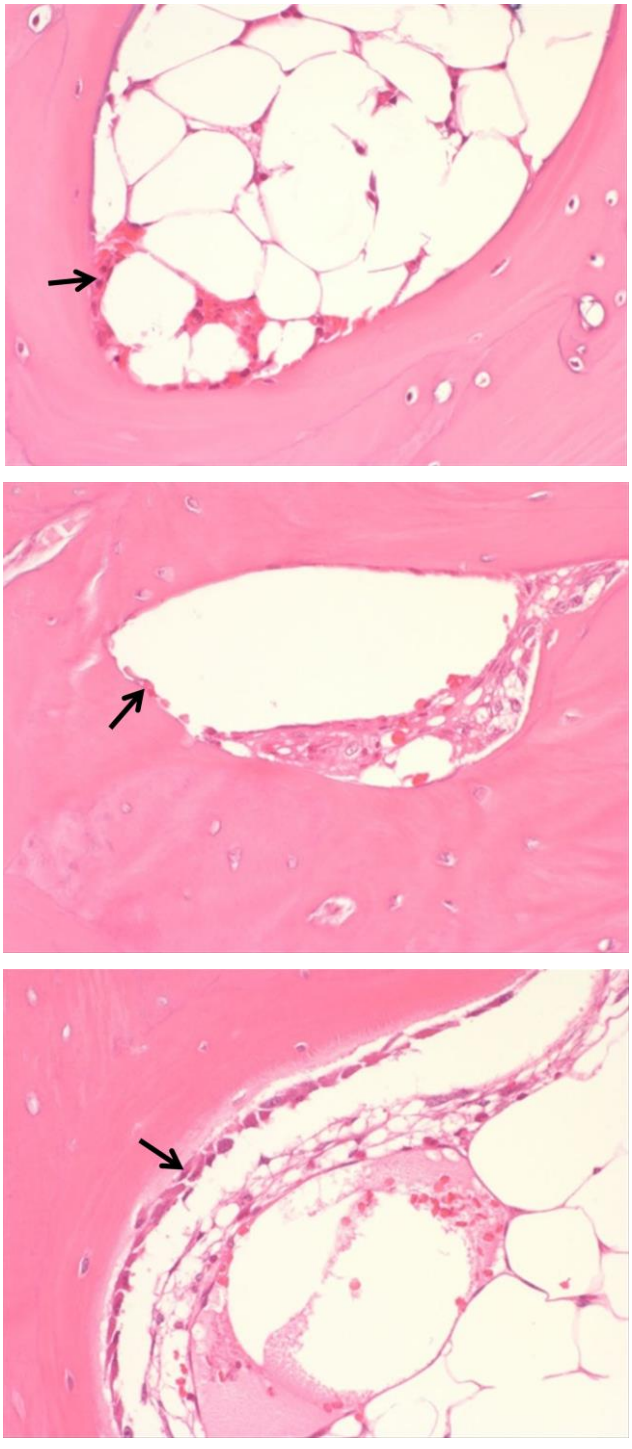

Figure 15. Microscopic findings of normal (upper), control (middle) and experimental (lower) group (x200). The marrow of the control and the experimental group reveals osteoblastic rimming (arrow). The number of osteoblasts of the experimental group increased significantly compared to the control and normal group.

