Immunohistochemical analysis on cortex-to-cortex heal following mandibular vertical ramus osteotomy in dynamic condition

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Immunohistochemical analysis on cortex-to-cortex heal following mandibular vertical ramus osteotomy in dynamic condition

Directed by Professor Park, Hyung-Sik

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The Graduate School

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June 2015

감사의 글

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실험에 큰 도움을 주고 실험 전반에 걸쳐 조언을 아끼지 않은 이종민 교수님, 이동준 선생님, 실험을 진행하면서 많은 도움을 아끼지 않은 김은정 선생님, 이민정 선생님, 윤경식 선생님 선생님께 감사를 드립니다. 그리고, 항상 옆에서 힘이 되는 의국 선후배 선생님, 구강악안면외과 식구들에게 감사의 마음을 전합니다.

항상 사랑과 믿음으로 지켜봐 주시고 지금까지 관심을 기울여 주신 부모님, 항상 저를 감싸주신 장인어른, 장모님께도 깊은 감사의 마음을 전합니다. 마지막으로 늘 곁에서 힘이 되어주고, 살아가는 이유가 되는 사랑하는 아내 혜원, 아들 준우, 딸 선우에게 고마움을 전하며 이 기쁨을 나누고 싶습니다.

2015년 6월

정휘동

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ABSTRACT

Immunohistochemical analysis on cortex-to-cortex heal following mandibular vertical ramus osteotomy in dynamic condition

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(Directed by Professor Park, Hyung-Sik, DDS, PhD)

Vertical ramus osteotomy (VRO) is a surgical method of performing an osteotomy from the sigmoid notch to the postero-inferior border of the ramus from the lateral aspect of the ascending ramus for the mandibular setback, followed by healing under dynamic condition which allows movement of bony segment. This study aimed to evaluate specific cytokines among the TGF-β superfamily at each period following VRO, and to compare those results with fibular fracture heal. The 4 beagle dogs were used for this experiments, and euthanized at 1, 2, 4, and 8 weeks postoperatively. 6 specific antibodies were used in this immunohistochemical analysis: bone morphometric protein -2/4, -7, (BMP -2/4, -7) matrix metalloproteinase-3 (MMP-3), transforming growth factor-beta 2, 3 (TGF-ß2, -ß3), and vascular endothelial growth factor (VEGF)

The results are followed:

1. In VRO heal, inflammatory cell infiltration and resorption of cortical bone at 1 week, cartilage formation by chondrocyte at 2 weeks, and cartilage resorption, primary bone formation, and vascularization at 4 and 8 weeks were observed in HE staining. In Fibular fracture heal, no remarkable differences were seen in HE staining compared to VRO heal at each period.
2. In general upregulation of BMP-2/4 was observed in whole heal periods in VRO and fibular fracture, while the strongest expression of BMP-2/4 was observed at 2 weeks postoperative following VRO in contrast to a relatively constant expression of BMP-2/4 following fibular fracture.
3. The strongest expression of BMP-7 was observed at 1 week following VRO, in contrast to constant up-regulation following fibular fracture. The decreasing pattern in VRO may be caused by dynamic movement of mandible.
4. The strongest expression of TGF-ß2 in VRO was observed at 8 weeks, the expression showing increasing pattern, meaning remodeling activity accelerated over time.
5. Strong expression of TGF-β3 was observed at 1 and 4 weeks in VRO, and at 1 and 8 weeks in fibular fracture. Up-regulation at 1 week is explained by degranulation of platelets, and up-regulation at 4 weeks in VRO and at 8 weeks in fibular fracture means indicates cartilage formation and periosteal response are activated after 4 weeks.
6. The strong expression of VEGF was observed at 1 week in VRO and 4 and 8 weeks in fibular fracture. This result means that the mandibular ramus has sufficient vascularity before injury, and that the needs for angiogenesis and vasculogenesis increased later in the case of fibular fracture.

Based on the above findings, the expressions of specific cytokines differed following VRO and fibular fracture although the healing process following VRO was similar at each period. Dynamic jaw movement is thought to be a contributing factor for differential expression of BMP-7 and TGF-β2, and anatomical factors including preexisting vascularity account for differences in expression of VEGF.

Key words: Vertical ramus osteotomy, Fibular fracture, VRO, Bone healing, TGF-beta superfamily

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I. INTRODUCTION

Vertical ramus osteotomy (VRO) is a surgical technique used for mandibular setback (Akin and Walters, 1975; Tornes and Gilhuus Moe, 1987), widely used in Asia because of the high incidence there of mandibular prognathism and class III malocclusion (Lew et al., 1993). VRO has several advantages over sagittal split ramus osteotomy (SSRO), including a short operation time (Nordin et al., 1987), little risk for inferior alveolar nerve damage (Astrand and Ridell, 1973; Hall and McKenna, 1987), predictable and reliable postoperative stability (Jung et al., 2013), improvement of TMJ symptoms (Bell et al., 1990; Jung et al., 2009), and facilitated rehabilitation of mandibular movement postoperatively (Aragon et al., 1985; Boyd et al., 1991; Jung et al., 2012; Storum and Bell, 1986).

Bone healing states after injury are categorized as inflammation, cartilage formation, and periosteal response, cartilage resorption and primary bone formation, and secondary bone

formation and remodeling. However, none of these include the expression of cytokines, which is highly variable and depends on healing stage and biological process (Ai Aql et al., 2008). Unlike SSRO, VRO makes a lateral overlap of the proximal segment, immediately changing the position of the proximal segment and cortex-to-cortex contact between the segments. The healing process takes place in a unique environment that allows free movement of segments; previous studies to identify the healing process following VRO are limited to HE staining (Bell and Kennedy, 1976; Boyne, 1966; Huebsch and Wellington, 1967).

When VRO is performed on the mandibular ramus, healing proceeds with cortex-to-cortex contact and no internal fixation; we find no cytokine expression studies following cortex-to-cortex contact in dynamic condition. Thus, the aim of this study was to analyze and compare expression of specific cytokines following VRO, which is considered a dynamic condition, and fibular fracture, which is considered a static condition.

II. MATERIALS AND METHODS

All experiments were performed under protocols approved by the Institutional Animal Care and Use Committee at the CRONEX Co., Ltd (CRONEX-IACUC 201209001).

1. Animal care

Four 8-month-old male beagle dogs were used in the experiments, average weight 12.57 ± 0.57 kg (between 11.09 and 13.5 kg). Temperature and relative humidity was maintained at 22 ± 2˚C and 50 ± 10%. Air ventilation was done 10 to 15 times per day via 100% HEPA-filter. Illumination was maintained at 200 LUX with a contrast period of 12 hours per day (8:00 to 20:00). Noise level was adjusted below 40 dB, and the ammonia concentration was less than 20 ppm. Each subject was housed in a stainless steel cage (dimensions: 700W x 900D x 1000H (mm); feces were cleaned once every morning. The main feedstuff was meat soup, replaced every morning in washed bowls. The cage and bowl were cleaned or replaced immediately when contaminated with feces. The feeding water was supplied by automatic water nozzles after performing reverse osmosis filtering and ultraviolet light irradiation.

2. Anesthesia

2 ml zoletil and xylazine solution (6:4) was injected intravenously before endotracheal intubation; isoflurane-induced general anesthesia was maintained during the operation.

3. Vertical ramus osteotomy

Surgical approach for VRO was performed through extraoral approach. The surgical area was sterilized by 10% povidone-iodine solution after shaving and covered with an aseptic surgical drape in a conventional manner. Masseter muscle and attached periosteum were reflected after submandibular incision (5 cm), then the mandibular inferior border was exposed. The buccal aspect of mandibular ramus was dissected until the sigmoid notch was signified. Osteotomy was performed with a Stryker reciprocating saw and the osteotomy line was placed posterior to ligula to prevent inferior alveolar nerve damage. Anatomically, the coronoid process of dog is much bigger than the condylar process, inhibiting mandibular setback. An additional wedge-shaped ostectomy was thus performed on the sigmoid notch area to facilitate setback of the distal segment. Internal fixation was not performed after confirming proper cortex-to-cortex contact following mandibular setback (Figure 1). The incised wound was sutured layer by layer. The mouth was fixed with a biting roll and elastic bandage to maintain contact between the segments (Figure 2).

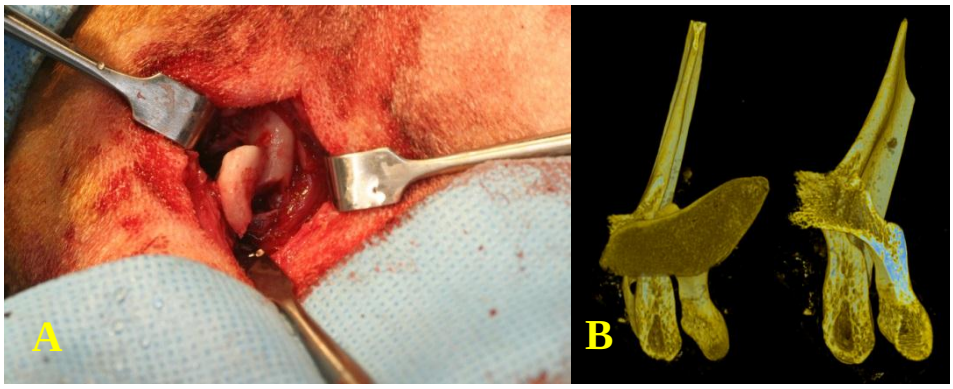

Figure 1. Vertical ramus osteotomy by submandibular approach (A). Distal segment was placed lateral to the proximal segment and cortex-to-cortex contact was confirmed (B).

Figure 2. Intermaxillary fixation with gauze roll biting and elastic bandage.

4. Fibular fracture

The right lateral calf was prepared in a sterile manner for artificial fibular fracture. A 3 cm long skin incision was performed and dissection was performed until the fibula was identified. Periosteum was reflected and oblique osteotomy was performed with reciprocating saw (Figure 3). Layer by layer wound closure was done and elastic bandage applied.

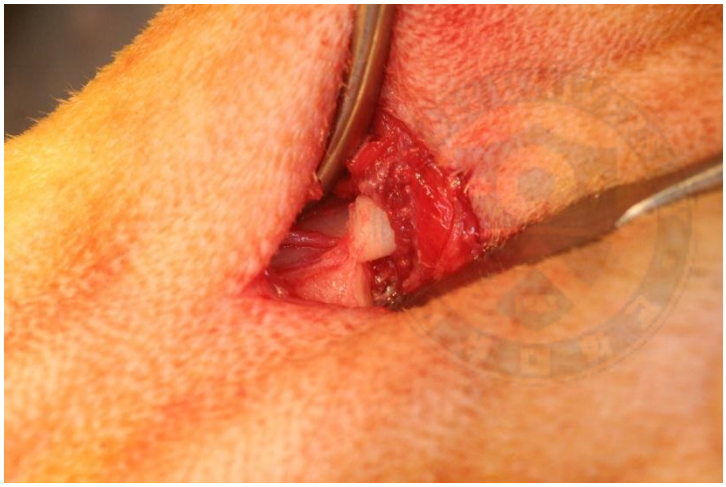

Figure 3. Displaced segment after fibular fracture.

