Effect of rhBMP-2 produced by E-coli expression system on bone formation in rat calvarial defects

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감사의 글

본 논문이 완성되기까지 부족한 저를 항상 격려해 주시고 사랑과 관심으로 논문지도를 해주신 조규성 교수님께 깊은 감사를 드립니다. 그리고, 많은 조언과 따뜻한 관심으로 지켜봐 주신 김종관 교수님, 채중규 교수님, 최성호 교수님께도 진심으로 감사 드립니다. 논문 심사를 맡아주신 이근우 교수님, 김형준 교수님께도 감사드립니다.

실험 및 연구를 도와준 채경준 선생님, 이지현 선생님 그리고 김민수 선생님을 비롯한 의국원 후배님들에게도 감사드립니다. 무엇보다도 늘 아낌 없는 사랑과 헌신적인 도움으로 든든하고 따뜻한 버팀목이 되어주고 부족한 나를 믿고 따라준 사랑하는 나의 아내와 사랑하는 딸 수린이 에게도 진정으로 사랑과 고마움의 마음을 전합니다. 항상 격려를 해주신 양가부모님과 친지들에게도 감사드리며 앞으로 더욱 좋은 모습으로 보답하겠습니다.

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Abstract

Effect of rhBMP-2 produced by E-coli expression system on Bone Formation in Rat Calvarial Defects

Bone morphogenetic proteins (BMPs) are being evaluated as potential candidates for periodontal and bone regenerative therapy. In spite of good prospects in BMP applications, there is economically inviable for clinical use in dental area. The purpose of this study was to evaluate the osteogenic effect of rhBMP-2 produced by E-coli expression system.

Eight-mm critical-size calvarial defects were created in 48 male Sprague-Dawley rats. The animals were divided into 6 groups of 8 animals each. Each group received one of the following: sham-surgery control(no material applied), positive control(ACS alone), experimental(ACS loaded with BMP). Defects were evaluated by histologic and histometric parameters following 2- and 8-week healing intervals.

The experimental group showed significantly defect closure at 2 and 8weeks than the sham surgery and positive control groups. Moreover, the experimental group showed significantly greater new bone and augmented area than the other groups at both 2 and 8weeks. rhBMP-2 produced by E-coli expression system may be effective for bone regeneration.

Key Words: Osteogenic effect; bone morphogenetic protein-2; E-coli expression system; ACS; rat calvarial defect model

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I. Introduction

The principal objective of periodontal treatments is to regenerate the original periodontal tissue. Several studies have been carried out to achieve this aim. Among the various procedures available to reconstruct bone, the application of bone morphogenetic proteins (BMPs) is believed to be a promising advancement. Urist reported that demineralized bone matrix implanted in the extraskeletal sites created osseous tissue in rats (Urist, 1965). In 1971, Urist et al. identified the factors within the matrix responsible for the effects of bone morphogenetic proteins ((BMPs) Urist

et al., 1971). BMPs are considered members of the transforming growth factor-β superfamily through their characteristic amino acid sequences (Reddi et al., 1976; Wozney et al., 1988). BMPs contain potent growth and differentiation factors that have an effect on mesenchymal cells causing them to differentiate into mature osteoblasts, resulting in new bone formation (Reddi et al., 1976). Many studies have reported the biological activities of BMPs in a variety of cells in vitro, including the induction of osteoblastic phenotype expression during the course of osteoblastic differentiation. BMPs include alkaline phosphatase(ALP), type Ⅰ collagen, osteocalcin, osteopontin and bone sialoprotein (Hiraki et al., 1991; Katagiri et al., 1994; Knutsen et al., 1993; Kobayashi et al.,1999; Takuwa et al., 1991; Thies et al., 1992; Wang et al., 1993; Yamagichi et al., 1996). When BMPs are implanted in vivo, the osteoinductive effect of BMPs initiates a series of cellular events, culminating in bone formation (Sampath et al., 1992; Urist, 1965; Wozney et al., 1988). Therefore, BMPs need accessible and bioactive delivery systems. Carrier systems for delivering BMP should be both biocompatible and biodegradable in order to minimize the local tissue response and be replaced by newly formed bone(Aldinger et al., 1991). An absorbable collagen sponge ((ACS) Sampath et al., 1981; King et al., 1998; Barboza et al., 2000), and β-tricalcium phosphate (β-TCP) have been studied and used extensively as carriers for BMPs (Urist et al., 1984; Urist et al., 1987; Gao et al., 1996; Alam et al., 2001). A collagen carrier acts only as a temporary template for the

osteoinduction of BMP at the early stage of bone formation, and is unsuitable for maintaining bone defects because it is quite absorbable. β-TCP has a multiporous structure, which is believed to entrap BMP and protect it from diffusion(Alam et al., 2001; Laffargue et al., 1999; Gao et al., 1996). In addition, the effects of Fibrin-Fibronectin Sealing System as a carrier for rhBMP-4 have been studied in rat calvarial defects(Han et al.,2005; Hong et al.,2005).