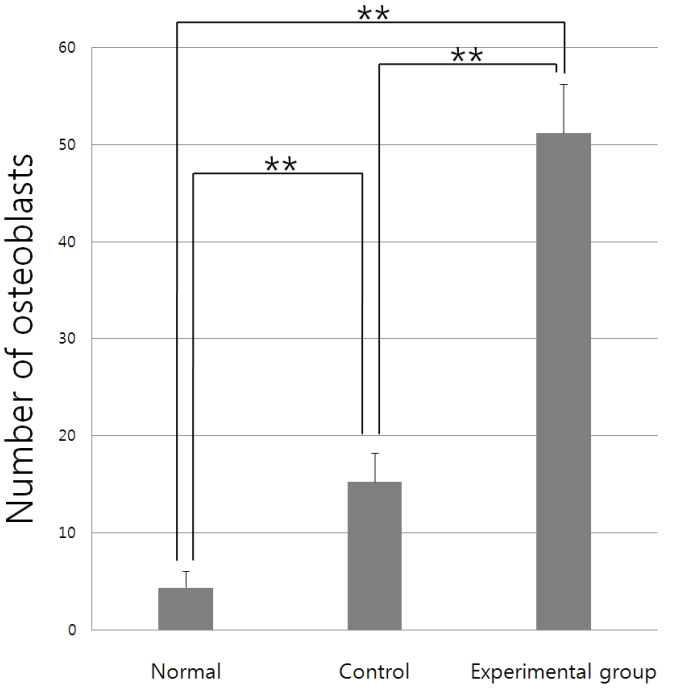

Figure 16. Comparison of the number of osteoblasts in the normal, control and experimental group. (x100) The experimental group had significantly increased osteoblasts compared to normal and the control group. (**$p < 0.01$) The control group also had increased number of osteoblasts compared to normal group. (**$p < 0.01$)

IV. DISCUSSION

Distraction osteogenesis (DO) has become useful treatment modalities in long bone lengthening and some membranous bone reconstruction, since Dr. Ilizarov's report.[6] To shorten the period of treatment and increase the efficacy, various refined approaches were introduced till recent years.

TDDO is the latest technique among them. Because regeneration of bone for large defect should be achieved using relatively small bare bone segment, it had less reliability on efficacy of bone formation and strength. This may be the reason that there were few reports about this technique since its introduction.

The author believes three factors are important for the successful TDDO treatment. The first is the ratio of transport disc to bone defect. Small calvarial defect can be treated by traditional bone graft or biomaterials with low morbidity at once. Large defect difficult to be treated by traditional method are the candidate for TDDO treatment. In the previous reports, the ratio of transport disc to calvarial defect was ranging from 19~50 %.[1,2,5,11-16] In this study, we designed the defect and transport disc size at the smallest ratio, 18%. (Table 3) The length of our transport disc was 8 mm and it was the minimal size for the fixation to the distraction device. In the top view, the defect size in this study was nearly one third of whole cranium, and this condition could provide useful information for application to human. However, appropriate size of transport disc was not determined till now. Maximal bone regeneration with minimal transport disc size may be ideal in clinical setting and optimal ratio of transport disc to defect size should be determined for human application in the future.

The second factor is the distraction device. Various types of device were reported in previous literatures. Nearly half of previous studies of TDDO for calvarial defect used external type of distractors, but in recent years, internal distractors were used more frequently. (Table 3) In this study, we used internal distractor, based on Hong' report, with guide rails, upgraded from the distractor of Yun's experiment.

Table 3. Previous reports about transport disc distraction osteogenesis for calvarial defect

	Bouletreau et al[5]	Hirano et al.[13]	Kramer et al.[11]	Muller et al.[12]	Hong et al.[14]	Durmus et al.[15]	Icekson et al.[1]	Cho-Lee GY et al.[2]	Yun et al.[16]	Steinbacher et al.[17]	This study
Subject	rabbit	rabbit	sheep	sheep	dog	sheep	sheep	human	dog	rabbit	dog
Distractor type	External	Internal	External	External	External	External	Internal	Internal	Internal	Internal	Internal
Defect size (D) (mm)	15x15	14x7	40x60	50x60	15x33	50x60	20x20	50x85	15x35	16x16	33x35
Transport disc size (T) (mm)	15x10[1]	(7~10) x 10 (trapezoid)[1]	40x20	40x20	15x7	40x20	20x10	50x20	14x7	10x16[1]	30x7
Disc ratio (T/D) (%)	40	36	33	27	21	27	50	24	19	38	18

1: additional craniectomy was performed outside of the defect for preparation of transport disc

It had increased durability and stability and might allow more bone regeneration.[14,16]

The third is the humoral factor to facilitate or accelerate bone regeneration. It can determine applicability of TDDO for human. Before application to human, enhancement of bone regeneration by TDDO combined with some humoral factors should be examined and confirmed by animal experiment. This study is relevant in this aspect. And our attempt of combined therapy showed superior results in bone regeneration of calvarial defect.