5. Postoperative care and preparation of specimens

After operation, the subjects received antibiotics (enrofloxacine 0.2 ml/kg, Komipharm International Co., Ltd., Korea), administered intramuscularly for 3 days to prevent postoperative infection. Elastic bandage was applied for 7 days as well as a neck collar to prevent loosening the bandage by front foot. The bandage was removed at 8^{th} day postoperatively and solid feed was mixed to water for feeding. The subjects were euthanized with suxamethonium chloride hydrate 50 mg/kg (Komipharm International Co., Ltd., Korea) intravenous injection at 1, 2, 4, and 8 weeks postoperatively. The experimental specimens were carefully harvested en bloc including their adjacent soft tissue, then fixed with 10% paraformaldehyde for 2 weeks. 0.5 mol EDTA was used for decalcification, the solution being changed every week.

6. Immunohistochemistry

IHC staining was performed on 6 μm paraffin-embedded sections. The sections were treated by heat-induced epitope retrieval with citrate buffer pH6.0 (Invitrogen). The slides were incubated with antibodies against VEGF (1:200 diluted, sc-507, Santa Cruz, USA), BMP7 (1:200 diluted, sc-9032, Santa Cruz, USA), MMP3 (1:200 diluted, sc-6839, Santa Cruz, USA), TGFβ2 (1:200 diluted, sc-90, Santa Cruz, USA), TGFβ3 (1:60 diluted, MAB243, R&D systems, USA), or BMP-2/4 (1:100 diluted, sc-9003, Santa Cruz, USA) at 4 ℃ overnight. The specimens were sequentially incubated with secondary antibody and streptavidin peroxidase. Finally, the results were visualized following staining using a diaminobenzidine (DAB) reagent kit (Invitrogen, USA). The sections

were counterstained with Mayer's hematoxylin. All specimens were observed by stereomicroscope (MD5500D; Leica, camera: DFC495; Leica, Lens: HCX PL APO 409; Leica). The positive pixels of images were counted using the software Leica Application to evaluate IHC staining (Leica Microsystems, Germany). All parameters of image acquisition were kept the same to allow accurate comparison.

III. RESULTS

1. VRO (HE staining)

Inflammatory cell infiltration and resorption of cortical bone was seen 1 week postoperatively. Cartilage formation by chondrocyte was observed at 2 weeks. Cartilage resorption, primary bone formation, and vascularization was observed at 4 weeks, and the process of maturing was maintained 8 weeks postoperatively.

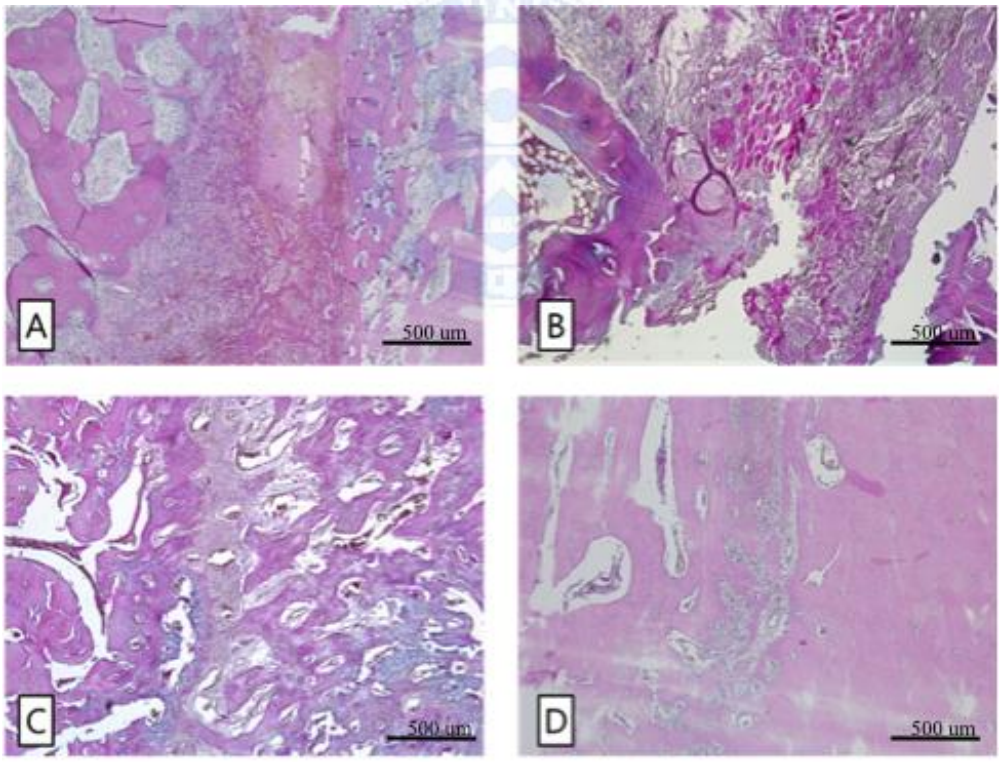

Figure 4. Vertical ramus osteotomy, HE staining.

A. 1 week, B. 2 weeks, C. 4 weeks, and D. 8 weeks postoperatively.

2. VRO (Immunohistochemical analysis)

Six antibodies including bone morphogenetic protein-2/4, -7 (BMP-2/4, -7), matrix metalloproteinase-3 (MMP-3), transforming growth factor-beta 2, 3 (TGF-ß2, -ß3), and vascular endothelial growth factor (VEGF) were used for immunohistochemistry. Expression results are followed by figures.

A. BMP-2/4

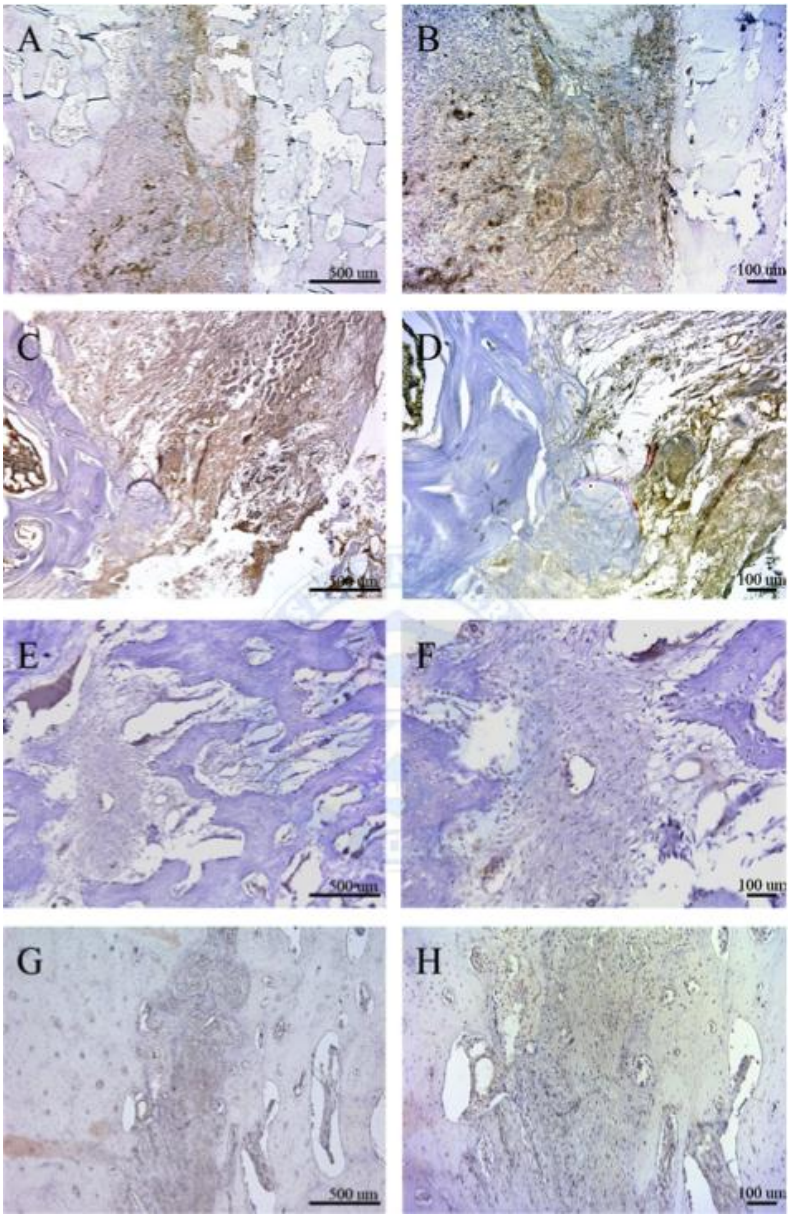

Figure 5. Expression of BMP-2/4 following VRO.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

B. BMP-7

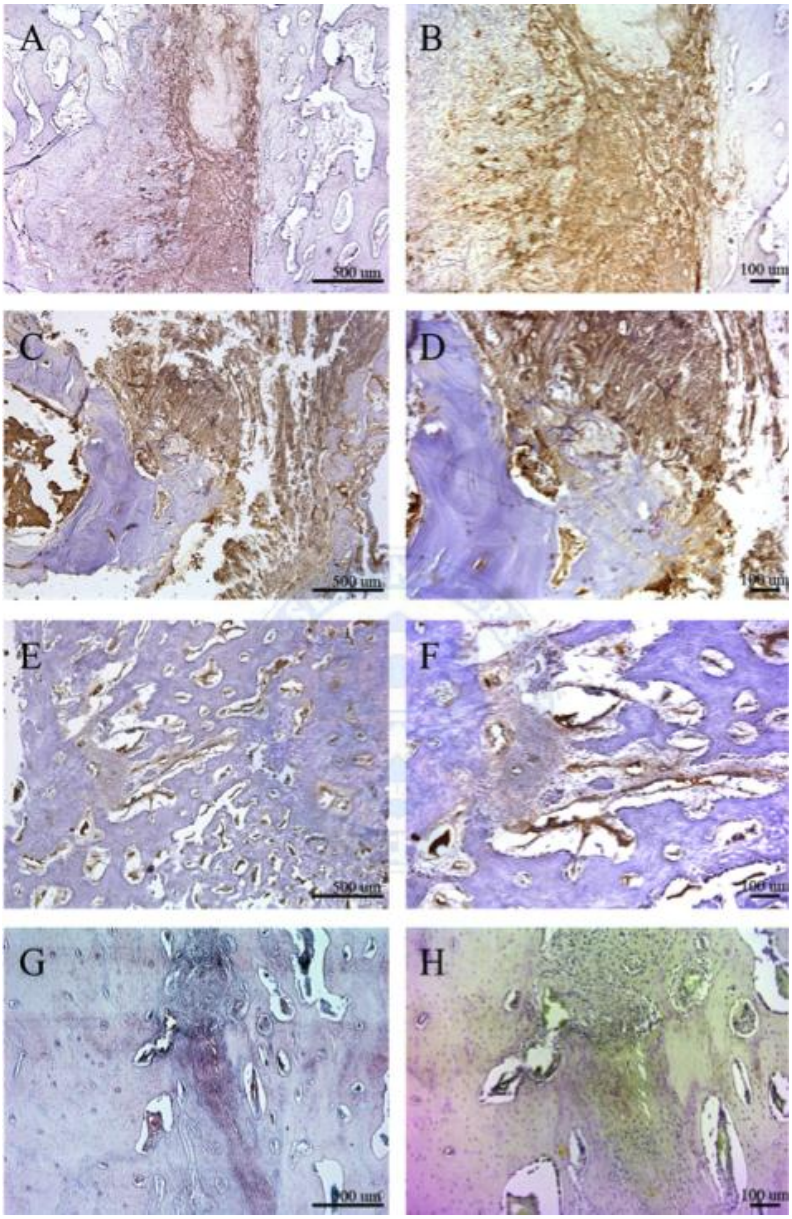

Figure 6. Expression of BMP-7 following VRO.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