Therefore, BMPs are considered to be the most suitable osteoinductive material and are expected to be used clinically for bone regeneration and reconstruction. In 2007, the FDA approved the use of rhBMP-2(Infuse) in the dental area. Although there are good effects after applying BMPs, there is still a gap between research and the realistic clinical use of BMP, due to the difficulty of a large quantity production. This difficulty results in the high cost of BMP production, which makes uneconomically viable for use in the dental area.

BMP-2 is produced from mammalian cell cultures, albeit in low yield (Wang. et al.,1990). Biologically active BMP-2 has been produced from E-coli through the in vitro refolding of inclusion bodies. However, the refolding procedure is complicated and the overall yield is either low or the refolding buffer contains expensive regents. One method involves several time consuming purification and concentration steps and produces a low yield of 0.2mg active BMP-2 per gram cell wet weight(R.Ruppert et al.,1996). Although another procedure improved the yield of the active BMP-2

dimer to 10mg/g cell wet weight, a high concentration of expensive reagent was required (Vallejo et al., 1996). Therefore, new, simple and inexpensive methods are needed to make them economically viable.

rhBMP-2 produced by an E-coli expression system was reported to solve these productivity and economic viability issues (Choi et al., 2008). The aim of this study was to evaluate the osteogenic effect of rhBMP-2 produced by an E-coli expression system in a rat calvarial defect model.

II. Materials & methods

1. Animals

Forty-eight male Sprague-Dawley rats (weight 250-300g) were used in this study. The rats were maintained in plastic cages in a room with a 12 h-day/night cycle and at 21°C. The rats were given access to water and standard laboratory pellets *ad libitum*. Animal selection and management, surgical protocol, and preparation were carried out in accordance with the routines approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

2. rhBMP-2 implants

The rhBMP-2 produced by the E-coli expression system* was reconstituted and diluted in a buffer to produce a concentration of 0.1 mg/ml. A sterile 8-mm diameter ACS was then loaded with 0.1 ml of the rhBMP-2 solutions. The buffer was used alone for the control experiments. No material was applied in sham surgery. The rhBMP-2 implants were fitted to a calvarial defect after a 5-minute binding period.

3. Surgical Procedures

The animals were anaesthetized by an intramuscular injection (5 mg/kg body wt.) of a 4:1 solution of ketamine hydrochloride[**]:Xylazine[††]. Routine infiltration anaesthesia[‡‡] was used at the surgical site. An incision was made in the sagittal plane across the cranium and a full thickness flap was reflected, exposing the calvarial bone. A standardized, circular, transosseous defect, 8 mm in diameter, was created on the cranium using a saline-cooled trephine drill [#]. The animals were divided into 6 groups containing 48 animals each. The wounds were allowed to heal for 2 (8 rats) or 8 (8 rats) weeks. Each animal received 1 of 3 experimental treatments. The periosteum and skin were closed and sutured with 4-0 coated Monosyn sutures[§§] for primary intention healing. Table 1 gives a summary of the study design

Table 1. Study design

Group(n)	Treatment
Sham surgery 2,8week(8,8)	no material apply
Positive control 2,8week(8,8)	absorbable collagen sponge alone
Experimental 2,8week(8,8)	absorbable collagen sponge loaded with rhBMP-2

4. Histologic and Histometric Procedures

The animals were sacrificed by CO_2 asphyxiation at 2 and 8 weeks post-surgery. Block sections, including the experimental sites, were removed and fixed in a 10% neutral buffered formalin solution for a 10 day period. The samples were decalcified in a 5% formic acid solution over a 14 day period and embedded in paraffin. Serial sections, 5μm in thickness, were prepared at 80 μm intervals, stained with hematoxylin/eosin (H-E), and examined by optical microscopy. The most central sections from each block were selected for the histological evaluation.