DO with BMP treatment has been one of the most well known regimen to improve the results of bony regeneration. BMP is a strong osteoinducer and plays a pivotal role in the molecular signaling cascade leading to bone regeneration and remodeling in a DO procedure.[23] Especially, BMP-2 is one of the most powerful growth factor for bone regeneration, widely studied. In 2007, the FDA approved BMP-2 delivered with an absorbable collagen sponge carrier for clinical use in craniofacial deformities.[35] InductOs® (Pfizer, former Wyeth, Berkshire, UK), Infuse Bone Graft™ (Medtronics, Minneapolis, MN, U.S.A.) and Amplify® (Medtronics, Minneapolis, MN, U.S.A.) are commercially available rhBMP-2 for clinical uses now. However, we used rhBMP-2 produced by other company. (BioVision, Milpitas, CA, U.S.A.)

In the long bone, many experiments were performed using DO and BMP-2. Sailhan et al. demonstrated that local application of rhBMP-2 with collagen sponge after osteotomy followed by DO significantly increased ossification and mineral content with a dose effect.[34] Lesaichot et al. also reported that rhBMP-2 with collagen sponge carrier at the end of distraction area in rabbit tibia model decreased consolidation period.[23]

In the membranous bone, mandible reconstruction with DO was widely studied. Issa et al. reported rhBMP-2 with monoolein gel carrier could enhance bone formation in acute DO and might potentially reduce the treatment period.[20] On the other hand, some researchers used mesenchymal stem cell (MSC) based gene

therapy for continuous BMP release and during distraction and consolidation period and maximizing bone regenerative effect. Long et al. demonstrated BMP-2 gene therapy using transfected bone marrow MSCs with adenovirus promoted new bone formation during mandibular DO and could even reduce distraction period.[36] Castro-Govea also reported similar results with 3-component graft (autologous mesenchymal stem cells transduced *ex vivo* with adenovirus expressing BMP-2 and demineralized bone matrix) on mandibular distraction of mongrel dog model.[37] Zhang et al. reported MSCs encoding BMP-2/7 could increase bone healing in even irradiated mandible. But these favorable effects were mainly about mandible and DO.[24]

Less study was conducted in TDDO model with BMP-2. Cakir-Ozkan et al. compared the effects of TDDO and bone graft in sheep model and reported that expression of BMP-2, 4 and TGF-β were more increased in DO model.[38] So, we can guess role of growth factor including BMP might be important in bone regeneration with TDDO.

Icekson et al. showed TDDO without growth factor can be effective tool for closing full-thickness calvarial defect in sheep. But they used only 4 animals and transport disc to defect size were 50%, relatively bigger than this study.[1] Moreover, one case of the control group healed completely without DO while other three cases of the same group were not healed at all.[1] Similar phenomena were encountered in other reports and in our study.[14,39] One of the possible explanations of these results would be the age of animals and the ratio of transport disc to defect size. If age is young enough to heal the defect, nearly whole critical size calvarial defect can be recovered spontaneously. Increased ratio of disc to defect will also contribute accelerated bone regeneration because disc itself can contribute to healing as a bone graft. But this hypothesis explains only small part of this unpredictability and there seemed to exist another factor determining healing potential which is still unknown.

In the comparison of osteogenesis area, the average of ratio of regenerated bone

to original defect was higher in the experimental group with statistical significance. Moreover, area ratio of regeneration was 94.64% in the experimental group. We think this result itself has a meaning for clinical application because large defect was nearly covered. In addition, variability of results was much reduced after combining rhBMP-2 treatment. In other words, area ratio of regeneration was increased in general with small standard deviation in the experimental group. This fact can indicate that variable production and secretion of humoral factors in each organism can be a possible reason of unpredictable bone defect healing. And, according to these results, TDDO combined with rhBMP-2 have a possibility to help more consistent or predictable bone regeneration, although we cannot draw a definite conclusion by our animal study with small numbers.