C. MMP-3

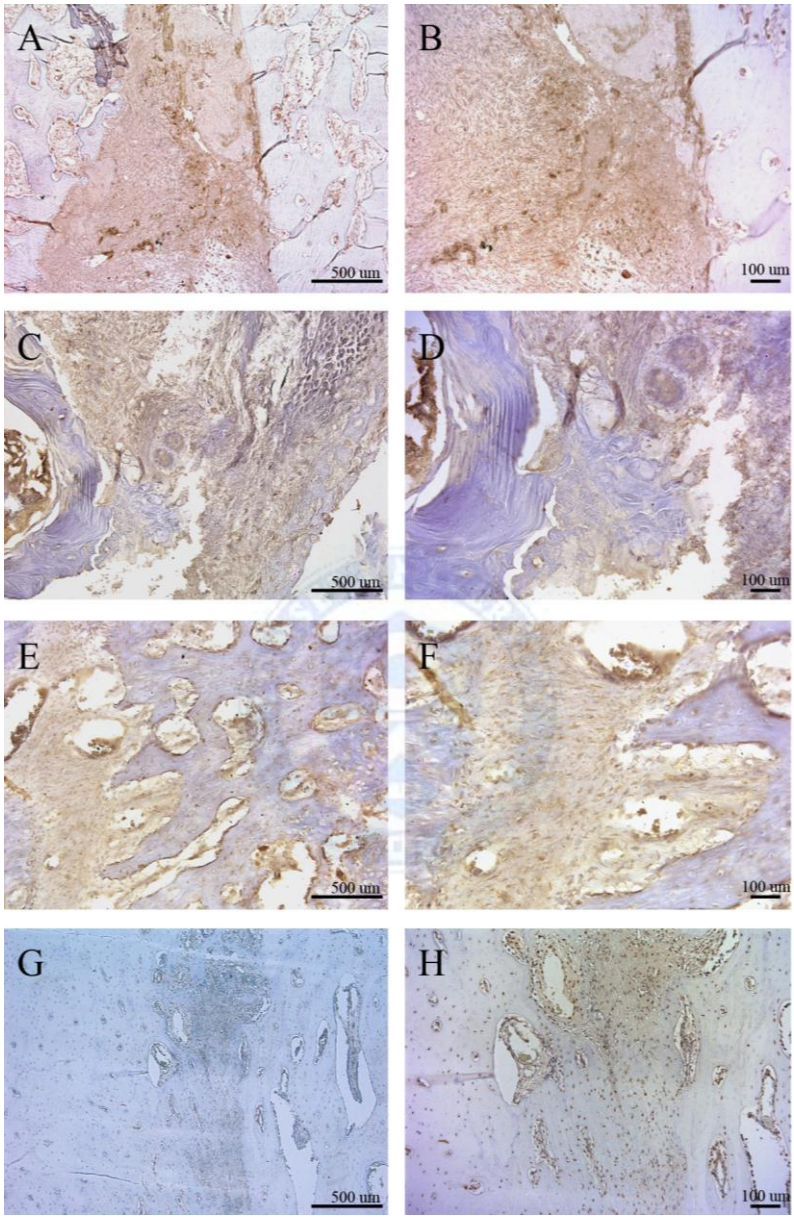

Figure 7. Expression of MMP-3 following VRO.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

D. TGF-β2

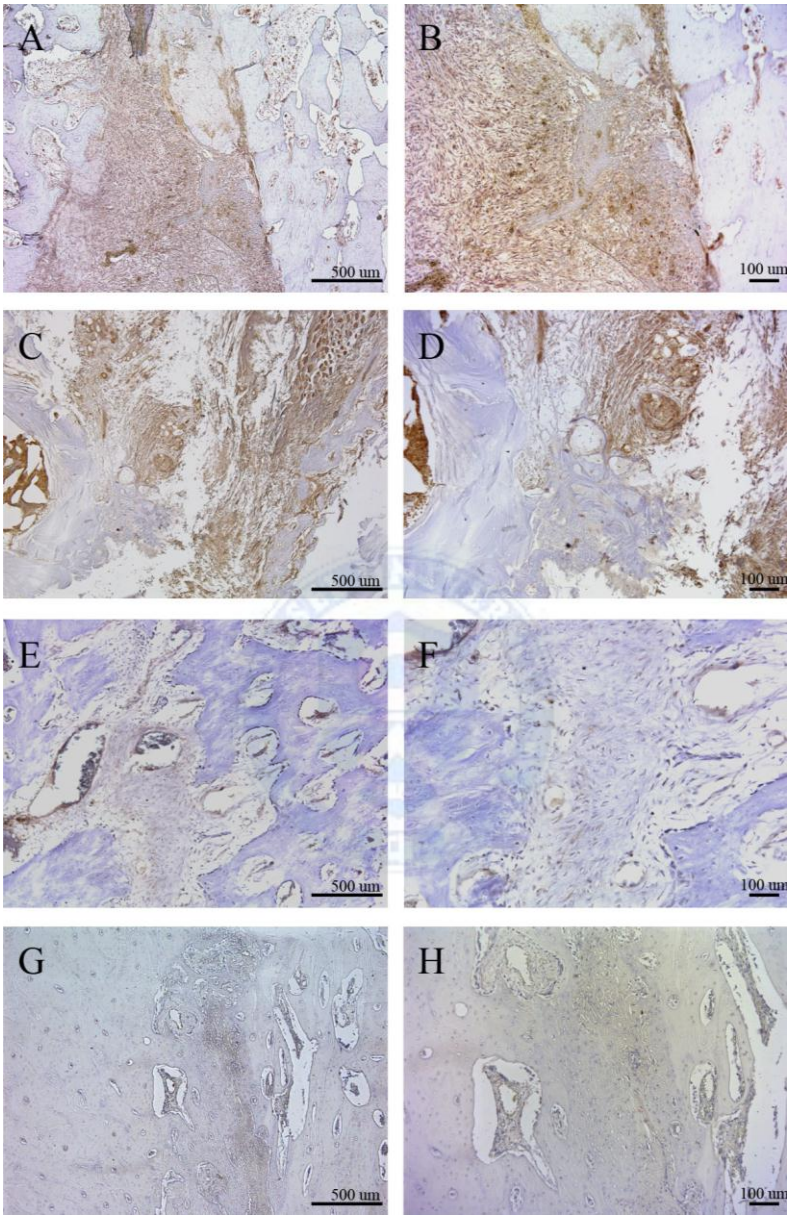

Figure 8. Expression of TGF-ß2 following VRO.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

E. TGF-β3

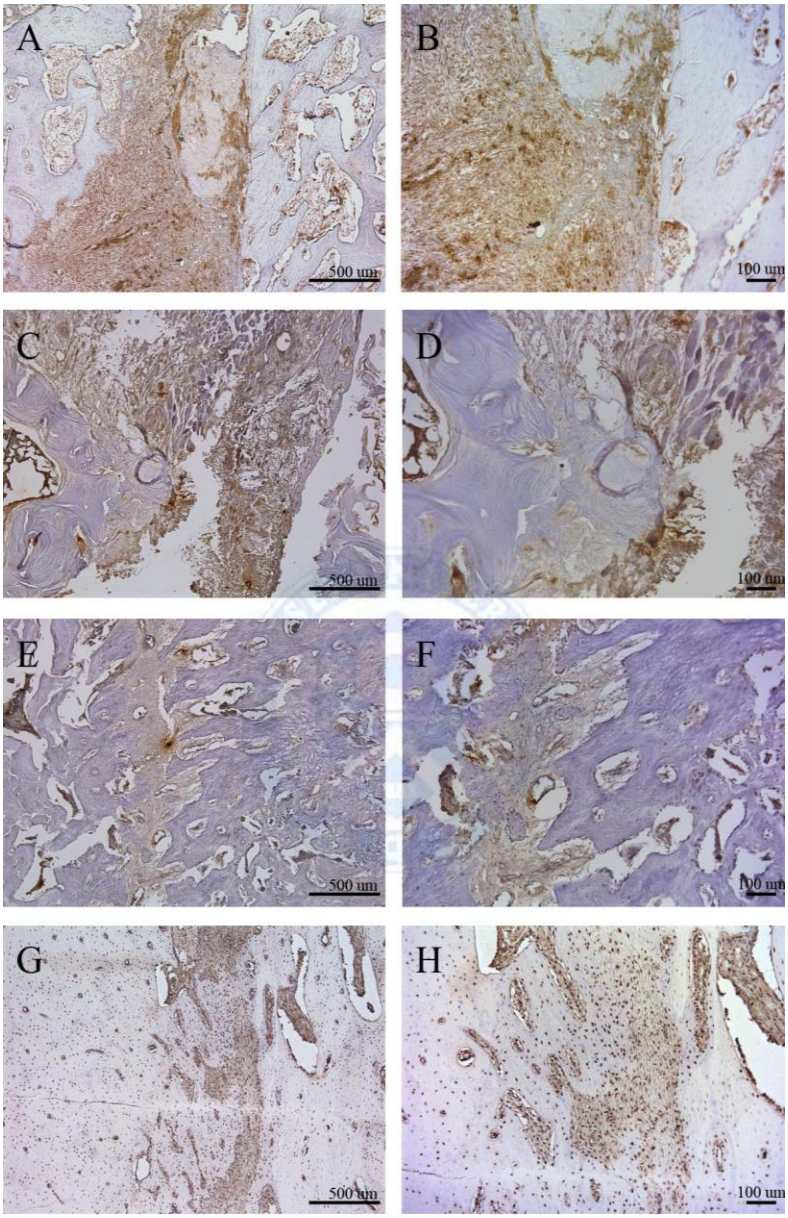

Figure 9. Expression of TGF-ß3 following VRO.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