Computer-assisted histometric measurements were obtained using an automated image analysis system[¶] coupled with a video camera on the optical microscope[¶¶]. The sections were examined at x 20 and x 100 magnifications. The histometric parameters are defined as follows (Figure 1). :

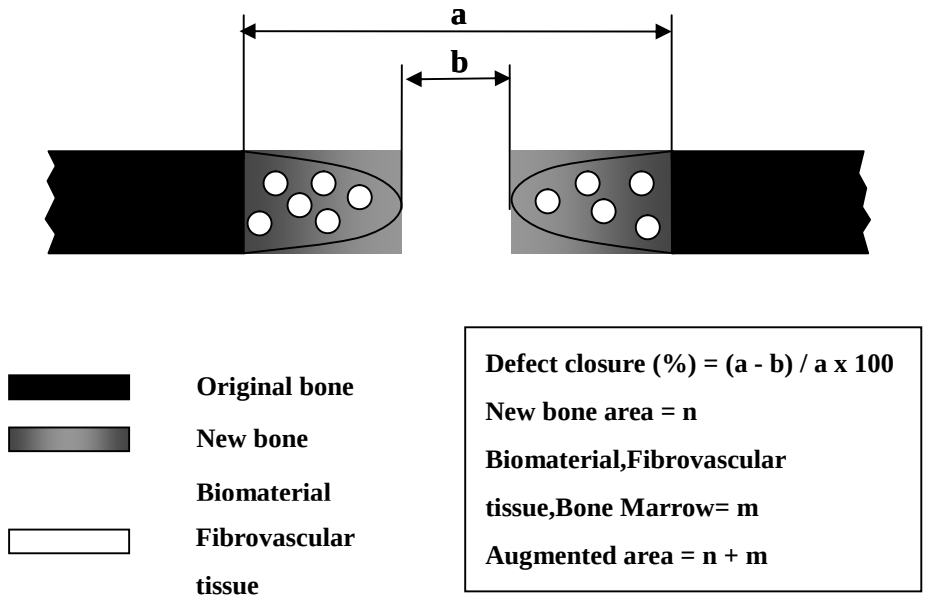

Figure 1. Schematic diagram of the calvarial osteotomy defect showing the histometric analysis

The level of defect closure was determined by measuring the distance between the defect margin and new bone margin, and is expressed in mm and as a percentage of the total defect width. The augmented bone area (mm^2) was measured including all the tissues within the boundaries of the newly formed bone, i.e., mineralized bone and fatty marrow and fibrovascular tissue/marrow and residual biomaterial.

5. Statistical Analysis

Histometric recordings from the samples were used to calculate the mean and standard deviations (m±SD). Two-way analysis of variance was used (two-way ANOVA) to detect the interactions between the healing interval and treatment conditions. ANOVA and post hoc t-tests were used to analyze the differences between the treatment groups at each healing interval. A paired t-test was used for the comparisons between the 2- and 8-week healing interval within the same group. A p-value <0.05 was considered significant.

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* Cowellmedi co.Ltd. Busan,Korea

‖ Collatape®, Calcitek, Carlsbad, CA, USA

** Ketalar®, Yuhan Co., Seoul, Korea

†† Rompun®, Bayer Korea, Seoul, Korea

‡‡ 2% lidocaine, 1:100,000 epinephrine, Kwangmyung Pharm., Seoul, Korea

3i, Palm Beach Gardens, FL, USA

§§Glyconate monofilament, absorbable. VIOLET, BRAUN Int.

¶Image-Pro Plus®, Media Cybernetics, Silver Spring, MD, USA

¶¶ Olympus BX50, Olympus Optical Co., Tokyo, Japan

III. Results

1. Clinical Observations

The level of wound healing was similar in all groups. There was no material exposure or other complications observed at the surgical sites.

2. Histologic Observations

Sham-surgery control group: At 2 and 8 weeks after surgery, the defects were filled with thin fibrous connective tissue. There was a minimal amount of new bone formation originating from the defect margins, and the defect center appeared to have collapsed. Moreover, there was minimal inflammatory cell infiltration at the defect site.(Figure 2,3)

Positive control group: At 2 weeks after surgery, there was dense, fibrous connective tissue at the defect site and the ACS had partially degraded but still present. There was a small amount of new bone formation adjacent to the defect margins, and obvious host bone-to-new bone interface. No inflammatory cell infiltration was

observed at the defect site. At 8 weeks, a similar pattern to that observed at 2 weeks was noted. However, the ACS was completely degraded.(Figure 4,5,6)

Experimental group: At 2 weeks after surgery, there was not only a consolidation of woven bone along the dural aspect but also marked bone regeneration. The degradation of the ACS had advanced considerably without significant adverse reactions, and some degraded ACS fragments were embedded within the new bone, without connective tissue intervention. At 8 weeks, the quantity of the new bone was greater than that observed at 2 weeks, and the appearance of the new bone was more lamellar than that observed at 2 weeks. No remnants of the ACS could be detected. There was no clear border between the preexisting bone and new bone.(Figure 7-13)