Many of previously described attempts to improve the efficacy of DO used BMP-2 with carriers. And, some researchers used gene therapy model for continuous release of BMP-2. But gene therapy is difficult to use clinically till now due to unpredictable expression, variability in effective period, complexity in gene transfection process and unknown hazard of adenoviral vector.[40]

Likewise, previous studies about DO combined with BMP-2 were performed using local application, sponge carrier and virus-delivery model. However, our experiment is differentiated from these reports in aspect of periodical injection of rhBMP-2 during whole distraction period. In this study, new distractor for TDDO allowing injection of liquid-type humoral factor like rhBMP-2 was devised. This device was designed to deliver rhBMP-2 on the lately distracted zone. The use of drug delivering distractor for TDDO has advantages of simplicity, easy dose calculation and increased predictability. First drug releasing distractor was devised by Grayson et al. in 2001. They made external distractor with cannulated pin where regimens can be injected.[41] But their device had some space between injection area and distraction zone. So, drug delivery might be not effective. Recently, Konas et al. also reported DO experiment with drug releasing internal distractor. They demonstrated intermittent rhBMP-2-chitosan hydrogel infusion

with their newly developed device facilitated ossification of femur of rats.[39]

However, their studies used DO, not TDDO and conducted in mandible or femur. Moreover, our distractor had guide rail structures at both side of the device for stable movement of transport disc. And this design had special advantages because it can be applied even when the defect area was curved. Because skull had a round surface, transport disc should pass along the curved area in substantial cases of large calvarial defect. This is hard to perform by conventional one-roded internal distractor and applicability to curved surface may be additional merits of our newly designed device.

Two animals of the experimental group was excluded form study because of infection. This infection occurred at the site of distraction zone. The inlet for injection was sealed with silicone membrane, and rhBMP-2 was delivered with syringe with 28-gauge needle. So, retrograde infection may not occur. Instead, we guess that the exposed rod of distraction device may be an alternative route of the infection. Compared with Hong's experiment, our study showed similar infection rate (25 % in our study versus 27% in Hong's study) and low extrusion rate (0% in our study versus 9% in Hong's study).[14] However, compared with Yun's experiment, infection rate was much increased. (0% in Yun's study[16]) This may be due to small numbers of experimental animals of both study and periodic injection of regimen from outside for a long time which also can make increased chances of contamination. Research should be continued to prevent peri- and postoperative infection because internal distractor should be placed in the deep and vital structures of cranium.

Strength of regenerated bone from the experimental group was similar with normal bone. Curve of compression test showed slope and ultimate load were increased in the regenerated bone of the experimental group and decreased in that of control group. Some samples of each group did not have ultimate load and showed just a linear curve. This might be progressive destruction of bony sample and we could determine assumptive ultimate load by detecting changing point of

slope of curve. Based on this hypothesis, all samples of the control and experimental group could be analyzed. As previously described, the regenerated bone had a tendency of lower slope and ultimate load compared to normal bone. On the other hand, the regenerated bone of the experimental group had a tendency of higher slope and ultimate load compared to normal bone. However, there was heterogeneity in the characteristics of each regenerated bone of the control and experimental group. Curves of the regenerated bones did not have an ultimate load in more than half of the samples in both control and experimental group. On the other hand, curves of normal bones of each group had an ultimate load in half of the tests.

Our study used 10 μg of rhBMP-2 daily. The amount of rhBMP-2 for consistent induction cartilage and bone formation was known to be 600 ng.[42] This is relatively high dose than previous report.[39] But, preclinical toxicity study have shown that even direct injection of high doses of rhBMP-2 (5.3 mg/kg) into the blood stream did not have adverse effect due to its short half life.[43,44] So, to maximize the bone regenerating effect of TDDO, considerable amount of rhBMP-2 can be injected locally. However, proper dose according to defect size and patient age should be calibrated via further study.

Distraction rate of this study was 2 mm/day. It was relatively faster than usual DO. As previously described, rhBMP-2 could shorten the distraction period.[22] And in preliminary study, we also experienced similar results in TDDO by use of rhBMP-2. One of the disadvantages of DO is long treatment period. We guess that this disadvantage can be overcome in the near future.

We detached periosteum and dura mater from transport disc. But, in some report, vascularized transport disc is emphasized for successful DO.[2] This is theoretically reasonable because ensuring viability of disc is important for bone regeneration. Especially, it is well known that dura mater has a paramount importance in calvarial bone regeneration.[45,46] However, preservation of dura mater with transport disc is not easy considering human application, so, the dura mater was

detached from the transport disc in this study. This allowed a greater degree of freedom in movement of transport disc during distraction.