F. VEGF

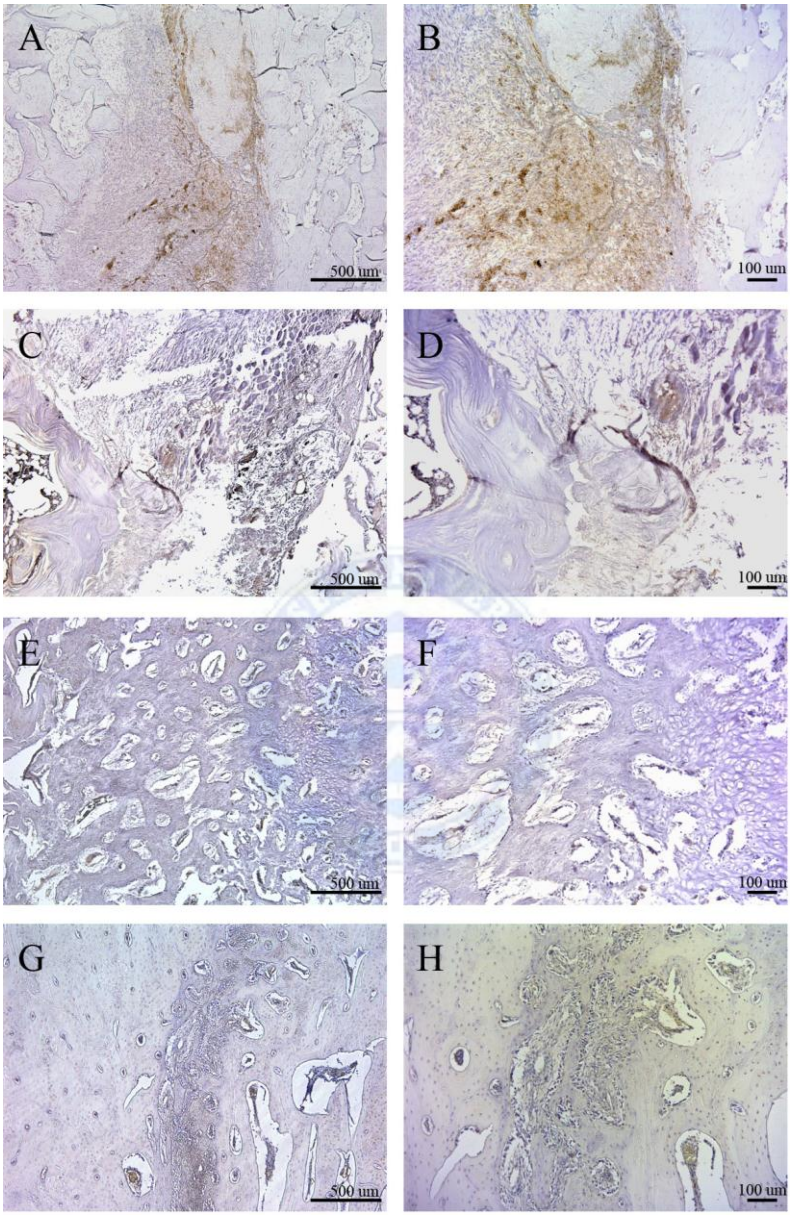

Figure 10. Expression of VEGF following VRO.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

3. Fibular fracture (HE staining)

Inflammatory cell infiltration and resorption of cortical bone was seen 1 week postoperatively. Cartilage formation by chondrocyte was observed at 2 weeks. Cartilage resorption, primary bone formation, and vascularization were observed at 4 weeks, and the process of maturing was maintained 8 weeks postoperatively. One segment was removed while making slides of 1 and 2 week samples.

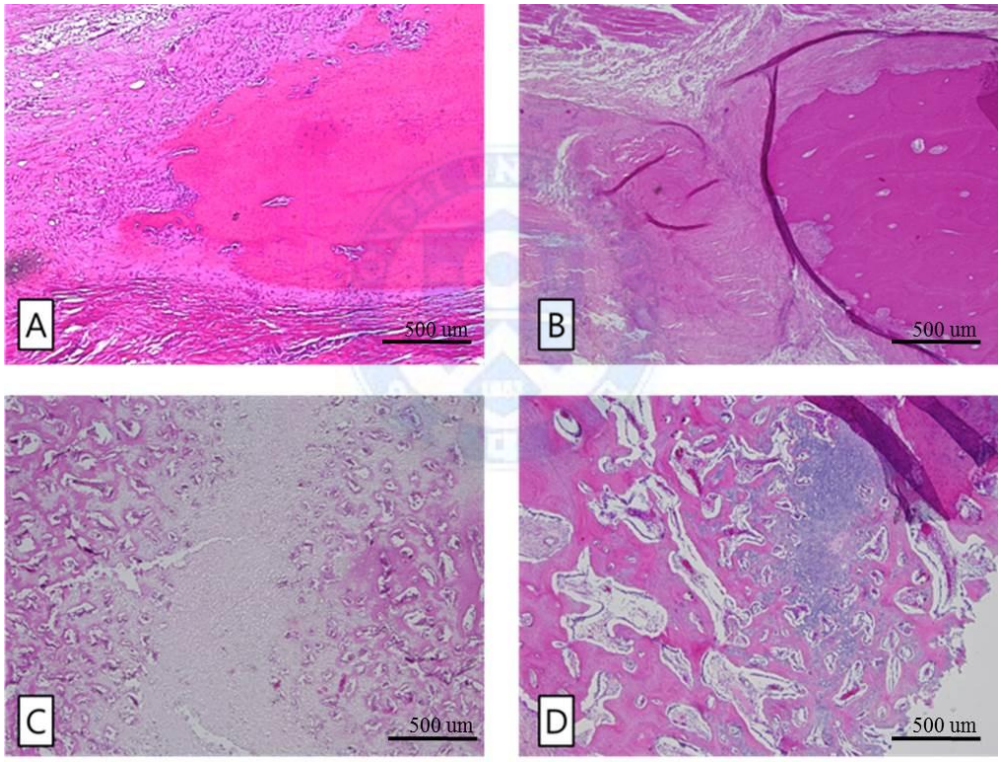

Figure 11. Fibular fracture, HE staining.

A. 1 week, B. 2 weeks, C. 4 weeks, and D. 8 weeks postoperatively.

4. Fibular fracture (immunohistochemical analysis)

Six antibodies including bone morphogenetic protein-2/4, -7 (BMP-2/4, -7), matrix metalloproteinase-3 (MMP-3), transforming growth factor-beta 2, 3 (TGF-ß2, -ß3), and vascular endothelial growth factor (VEGF) were used for immunohistochemistry. Expression results are followed by figures.

A. BMP-2/4

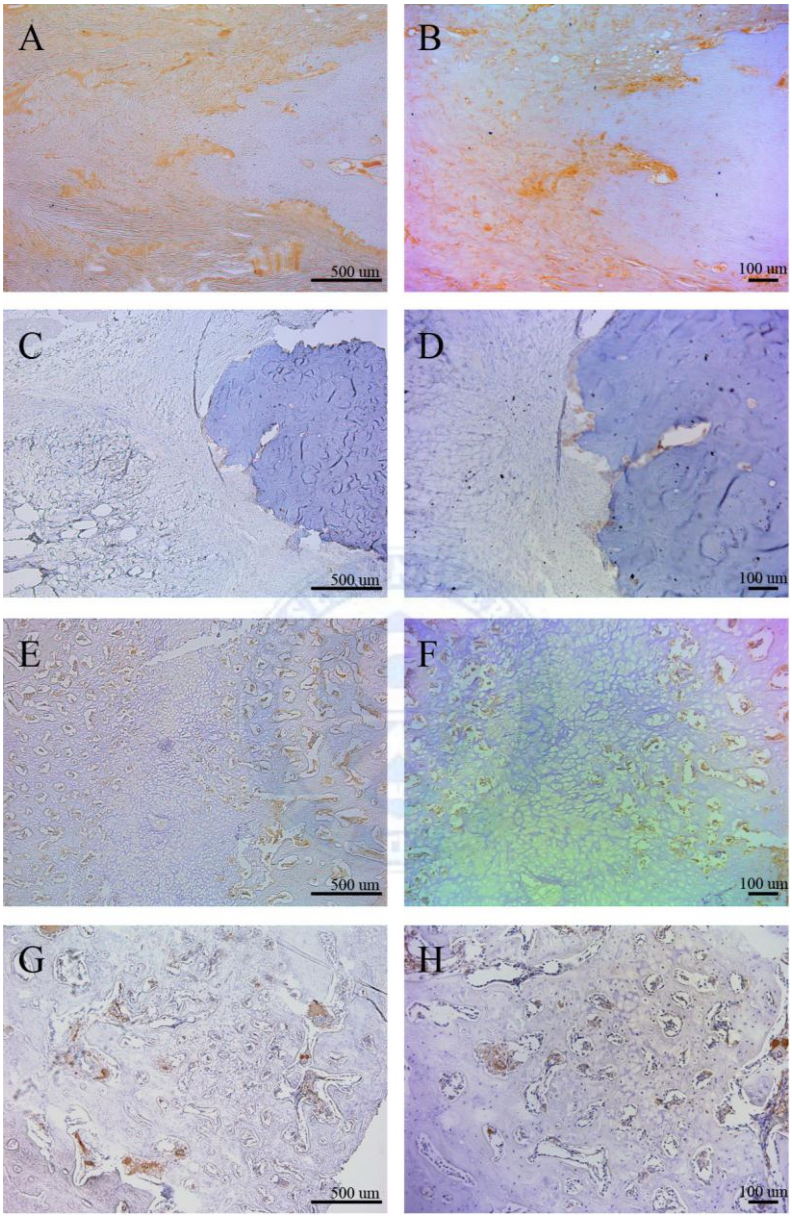

Figure 12. Expression of BMP-2/4 following fibular fracture.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