3. Histometric Analysis

Table 2-4 show the results of the histomorphometric analysis are shown in. At 2 weeks after surgery, the mean defect closure (±SD) for the sham surgery control, positive control, experimental groups was 13.53±3.58%, 17.98±.7.66%, 85.9±16.25%, respectively, with a significant difference being observed between the groups ($P<0.01$). At 8 weeks after surgery, the corresponding values were 18.3±8.65%, 21.88±8.34%, 100%, respectively, with significant difference observed between the

groups (P<0.01). The experimental group had a significantly longer closure length at both 2 and 8 weeks than the other groups (P<0.05). The defects were completely closed in the experimental group at 8 weeks. (Table 2.)

The results of the new bone and augmented bone areas were similar to the defect closure result. The experimental group had significantly greater new bone and augmented areas at both 2 and 8weeks than the other groups.(Table 3,4.)

Two-way ANOVA revealed interaction between the healing interval and treatment conditions in the defect closure and new bone area (p <0.01). The treatment had a strong influence on defect closure, new bone area and augmented area (p <0.01), whereas the healing interval had an influence on defect closure and the new bone area (p <0.01).

Table 2. Defect closure (group means ± SD; n=8, %)

	2 weeks	8 weeks
Sham-surgery control	13.53 ± 3.58	18.30 ± 8.65
Positive control	17.98 ± 7.66	21.88 ± 8.34
Experimental	85.90 ± 16.25*¶	100*¶ʃ

*: Statistically significant difference compared with the sham-surgery control group (P<0.01)

¶: Statistically significant difference compared with the positive control group (P<0.01)

ʃ : Statistically significant difference compared with that observed at 2 weeks (P<0.05)

Table 3. New bone area (group means ± SD; n=8, mm^2)

	2 weeks	8 weeks
Sham-surgery control	0.18 ± 0.04	0.81 ± 0.27 $^{\oint}$
Positive control	0.23 ± 0.13	0.46± 0.07
Experimental	1.73 ± 0.64 *¶	7.4±1.56 $^{*¶\oint}$

*: Statistically significant difference compared with the sham surgery control group (P<0.01)

¶: Statistically significant difference compared with the positive control group (P<0.01)

$\oint$: Statistically significant difference compared with that observed at 2 weeks (P<0.05)

Table 4. Augmented area (group means ± SD; n=8, mm^2)

	2 weeks	8 weeks
Sham-surgery control	0.22 ± 0.07	0.89 ± 0.32 $^{\oint}$
Positive control	2.00 ± 1.01 *	3.5± 0.30 $^{*\oint}$
Experimental	5.09± 1.13 *¶	9.8± 1.56 $^{*¶\oint}$

*: Statistically significant difference compared with the sham surgery control group (P<0.01)

¶: Statistically significant difference compared with the positive control group (P<0.01)

$\oint$: Statistically significant difference compared with that observed at 2 weeks (P<0.05)

Ⅳ. Discussion

The aim of this study was to evaluate the bone regenerative effect of rhBMP-2 produced by the E-coli expression system and delivered with an absorbable collagen sponge (ACS). Previous studies reported the osteoinductive effects of rhBMP-2 both in vivo and in vitro. In 2005, Hong et al. examined effect of a fibrin-fibronectin sealing system in combination with ß-Tricalcium Phosphate as a carrier for recombinant human bone morphogenetic protein-2 on the bone formation effects in rat calvarial defects(Hong et al.,2005).

In this study, 6 groups containing 48 animals each received one of the following: sham-surgery control(No material applied), positive control(ACS alone), experimental(ACS loaded with BMP). These groups were evaluated using the histologic and histometric parameters after a 2- and 8-week healing period. The BMP produced by the E.coli expression system at a concentration of 0.1 mg/ml was used. Preexisting BMP studies mainly used a BMP concentration of 0.025~0.05 mg/ml. However, 0.1 mg/ml of BMP was used in this study because the biological activity concentrations of the rhBMP-2 obtained from CHO cell and E-coli are different.

The experimental model used in this study was based on the model reported by Takagi and Urist (Takagi et al., 1982). A critical-size rat calvarial defect was used

because of its relative accessibility, simplicity and reproducibility. This model is convenient for evaluating the bone regenerative effects of biomaterials. There was no spontaneous healing in the control specimens (Frame, 1980; Schmitz et al., 1986).