In histologic examination, prominent osteoblastic rimming was observed in the bone marrow of the experimental group. This is the hallmark of bone regeneration. And the number of osteoblasts was increased in order of the normal, control and experimental group. This finding reflects vigorous bony regeneration occurred by TDDO itself. And it also indicates that application of rh-BMP-2 into distraction zone can augment this process additionally. It is reasonable because BMP promote bone formation, bone remodeling and bone marrow differentiation by directing cell differentiation of mesenchymal stem cells to osteoblasts and osteocytes, as previously described.[25-27]

Non-steroidal anti-inflammatory drug (NSAIDs), ketorolac, was used to relieve pain of the experimental animals in this study. There were some reports that ketorolac could interfere negatively with new bone formation in animal and human.[47-50] This may be due to the inhibition of cyclooxygenase (COX) activity which was known to be essential in fracture healing.[51-53] However, the exact role of NSAIDS in this unfavorable effect was not clearly elucidated till now and only some theories or hypothesis were introduced. One is the possibility of early weight loading to injured area by pain relieving effect of NSAIDS. Another is that COX-2 function is essential for mesenchymal stem cell differentiation into osteoblasts. In addition, there was also a report that COX-2 dependent prostaglandins promote angiogenesis required for fracture healing.[54] On the other hand, Martin et al. reported that rhBMP-2 could overcome the detrimental effect of ketorolac in rabbit model of posterolateral lumbar spinal fusion. They used subcutaneously implanted mini-osmotic pumps for administration of rh-BMP-2 and confirmed the addition of rh-BMP-2 to the autologous bone graft was able to compensate for the inhibitory effect of ketorolac on bone formation. In this study, we can infer rhBMP-2 has similar effect on calvarial bone reconstruction using TDDO. However, because ketorolac was also used in the control group, the exact effect of

NSAIDs in TDDO for calvarial defect should be investigated by properly designed study in the future.

In this study, consolidation period was 3 months similar with previous studies. There was a report that BMP-2 reduced not only distraction period but also consolidation period.[23,34] There was a possibility that consolidation period could be reduced in our study but we did not perform serial analysis. However, we can guess consolidation period might be reduced in our experiment because bone strength was significantly increased in the experimental group compared to the control group. Optimal consolidation period should be determined to reduce patient treatment period in the future study.

Previous animal studies were designed with relatively small critical sized calvarial defect. (Table 3) Comparison of absolute size cannot be proper in some aspect. Because, although defect size is equal, relative ratio can be larger in small animal model. However, considering application to human, large defect in large animal may be more suitable in this kind of study. Our experiment was performed with relatively large defect, 33 x 35 mm. Clinically, we can often encounter large calvarial defect after neurosurgical operation, which is challenging to be treated with traditional method. Moreover, as previously described, curvature of cranium is another obstacle even in traditional DO treatment, especially in large defect. In this study, we could demonstrate that TDDO treatment combined with rhBMP-2 injection could successfully cover the large calvarial defect with strength.

The result of regenerated bony area was about 95% in average in the experimental group. And the strength was nearly equal (1.08) to normal bone and, of course, higher than the control group. It is clinically useful because we could achieve these results despite some harsh condition for distraction osteogenesis including the use of bare bone as transport disc detached from dura mater and perisoteum, fast rate of distraction and the use of ketorolac. Moreover, this study showed the possibility to shorten the whole treatment time. Authors think that this result would be better experimental basis for human application than ever.

V. CONCLUSIONS

Transport disc distraction osteogenesis with internal distraction device of delivering bone morphogenic protein-2 can enhance bone regeneration of calvarial defect in dog model. The analysis of area and strength of regenerated bone indicates that the clinical human application is possible.