B. BMP-7

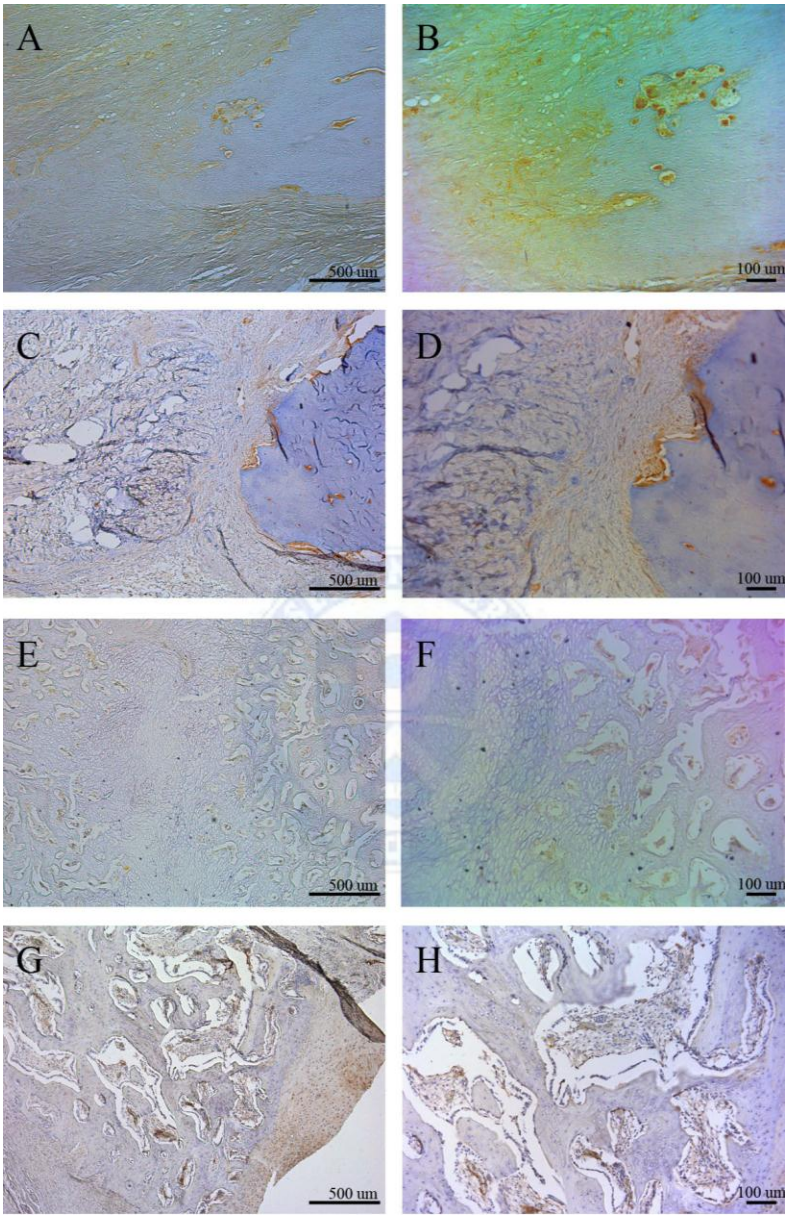

Figure 13. Expression of BMP-7 following fibular fracture.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

C. MMP-3

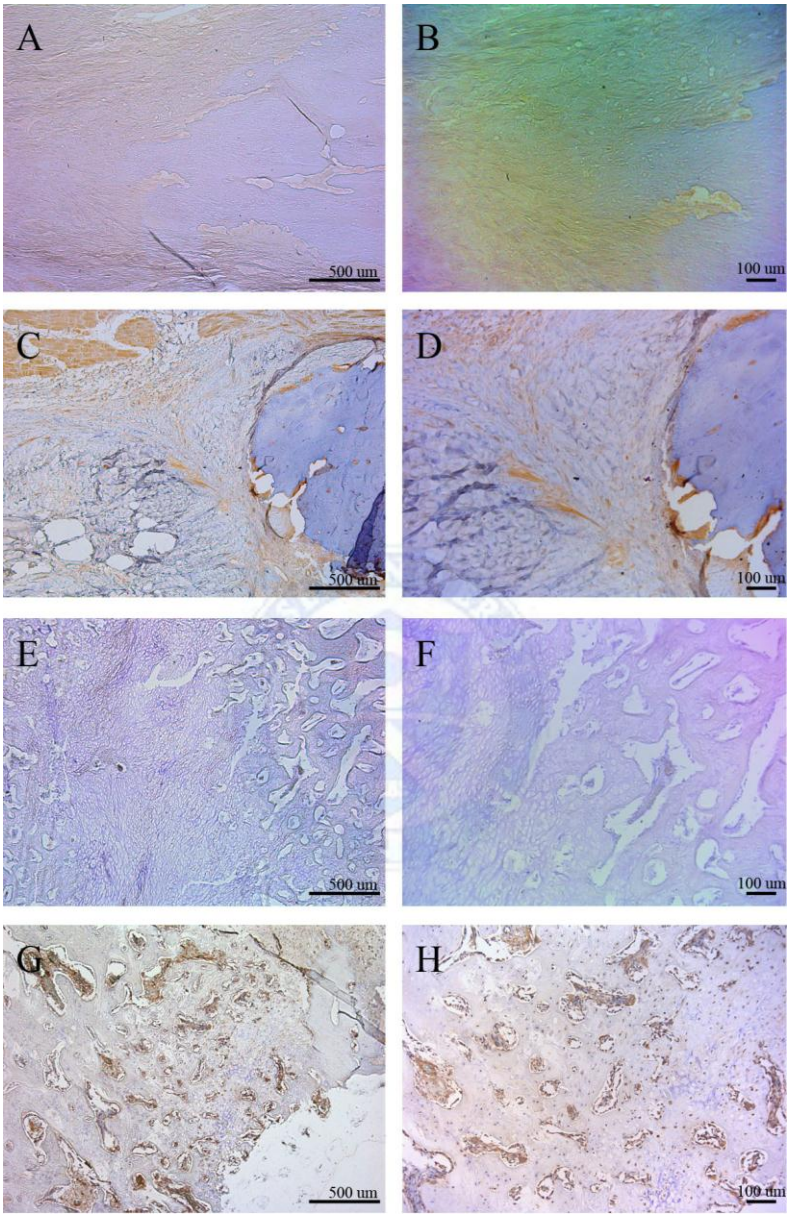

Figure 14. Expression of MMP-3 following fibular fracture.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

D. TGF-β2

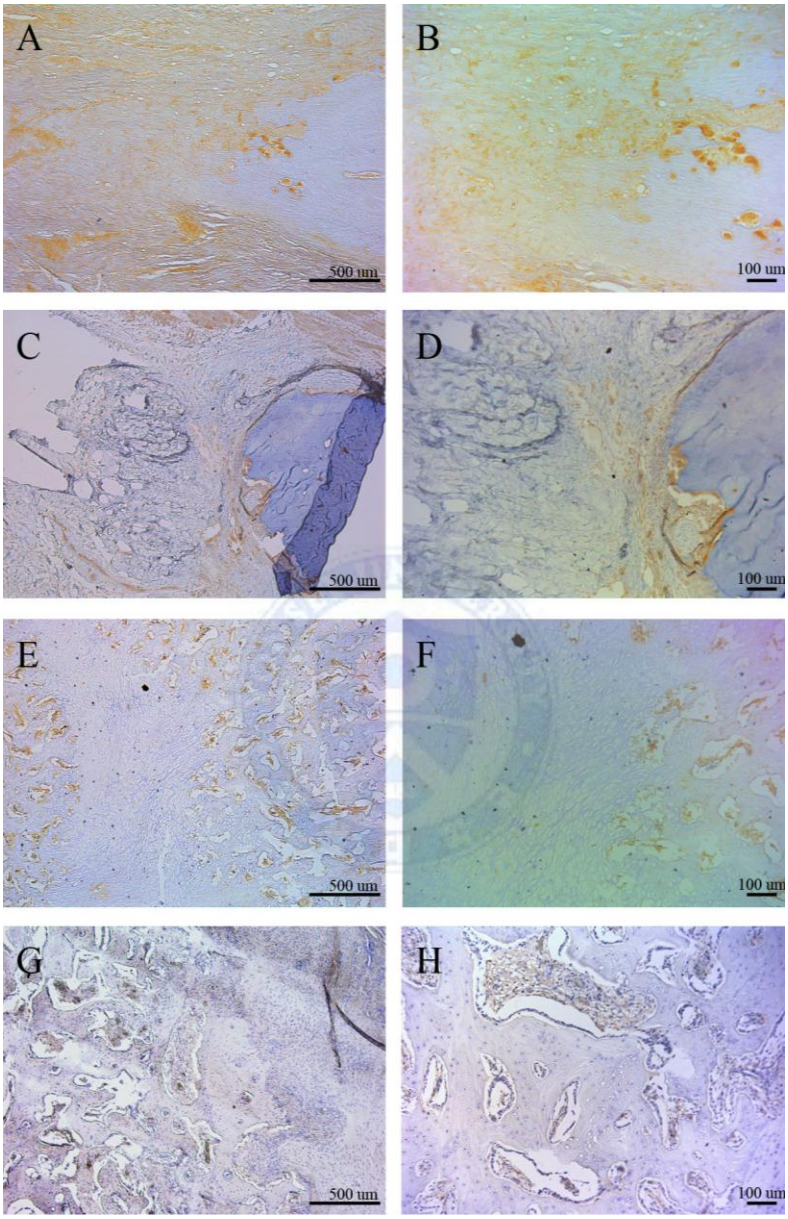

Figure 15. Expression of TGF-ß2 following fibular fracture.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

E. TGF-β3

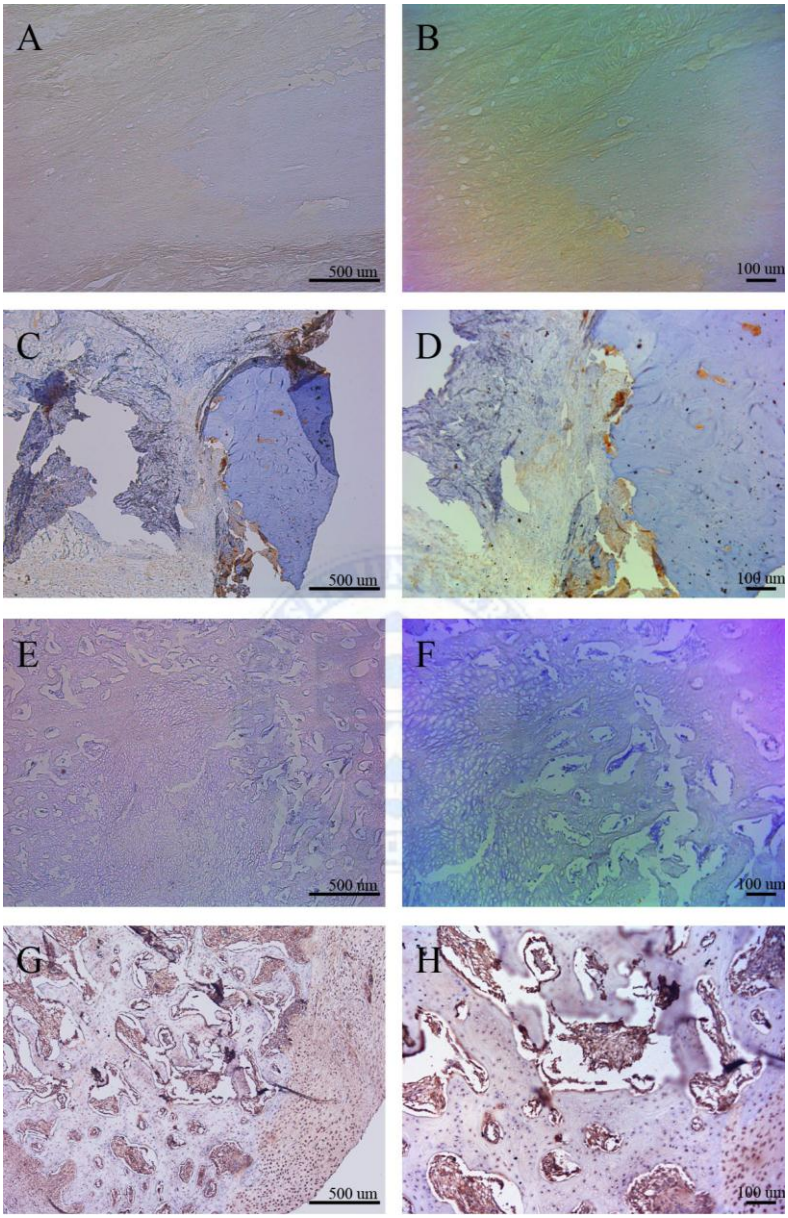

Figure 16. Expression of TGF-ß3 following fibular fracture.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