BMPs were first identified by Urist(Urist.,1965) in partially purified form from a rabbit and bovine demineralized bone matrix, and were isolated by Urist and coworkers in the 1980s(Mizutani H.et al, 1982; Urist et al, 1982). BMPs form a set of growth and differentiation factors that act on the early osteoprogenitor cells so they can differentiate into mature osteoblasts, resulting in the formation of new bone and cartilage when implanted in animals(Gerhart T.N.et al., 1993; Ferguson D.et al , 1987)

In order to observe the osteoinductive effect of BMP, an appropriate carrier system is essential for the delivery, retention, and release of BMPs at the implantation site (Wikesjö et al., 2001). Ahn.et al.(2002) reported that an ACS was a superior carrier to β-TCP for the bone regenerative effect of rhBMP-4(Ahn. et al., 2002). Pang et al.(2004) reported that rhBMP-4 using ACS or ß-TCP carrier technologies have significant potential to induce bone formation in the rat calvarial critical size defect model, and both ACS and ß-TCP might be effective carriers for rhBMP-4 (Pang. et al., 2004). In this study, ACS was used as the BMP carrier.

BMP is produced from mammalian cells. BMP-2 was also found to be expressed from Chinese hamster ovary (CHO) cells. However, the BMPs used thus far have a

limitation in mass production, which results in the high cost of BMP production giving them no economic value when used in dentistry. More economical and less difficult methods are being examined in an attempt to solve this problem. rhBMP-2 produced by E-coli expression system solves these productivity and economic viability issues (Choi et al., 2008). rhBMP-2 produced from E-coli is different from the rhBMP-2 produced from CHO cells without glycosylation but there is no difference in biological activity between both rhBMP-2 (Vallejo et al.,2002).

In this study, the experimental group showed significantly greater defect closure at 2 and 8weeks than the sham surgery and positive control groups. In particular, the defects in the experimental group had closed completely at 8 weeks, which is unlike that observed in the sham surgery and positive control groups (18.30±8.65% and 21.88±8.34%, respectively).

The new bone and augmented bone areas were similar to the defect closure results. The experimental group had significantly larger new bone and augmented area than the other groups at both 2 and 8weeks. The new bone area in the experimental group at 8weeks was significantly greater than at 2 weeks ($p<0.05$). These new bone inductive effects of rhBMP-2 produced by the e-coli expression system are similar to the preexisting rhBMP-2 produced by CHO cells (Hong et al.,2005).

These results suggest that the rhBMP-2 produced from E-coli is effective in new bone formation. In addition, rhBMP-2 produced by the E-coli expression system has economic value and can be applied to dentistry. Compared with CHO cell-expressed rhBMP-2, a 0.1mg/ml concentration was used in this study because the activity of the E.coli-expressed rhBMP-2 was approximately five times lower. Future experiments will examine various concentrations of rhBMP-2 produced by the E-coli expression system.

V. Conclusion

This study evaluated the bone regenerative effect of rhBMP-2 produced by an E-coli expression system delivered with an absorbable collagen sponge (ACS).

An 8-mm critical-size calvarial defect was created in 48 male Sprague-Dawley rats. The animals were divided into 6 groups containing 8 animals each. The defects were treated with rhBMP-2, a positive control (Absorbable Collagen Sponge only) or were left untreated as a sham-surgery control. The defects were evaluated using the histologic and histometric parameters after a 2- and 8-week healing interval (8 animals/group/healing intervals).

The experimental group showed significantly more defect closure at 2 and 8weeks than the sham surgery and positive control groups. Moreover, the experimental group showed a significantly greater new bone and augmented area than the others groups at both 2 and 8weeks. There was greater new bone area in the experimental group at 8weeks than at 2 weeks ($p<0.05$). Overall, rhBMP-2 produced by an E-coli expression system may be effective for bone regeneration.

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FIGURES

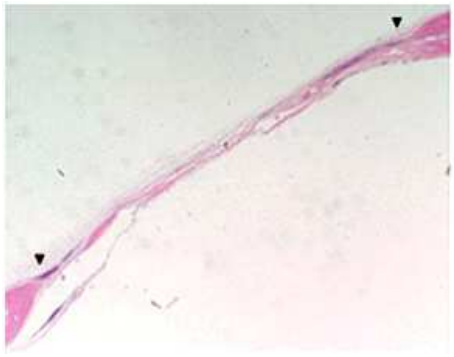

Figure 2. Sham surgery control group (2 week, x20 magnification)

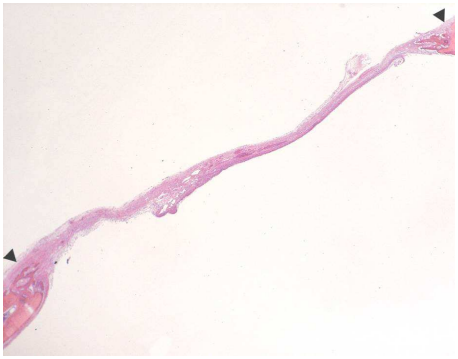

Figure 3. Sham surgery control group (8 week, x20 magnification)