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ABSTRACT(IN KOREAN)

이동형 골신장술과 골형성단백질-2을 이용한 광범위 두개골 결손의 재건

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광범위 두개골 결손은 외상이나 수술, 선천성 기형으로 발생할 수 있다. 이러한 상태는 외부 충격에 의해 보다 쉽게 뇌손상이 생길 수 있으므로 위험하다. 이러한 결손의 재건을 위해 전통적으로는 자가골조직이나 생체재료가 이용되어 왔으나, 공여부 합병증과 감염 등과 같은 문제들이 보고된 바 있다. 한편, 골재건에 이동형 골신장기를 이용하려는 노력도 계속되어 왔으나 아직까지 재현성과 신뢰성이 부족한 실정이었다. 저자는 이러한 한계를 극복하기 위해 외장형 골신장기에 비해 안정적인 내장형 골신장기를 사용하고, 골성장인자 중 골형성단백질-2를 함께 투여하여 충분한 골재생을 유도하고자 하였다. 골형성단백질-2는 골경화를 촉진시키고, 신연연장술의 기간을 단축시켜서 골재생을 촉진하는 것으로 알려져 있다. 결국 본 연구에서는 기계적 요소 (이동형 골신장술)와 기계적 요소 (이동형 골신장술)와 성장인자 (골형성단백질-2)가 함께 투여하여

골재생의 정도를 여러 가지 방법을 이용하여 비교하였다.

동물실험을 위해 14마리의 잡견이 이용되었다. 6마리는 대조군이었으며, 8마리는 실험군이었다. 본 실험을 위해 특별히 고안된 내장형 골신장기가 이용되었다. 이 기구는 골신장술 기간 동안 안정성을 향상시키는 두 개의 궤도를 가지고 있으며, 회전부가 관의 형태로 가공되어 이 공간을 통해 약물의 투여가 가능하도록 설계되었다. 5일 간의 잠재기를 거친 후, 골신장술이 시작되었다. 속도는 하루에 2 mm였고, 골신장술이 끝나기까지 총 13일이 소요되었다. 하루에 10 μg/ml의 골형성단백질-2가 투여되었다. 경화기간은 3개월이었다. 골형성면적은 3차원 전산화단층촬영술에서 얻은 영상을 바탕으로 원래의 결손 면적대비 재생된 골의 면적의 비율로 계산되었다. 재생된 골의 경도는 경도시험기를 이용하여 같은 개체의 정상골 대비 재생된 골의 경도 측정치의 비율로 비교되었으며, 대조군과 실험군에서 얻는 대생된 골조직의 조직학 검사도 시행되었다.

실험 중 두마리의 동물이 감염으로 사망하여, 결국 대조군 중 6마리와 실험군 중 6마리가 결과 분석에 이용되었다. 골형성면적 비교에서 대조군은 68.04 ± 18.07%, 실험군은 94.64 ± 5.29%를 나타내어 통계적으로 유의하게 실험군에서 더 많은 골재생이 일어났음을 확인할 수 있었다. ($p < 0.01$) 경도 비교에서는 대조군이 평균 23.85 ± 6.19 N/mm^2, 실험군이 평균 53.75 ± 18.66 N/mm^2을 나타내었다. 같은 개체의 정상골의 경도에 대한 재생된 골의 경도의 비율을 이용한 비교에서 실험군의 재생된 골이 통계적으로 유의하게 더 높은 경도를 나타내었다. ($p < 0.05$) 조직학 검사에서 대조군은 피질의 비율이 적고 층판이 덜 성숙된 무층골에 가까운 양상을 보였고, 실험군은 보다 정상 해면골과 유사한 양상을 보였다.

대조군과 실험군의 골수에서 정상골에 비교하여 골모세포의 부연법이 관찰되었다. 특히 실험군에서 통계적으로 유의하게 대조군보다 많은 수의 골모세포가 골수에서 관찰되었다. ($p < 0.01$)

이동형 골신장술에 골형성단백질-2를 투여할 수 있는 골신장기를 이용하여 병합치료한 결과 실험 동물 모델에서 두개골 재생이 촉진됨을 확인할 수 있었다. 실험군에서 재생된 골은 최초의 만들어진 결손부를 거의 덮었으며, 경도 분석에서도 정상골과 유사한 경도를 나타내었다. 이러한 결과는 골신장술과 골형성단백질-2의 병합치료가 인간에게도 적용될 수 있음을 시사한다.

핵심되는 말 : 신연연장술, 이동형 골신장술, 이동 신연연장술, 두개골 결손, 두개골 재건, 골형성단백질-2

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