F. VEGF

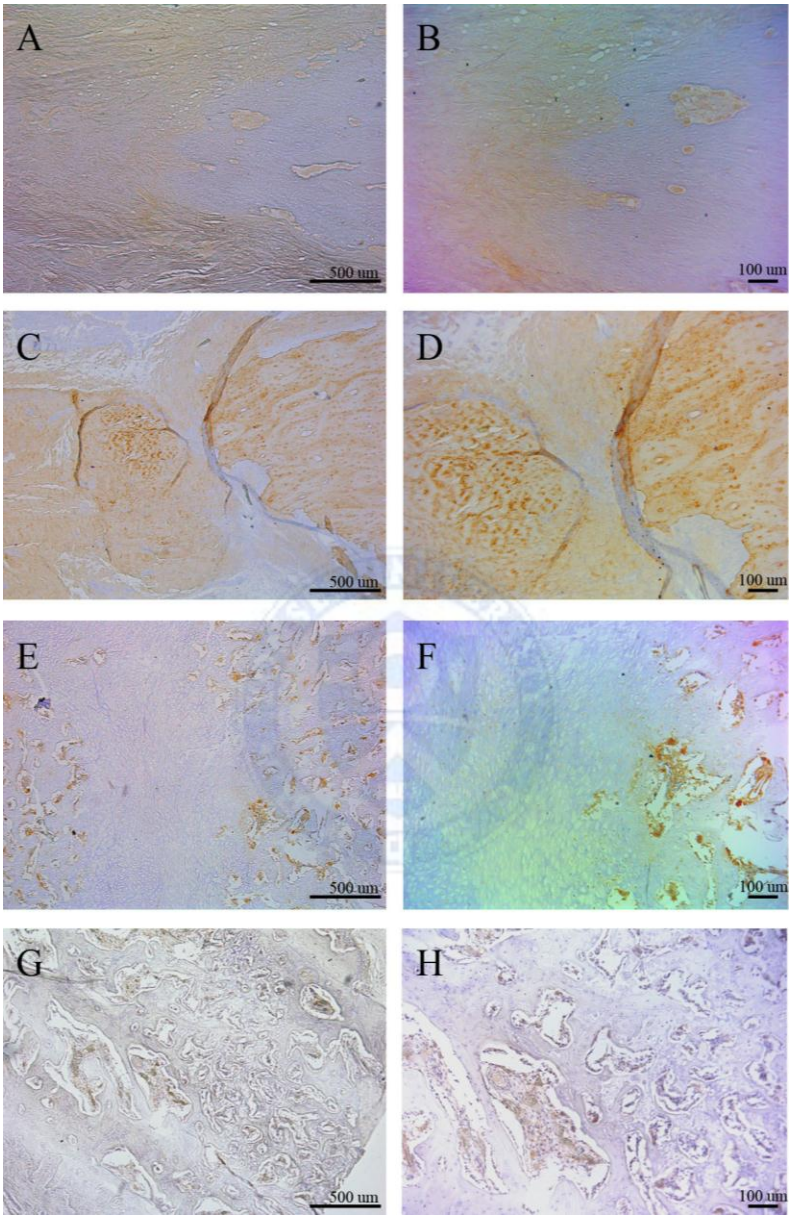

Figure 17. Expression of VEGF following fibular fracture.

A, B. 1 week, C, D. 2 weeks, E, F. 4 weeks, and G, H. 8 weeks postoperatively.

5. Evaluation of immunohistochemical analysis

The positive pixels of images were counted using the software Leica Application to evaluate IHC staining (Leica Microsystems, Germany). The strongest expression of BMP-2/4 was observed at 2 weeks postoperatively in VRO, a relatively constant expression of BMP-2/4 being observed in fibular fracture. A decreased pattern of BMP-7 was observed in VRO, significant down-regulation being observed at 4 and 8 weeks postoperatively. In contrast to VRO, the expression pattern of BMP-7 was constant in fibular fracture. An increased pattern of TGF-β2 was observed in VRO, the expression pattern remaining constant in fibular fracture. Up-regulation of TGF-β3 was observed at 1 and 4 weeks in VRO, and at 1 and 8 weeks in fibular fracture. A decreased pattern of expression of VEGF was observed in VRO, up-regulation of VEGF being observed at 1 week in VRO, and at 4 and 8 weeks in fibular fracture. The results showed in figure 18 and 19.

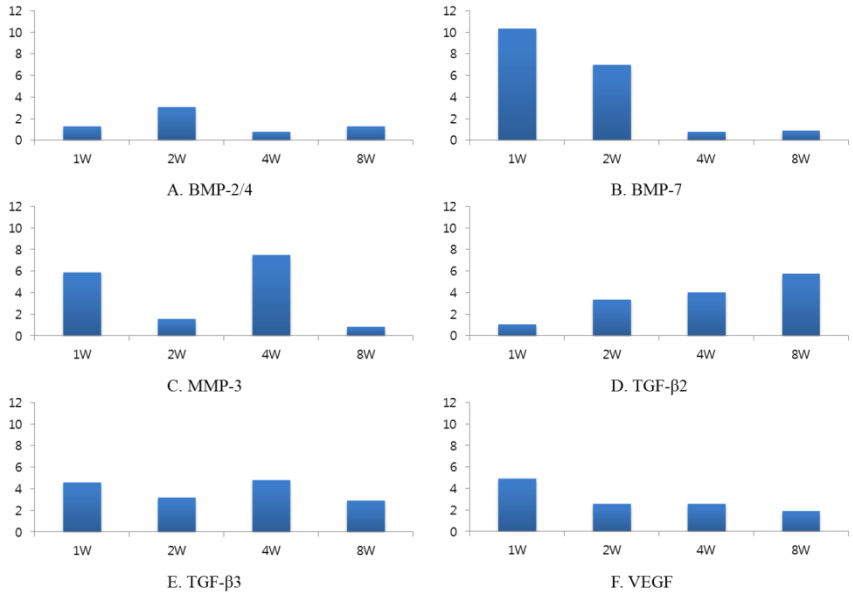

Figure 18. Evaluation of IHC staining in VRO.

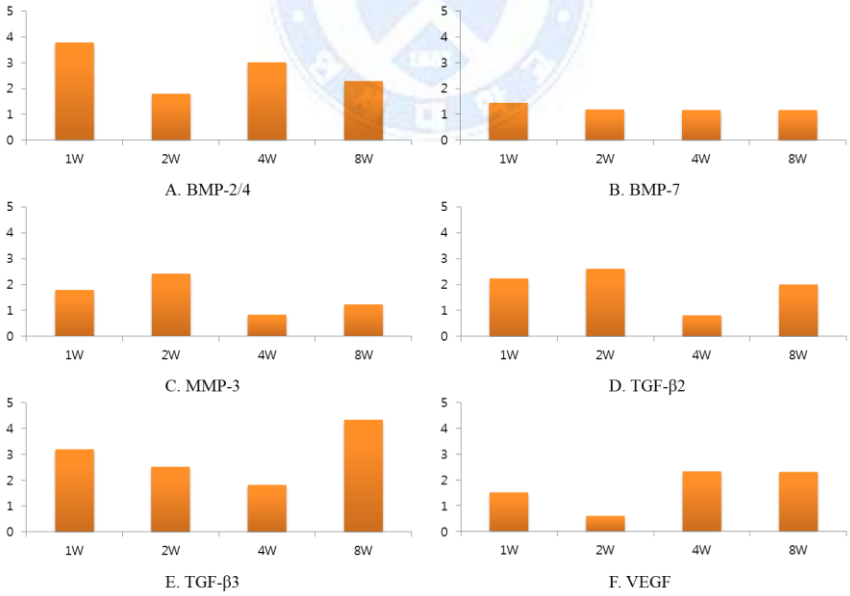

Figure 19. Evaluation of IHC staining in fibular fracture.

IV. DISCUSSION

The healing process following orthognathic surgery is based on the substantial capacity of bone for repair and regeneration in response to injury. Orthognathic surgery corrects conditions of the jaw and face related to structure, growth, sleep apnea, TMJ disorders, malocclusion problems owing to skeletal disharmonies, or other orthodontic problems that cannot be easily treated with braces. VRO is a surgical method for mandibular setback performed as vertical osteotomy from sigmoid notch to mandibular angle while avoiding the mandibular foramen. Conventionally, internal fixation is not performed following VRO, thus bone heal following VRO is considered secondary bone healing allowing dynamic movement of bony segments. The aim of this study was to determine the specific signaling molecules in each period following VRO and to compare the results with those from fibular fracture, which is considered a relatively static condition.

Bone repair in adults recapitulates the pathway of embryonic development, with the coordinated participation of several cell types originating from the cortex, periosteum, surrounding soft tissue, and bone marrow (Ferguson et al., 1999; Gerstenfeld et al., 2003). The majority of fractures heal through a combination of intramembranous and endochondral ossification. Endochondral bone formation usually occurs external to the periosteum, around the fractured site, whereas intramembranous ossification occurs internal to the periosteum at the proximal and distal edges (Dimitriou et al., 2005). Cortex-to-cortex contact is made following VRO, and bony gap also remains in fibular fracture, thus endochondral bone formation might be the main process in healing. The most important difference was mobility status; although dynamic movement was allowed by jaw movement in VRO, fibular fracture was relatively static because the fibula is not a weight-bearing bone.