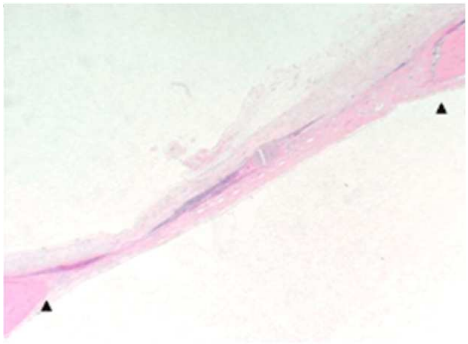

Figure 4. Positive control group (2 week, x20 magnification)

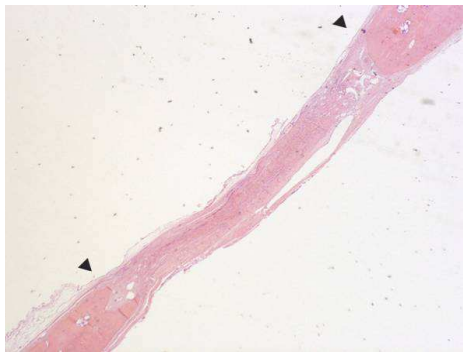

Figure 5. Positive control group (8 week, x20 magnification)

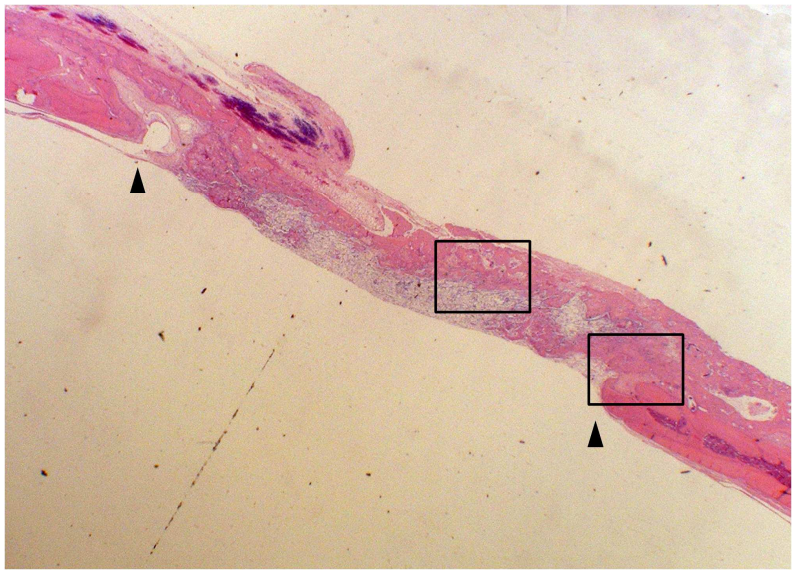

Figure 6. x20 magnification of 2 week group (Arrow : defect margin)

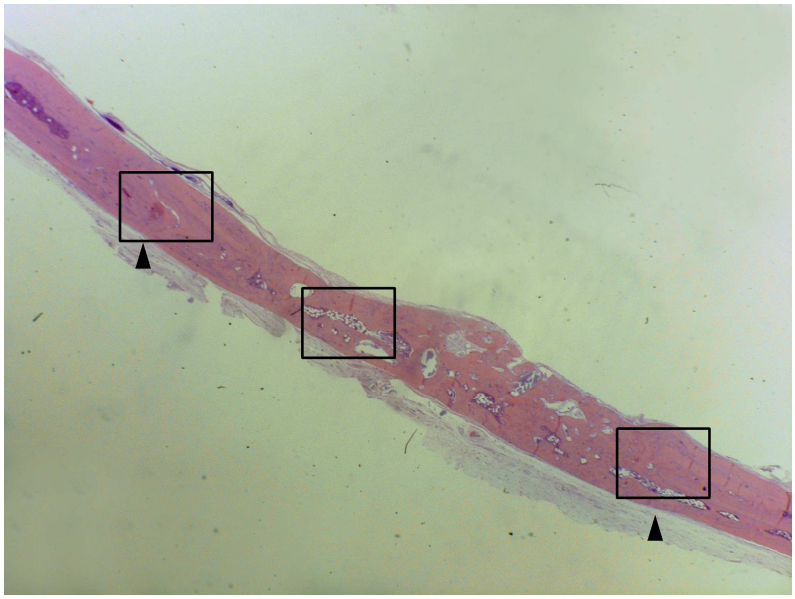

Figure 7. x20 magnification of 8 week group (Arrow : defect margin)

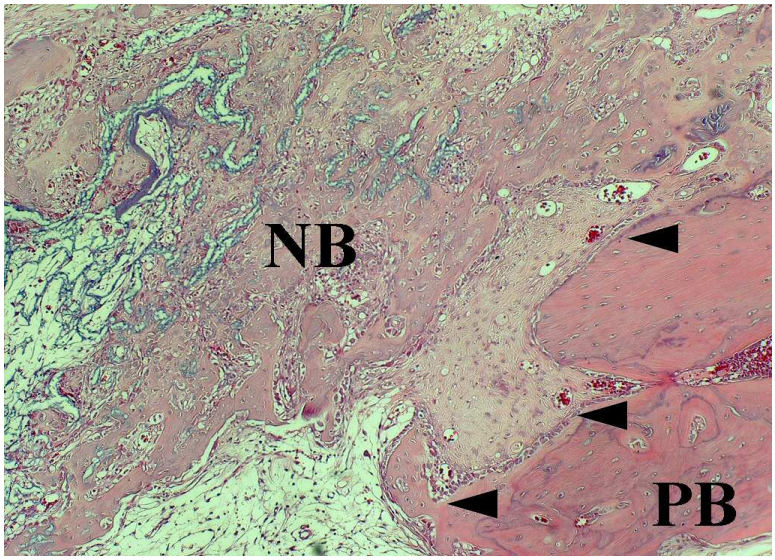

Figure 8. x100 magnification of 2 week group
(PB : pre-existing bone, NB : new bone, Arrow : defect margin)

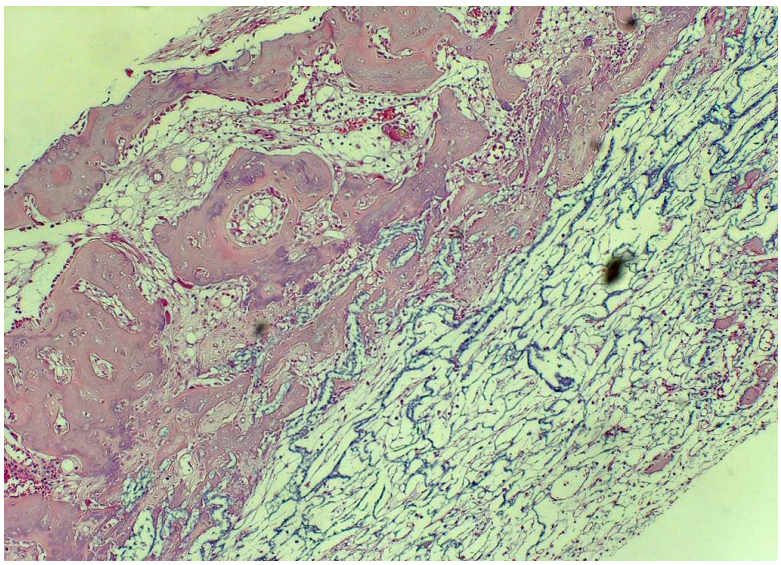

Figure 9. x100 magnification of 2 week group (central portion)

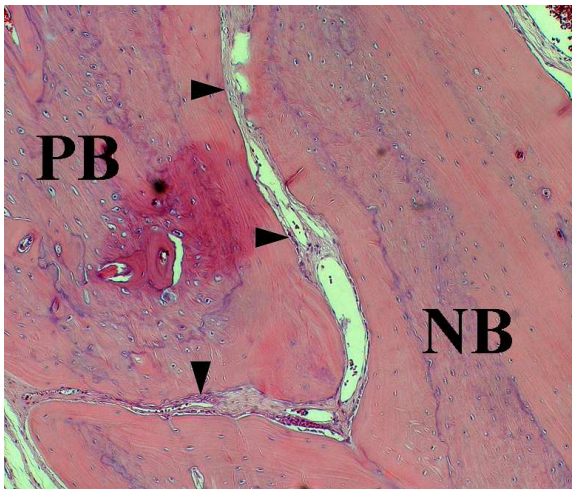

Figure10. x100 magnification of 8 week group
(PB : pre-existing bone, NB : new bone, Arrow : defect margin)