Bone healing is categorized in terms of inflammation, cartilage formation and periosteal response, cartilage resorption and primary bone formation, and secondary bone formation and remodeling. In this study, no remarkable difference in HE staining was observed between VRO and fibular fracture. The histologic pattern observed was inflammatory phase in 1 week; cartilage formation and periosteal response in 2 weeks; and cartilage resorption and primary bone formation in 4 and 8 weeks.

Inflammatory cytokines are produced and function immediately after injury for a limited time period, initiating the repair cascade following injury. Interleukins-1 and -6 (IL-1 and IL-6) and tumor necrosis factor-alpha (TNF-α) have been shown to play a role in initiating the repair cascade. They are secreted at the injury site by macrophages, inflammatory cells, and cells of mesenchymal origin, their expression peaking within the first 24 hours and then declining rapidly (Cho et al., 2002). They induce a downstream response to injury by recruiting other inflammatory cells, enhancing extracellular matrix synthesis, and stimulating angiogenesis (Kon et al., 2001).

The TGF-β superfamily consists of a large number of growth and differentiation factors that include BMPs, TGF-β, GDFs, activins, inhibins, and Müllerian inhibiting substance. Among them, BMPs (2-8), GDF (1,5,8, and 10), and TGF-β 1-3 specifically promote various stages of intramembranous and endochondral bone ossification during bone healing (Cho et al., 2002). Different BMPs act to trigger a cascade of events that promote the formation of cartilage or bone, functioning independently or in collaboration with each other as well as with other members of the TGF-β superfamily. BMPs are produced by mesenchymal cells, osteoblasts, and chondrocytes. Cellular processes stimulated include chemotaxis, mesenchymal cell proliferation and differentiation, angiogenesis, and synthesis of extracellular matrix (Reddi, 2001; Sakou, 1998).

BMP-2 is known as an initiator of the repair cascade and a controller for the expression of several other BMPs. It is known as an essential element for postnatal bone repair and is genetically associated with the maintenance of normal bone mass (Tsuji et al., 2006; Xiong et al., 2006).

When its activity is blocked, marrow stromal stem cells fail to differentiate into osteoblasts (Edgar et al., 2007). Up-regulation of BMP-2 and -4 are observed in whole bone healing steps including inflammation; cartilage formation and periosteal response; cartilage resorption and primary bone formation; and secondary bone formation and remodeling (Cho et al., 2002; Dimitriou et al., 2005; Gerstenfeld et al., 2003; Kon et al., 2001). In this study, the strongest expression of BMP-2/4 was observed at 2 weeks postoperatively in VRO, whereas relatively continuous up-regulation was observed in fibular fracture.

BMP-7 plays a key role in the transformation of mesenchymal cells into bone and cartilage (Chen et al., 2004). Cho et al. reported that BMP-7 shows a restricted period (days 14 through 21) of expression during fracture healing, when the resorption of calcified cartilage and osteoblastic recruitment are the most active in murine experiment (Cho et al., 2002). In our results, the strongest expression of BMP-7 was observed at 1 week in VRO, then decreasing. However, a constant expression pattern was observed in fibular fracture. Tsuji et al. reported that the absence of locally produced BMP-7 has no effect on postnatal bone growth, articular cartilage formation, maintenance of bone mass, or fracture heal (Tsuji et al., 2006). Weak or nearly absent expression of BMP-7 was observed during distraction osteogenesis (Campisi et al., 2003; Sato et al., 1999; Yazawa et al., 2003). Thus, dynamic movement between bony segments may lead to decreased expression of BMP-7 following VRO.

Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix and during tissue remodeling in normal physiological processes. Further, MMP-3 can activate other MMPs such as MMP-1, -7, and -9, rendering MMP-3 crucial in connective tissue remodeling (Ye et al., 1996). In our results, up-regulation of MMP-3 was observed at weeks 1 and 4 in VRO, the strongest expression following fibular fracture being observed at 2 weeks post-operative.

TGF-β1-3 are produced by degranulated platelets after initial injury, which suggests their involvement in the initiation of callus formation (Bolander, 1992; Bostrom, 1998). They are also produced by osteoblasts and chondrocytes at later stages, enhancing the proliferation of these cells as well as that of mesenchymal cells and pre-osteoblasts (Lieberman et al., 2002). TGF-β, thought to play an important role in chondrogenesis and endochondral bone formation (Barnes et al., 1999), induces the expression of extracellular matrix proteins (Sandberg et al., 1993). In our results, the strongest expression of TGF-ß2 was observed at 8 weeks in VRO with increased pattern. Up-regulated expression was observed at weeks 1, 2, and 8 in fibular fracture. TGF-β2 functions as a local positive regulator of bone remodeling (Erlebacher and Derynck, 1996); Erlebacher et al. observed overexpressed TGF-β2 at the site of matured bone matrix formation, which is suspected to be a homeostatic response to increased bone resorption (Erlebacher et al., 1998). Thus, we can conclude that the remodeling activity is accelerated under dynamic condition following VRO based on the increase pattern of BMP-7 in VRO. Further, the strong expression of TGF-β3 at 1 week in VRO and fibular fracture may be caused by degranulated platelets, and the up-regulation at 4 weeks in VRO and at 8 weeks in fibular fracture may be caused by activation of the remodeling response (Cho et al., 2002; Dimitriou et al., 2005).

Optimal bone healing is dependent on adequate vascularization and therefore requires the development of new blood vessels; VEGF is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis. In this study, the strong expression of VEGF was observed at 1 week in VRO, and at 4 and 8 weeks in fibular fracture. The different patterns of up-regulation might be due to an anatomical factor: whereas the mandibular ramus has relatively greater blood flow than do extremities, the need for vasculogenesis and angiogenesis is greater in late stage following fibular fracture.

To achieve stable bone healing after VRO, one must understand the bone healing mechanism. In the present study, significantly different expression patterns were observed with respect to BMP-7, TGF-β2, and VEGF. Dynamic movement following VRO is strongly suspected as a factor for a decrease expression pattern of BMP-7, and BMP-7 might not be an essential factor for bone healing. The increase expression pattern of TGF-β2 means that the remodeling activity is accelerated over time following VRO. Moreover, sufficient preexisting vascularity in the mandibular ramus accounts for the earlier expression of VEGF in VRO.

V. CONCLUSION

Vertical ramus osteotomy (VRO) is a surgical method of performing an osteotomy from the sigmoid notch to the postero-inferior border of the ramus from the lateral aspect of the ascending ramus for the mandibular setback, followed by healing under dynamic condition which allows movement of bony segment. This study aimed to evaluate specific cytokines among the TGF-β superfamily at each period following VRO, and to compare those results with fibular fracture heal. The 4 beagle dogs were used for this experiment were euthanized at 1, 2, 4, and 8 weeks postoperatively. 6 specific antibodies were used in this immunohistochemical analysis: bone morphogenetic proteins -2/4, -7 (BMP -2/4, -7), matrix metalloproteinase-3 (MMP-3), transforming growth factor-beta 2, 3 (TGF-ß2, -ß3), and vascular endothelial growth factor (VEGF).

The results are as follows:

1. In VRO heal, inflammatory cell infiltration and resortion of cortical bone at 1 week, cartilage formation by chondrocyte at 2 weeks, and cartilage resorption, primary bone formation, and vascularization at 4 and 8 weeks were observed in HE staining. In fibular fracture heal, no remarkable differences were seen in HE staining compared to VRO heal at each period.

2. In general, upregulation of BMP-2/4 was observed in whole heal periods in VRO and fibular fracture, while the strongest expression of BMP-2/4 was observed at 2 weeks postoperative following VRO in contrast to a relatively constant expression of BMP-2/4 following fibular fracture.

3. The strongest expression of BMP-7 was observed at 1 week following VRO, in contrast to constant up-regulation following fibular fracture. The decreasing pattern in VRO may be caused by dynamic movement of mandible.

4. The strongest expression of TGF-ß2 in VRO was observed at 8 weeks, the expression showing increasing pattern, meaning remodeling activity accelerated over time.

5. Strong expression of TGF-β3 was observed at 1 and 4 weeks in VRO, and at 1 and 8 weeks in fibular fracture. Up-regulation at 1 week is explained by degranulation of platelets, and up-regulation at 4 weeks in VRO and at 8 weeks in fibular fracture indicates cartilage formation and periosteal response are activated after 4 weeks.

6. The strong expression of VEGF was observed at 1 week in VRO and 4 and 8 weeks in fibular fracture. This result means that the mandibular ramus has sufficient vascularity before injury, and that the need for angiogenesis and vascular genesis increased later in the case of fibular fracture.

Based on the above findings, the expressions of specific cytokines differed following VRO and fibular fracture although the healing process following VRO was similar at each period. Dynamic jaw movement is thought to be a contributing factor for differential expression of BMP-7 and TGF-β2, and anatomical factors including preexisting vascularity account for differences in expression of VEGF.

VI. REFERENCES

Ai Aql ZS, Alagl AS, Graves DT, Gerstenfeld LC, Einhorn TA: Molecular mechanisms controlling bone formation during fracture healing and distraction osteogenesis. *Journal of dental research* 87(2): 107-118, 2008.

Akin RK, Walters PJ: Experience with the intraoral vertical subcondylar osteotomy. *Journal of oral surgery* 33(5): 342-345, 1975.

Aragon SB, Van Sickles JE, Dolwick MF, Flanary CM: The effects of orthognathic surgery on mandibular range of motion. *Journal of oral and maxillofacial surgery* 43(12): 938-943, 1985.

Astrand P, Ridell A: Positional changes of the mandible and the upper and lower anterior teeth after oblique sliding osteotomy of the mandibular rami. A roentgen-cephalometric study of 55 patients. *Scandinavian journal of plastic and reconstructive surgery* 7(2): 120-129, 1973.

Barnes GL, Kostenuik PJ, Gerstenfeld LC, Einhorn TA: Growth factor regulation of fracture repair. *Journal of bone and mineral research* 14(11): 1805-1815, 1999.

Bell WH, Kennedy JW: Biological basis for vertical ramus osteotomies--a study of bone healing and revascularization in adult rhesus monkeys. *Journal of oral surgery* 34(3): 215-224, 1976.

Bell WH, Yamaguchi Y, Poor MR: Treatment of temporomandibular joint dysfunction by intraoral vertical ramus osteotomy. *The International journal of adult orthodontics and orthognathic surgery* 5(1): 9-27, 1990.

Bolander ME: Regulation of fracture repair by growth factors. *Proceedings of the Society for Experimental Biology and Medicine* 200(2): 165-170, 1992.

Bostrom MP: Expression of bone morphogenetic proteins in fracture healing. *Clin Orthop Relat Res.* (355 Suppl): S116-123., 1998.

Boyd SB, Karas ND, Sinn DP: Recovery of mandibular mobility following orthognathic surgery. *Journal of oral and maxillofacial surgery* 49(9): 924-931, 1991.