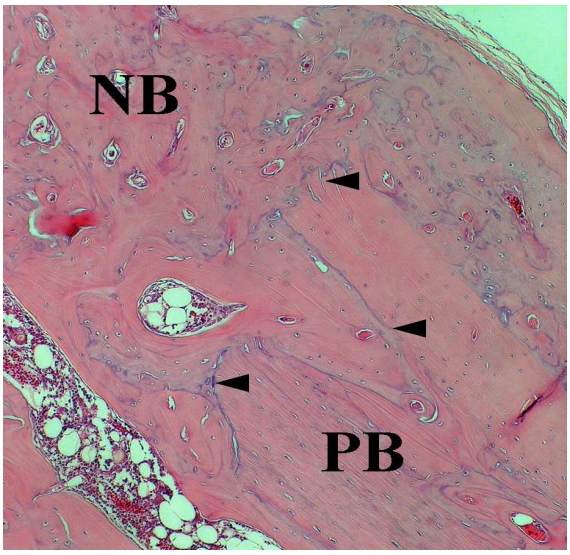

Figure 11. x100 magnification of 8 week group
(PB : pre-existing bone, NB : new bone, Arrow : defect margin)

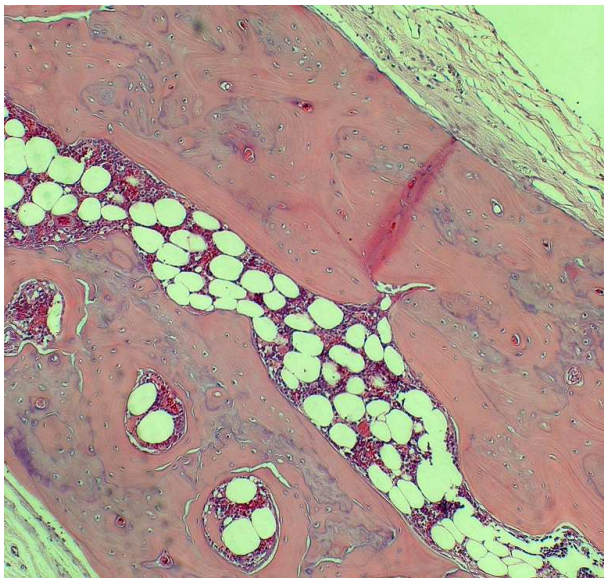

Figure 12. x100 magnification of 8 week group (central portion)

국문요약

백서 두개골 결손부에서 E-coli expression system 에 의해 생산된 rhBMP-2 의 골재생 효과

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권 석 훈

골형성 유도 단백질 (bone morphogenetic protein, BMPs)은 치주치료와 골 재생 치료를 위한 효과적인 골 대체 물질로 평가되어왔다. BMPs 는 그 자체로도 골형성을 유도할수 있는 충분한 능력이 있지만 수용부에 적절히 작용하기 위해서는 carrier 가 필요하다. BMPs 는 우수한 골형성 능력에도 불구하고 대량생산의 어려움 및 이로 인한 높은 생산비용으로 dental area 에서 사용이 제한적이었다. 기존의 mammalian cell 에서 생산된 BMPs 와 달리 E-coli 로부터 생산되는 BMPs 는 대량생산이 상대적으로 쉬워서 비교적 생산단가가 낮고 골재생 효과만 입증된다면 치과영역에서 활발이 사용될 수 있을 것이다. 본 연구의

목적은 rat calvarial defect model 에서 E-coli expression system 으로 생산된 rhBMP-2 의 골재생 효과를 알아보는 것이다.

48 마리의 웅성 백서에서 8mm 지름을 갖는 임계크기의 두개부 결손을 형성하였다. 8 마리씩 6 개의 군으로 나누고, 각 군은 아무것도 이식하지 않은 sham surgery 군, ACS(absorbable collagen sponge)만 이식한군, 0.1mg/ml rhBMP-2 를 soaking 한 ACS 를 이식한군으로 나누어 술 후 2 주와 8 주에 치유 결과를 조직학적, 조직계측학적으로 비교 관찰하였다. 조직학적 및 조직계측학적 관찰 결과 rhBMP-2를 이식한군에서 다른 군들보다 defect closure, new bone area, augmented bone area에서 유의성있게 증가함을 보였고, rhBMP-2를 이식한 군내에서도 8주가 2주에 비해서 모든 항목에서 유의성있게 증가함을 보였다. 백서 두개골 결손부에서 E-coli expression system으로부터 생산된 rhBMP-2를 사용하였을 때 결손부 폐쇄 및 신생골 형성에 유의한 효과가 있다고 사료된다.

핵심되는 말: 골형성 효과, 골형성 유도 단백질, E-coli expression system, ACS(absorbable collagen sponge), 백서 두개골 결손부