Boyne PJ: Osseous healing after oblique osteotomy of the mandibular ramus. *Journal of oral surgery* 24(2): 125-133, 1966.

Campisi P, Hamdy RC, Lauzier D, Amako M, Rauch F, Lessard M: Expression of bone morphogenetic proteins during mandibular distraction osteogenesis. *Plastic and reconstructive surgery* 111(1): 201-208; discussion 209, 2003.

Chen D, Zhao M, Mundy GR: Bone morphogenetic proteins. *Growth factors* 22(4): 233-241, 2004.

Cho T, Gerstenfeld LC, Einhorn TA: Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. *Journal of bone and mineral research* 17(3): 513-520, 2002.

Dimitriou R, Tsiridis E, Giannoudis PV: Current concepts of molecular aspects of bone healing. *Injury* 36(12): 1392-1404, 2005.

Edgar CM, Chakravarthy V, Barnes G, Kakar S, Gerstenfeld LC, Einhorn TA: Autogenous regulation of a network of bone morphogenetic proteins (BMPs) mediates the osteogenic differentiation in murine marrow stromal cells. *Bone* 40(5): 1389-1398, 2007.

Erlebacher A, Derynck R: Increased expression of TGF-beta 2 in osteoblasts results in an osteoporosis-like phenotype. *The Journal of cell biology* 132(1-2): 195-210, 1996.

Erlebacher A, Filvaroff EH, Ye JQ, Derynck R: Osteoblastic responses to TGF-beta during bone remodeling. *Molecular biology of the cell* 9(7): 1903-1918, 1998.

Ferguson C, Alpern E, Miclau T, Helms JA: Does adult fracture repair recapitulate embryonic skeletal formation? *Mechanisms of development* 87(1-2): 57-66, 1999.

Gerstenfeld LC, Cullinane DM, Barnes GL, Graves DT, Einhorn TA: Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *Journal of cellular biochemistry* 88(5): 873-884, 2003.

Hall HD, McKenna SJ: Further refinement and evaluation of intraoral vertical ramus osteotomy. *Journal of oral and maxillofacial surgery* 45(8): 684-688, 1987.

Huebsch RF, Wellington JS: Osseous healing in dog mandibles with and without decortication. *Oral surgery, oral medicine, oral pathology* 23(2): 236-240, 1967.

Jung H, Jung Y, Kim SY, Kim DW, Park H: Postoperative stability following bilateral intraoral vertical ramus osteotomy based on amount of setback. *The British journal of oral & maxillofacial surgery* 51(8): 822-826, 2013.

Jung H, Jung Y, Park H: The chronologic prevalence of temporomandibular joint disorders associated with bilateral intraoral vertical ramus osteotomy. *Journal of oral and maxillofacial surgery* 67(4): 797-803, 2009.

Jung H, Jung Y, Park JH, Park H: Recovery pattern of mandibular movement by active physical therapy after bilateral transoral vertical ramus osteotomy. *Journal of oral and maxillofacial surgery* 70(7): e431-437, 2012.

Kon T, Cho TJ, Aizawa T, Yamazaki M, Nooh N, Graves D, et al.: Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing. *Journal of bone and mineral research* 16(6): 1004-1014, 2001.

Lew KK, Foong WC, Loh E: Malocclusion prevalence in an ethnic Chinese population. *Australian Dental Journal* 38(6): 442-449, 1993.

Lieberman JR, Daluiski A, Einhorn TA: The role of growth factors in the repair of bone. Biology and clinical applications. *Journal of bone and joint surgery American volume* 84-A(6): 1032-1044, 2002.

Nordin T, Nyström E, Rosenquist J, Astrand P: Extraoral or intraoral approach in the oblique sliding osteotomy of the mandibular rami? Clinical experience and results. *Journal of cranio-maxillo-facial surgery* 15(5): 233-237, 1987.

Reddi AH: Bone morphogenetic proteins: from basic science to clinical applications. *Journal of bone and joint surgery American volume* 83-A Suppl 1(1): S1-6, 2001.

Sakou T: Bone morphogenetic proteins: from basic studies to clinical approaches. *Bone* 22(6): 591-603, 1998.

Sandberg MM, Aro HT, Vuorio EI: Gene expression during bone repair. *Clin Orthop Relat Res.* (289): 292-312., 1993.

Sato M, Ochi T, Nakase T, Hirota S, Kitamura Y, Nomura S, et al.: Mechanical tension-stress induces expression of bone morphogenetic protein (BMP)-2 and BMP-4, but not BMP-6, BMP-7, and GDF-5 mRNA, during distraction osteogenesis. *Journal of bone and mineral research* 14(7): 1084-1095, 1999.

Storum KA, Bell WH: The effect of physical rehabilitation on mandibular function after ramus osteotomies. *Journal of oral and maxillofacial surgery* 44(2): 94-99, 1986.

Tornes K, Gilhuus Moe OT: The surgical technique of vertical subcondylar osteotomy for correction of mandibular prognathism. A 10-year survey. *Acta odontologica scandinavica* 45(3): 203-211, 1987.

Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L, et al.: BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. *Nature Genetics* 38(12): 1424-1429, 2006.

Xiong D, Shen H, Zhao L, Xiao P, Yang T, Wang W, et al.: Robust and comprehensive analysis of 20 osteoporosis candidate genes by very high-density single-nucleotide polymorphism screen among 405 white nuclear families identified significant association and gene-gene interaction. *Journal of bone and mineral research* 21(11): 1678-1695, 2006.

Yazawa M, Kishi K, Nakajima H, Nakajima T: Expression of bone morphogenetic proteins during mandibular distraction osteogenesis in rabbits. *Journal of oral and maxillofacial surgery* 61(5): 587-592, 2003.

Ye S, Eriksson P, Hamsten A, Kurkinen M, Humphries SE, Henney AM: Progression of coronary atherosclerosis is associated with a common genetic variant of the human stromelysin-1 promoter which results in reduced gene expression. *Journal of biological chemistry* 271(22): 13055-13060, 1996.

ABSTRACT (IN KOREAN)

비고정성 하악골 상행지 수직 골절단술에서의 피질골 간 접촉 치유에 관한 면역형광염색 연구

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정 휘 동

하악골 상행지 수직 골절단술 (Vertical ramus osteotomy, VRO)은 Sigmoid notch로부터 상행지 후하방까지 수직 골절단술을 시행하는 술식으로 하악골 후퇴에 주로 이용되는 악교정 수술 기법이다. VRO 시행 후 근심골편은 원심 골편의 외측에 위치되어 피질골간 접촉이 형성되며, 골편간 고정이 시행되지 않고 능동적 움직임이 허용되는 독특한 환경에서 치유가 진행된다. 본 연구의 목적은 VRO 후 치유과정에서 나타나는 특정 cytoknoe의 변화 양상을 면역형광염색을 통해 관찰하고, 비골골절에서 VRO와 동일한 비고정, 피질골간 접촉 조건 하의 치유과정과의 차이를 규명하고자 함이다. 총 4 마리의 비글견을 이용하여 연구를 진행하였으며 각각의 개체에서 구외접근을 통한 VRO와 비골 골절 후 각각 1, 2, 4, 8 주에 희생하여 조직을 적출하였다. 각각의 시기에 cytokine의 발현을 확인하기 위하여 6개의 특정 항체를 이용한 면역형광염색을 시행하였다: Bone morphometric protein-2/4, -7, (BMP-2/4, -7) matrix

metalloproteinase-3 (MMP-3), transforming growth factor-beta 2, 3 (TGF-ß2, -ß3), and vascular endothelial growth factor (VEGF). 면역형광염색 결과는 Leica application을 이용하여 positive pixel counting을 시행하여 발현 정도를 평가하였다.

상기 연구 과정을 통하여 아래와 같은 결과를 얻었다.

1. VRO와 비골골절의 치유 양상은 HE 염색상 유사한 과정을 보였다. 염증세포 침윤 및 피질골의 파괴가 1주 경과 시 관찰되었으며, 연골형성이 2주에 관찰되었고, 연골 흡수, 일차 골형성, 혈관형성이 4주, 8주에서 관찰되었다.

2. BMP-2/4의 발현은 양측 모두에서 모든 시기에 나타났다. VRO의 경우 가장 강한 발현은 술후 2주에 나타났으며, 비골골절에서는 비교적 일정한 발현이 지속되는 양상으로 관찰되었다.

3. BMP-7의 발현은 양측에서 다른 양상으로 관찰되었다. VRO의 경우 술후 1주에 가장 강한 발현을 보이고 이후 점차 감소하는 양상을 보였으며, 비골골절에서는 비교적 일정한 정도의 발현이 지속되는 양상이 관찰되었다. VRO 후 허용되는 능동적 움직임이 BMP-7의 발현 감소의 원인으로 생각되었다.

4. TGF-β2의 발현은 양측에서 다른 양상으로 관찰되었다. VRO의 경우 점차 발현이 증가하는 양상을 보였으나, 비골골절에서는 특정한 패턴을 관찰할 수 없었다. VRO 후 발현이 증가하는 이유는 능동적 움직임에 기인한 것으로 판단되며, 시간이 경과할수록 골재형성이 가속화되는 것으로 판단되었다.

5. TGF-β3의 경우 VRO 에서는 1주, 4주의 시기에 가장 강한 발현을 보였으며, 비골 골절의 경우 1주, 8주의 시기에 가장 강한 발현을 나타냈다. 1주의 강한 발현

은 혈소판 탈과립화에 의한 것으로 판단되며, 4주 이후의 강한 발현은 골 재형성화에 기인한 것으로 생각되었다.

6. VEGF의 발현은 양측에서 다른 양상으로 관찰되었다. VRO에서는 1주에 강한 발현을 보였고 이후 감소된 채 유지되는 양상을 보였으며, 비골골절에서는 4주와 8주에 발현이 증가하는 양상을 보였다. 이는 해부학적으로 하악골 상행지의 혈행이 상대적으로 양호하여 술 후 1주를 제외하고 혈관 신생의 요구도가 낮고, 비골에서는 혈행이 상대적으로 불량하여 4주 이후 혈관 신생의 요구도가 높았기 때문으로 판단된다.

이상의 결과를 바탕으로 VRO와 비골골절 후 HE 염색상 각각의 시기에서 양측의 차이는 관찰되지 않음을 확인할 수 있었으며, 각각의 시기에 특정 cytokine의 발현은 차이를 보임을 확인할 수 있었다. BMP-7의 감소경향은 VRO후 허용되는 능동적 움직임에 기인한 독특한 양상으로 판단되었으며, TGF-β2의 증가경향은 VRO후 시간이 경과할수록 골재형성화가 가속화됨을 의미한다. 또한, VRO에서 1주에서만 나타난 VEGF의 강한 발현은 하악골 상행지의 혈행이 비골보다 유리했기 때문으로 판단되었다.

핵심되는 말: 상행지 수직 골절단술, 비골 골절, 골치유, TGF-beta