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Abstract: Background: Mandibular bone regeneration is stepped up by human recombinant bone morphogenetic protein-2 (BMP-2) whose application is also related to limited cementum and periodontal ligament regeneration, local root resorption, and ankylosis. The alveolar bone grafting without traditional autologous bone grafts, remains a challenge for plastic surgeons.
Method: Bilateral mandibular alveolar and periodontal defects were created over the premolar areas in nine mature male beagles. The defects were randomly assigned for either the advBMP-2 group with BMP-2-expressing mesenchymal stem cells (MSCs) or the control with MSCs alone. The regenerated periodontal attachment apparatus was evaluated histologically and the whole regenerated bone volume was scrutinized from three-dimensional computed tomography analysis.
Results: Periodontal apparatus regeneration was significantly better in the advBMP-2 group. New cementum and Sharpey's fibers were observed on the denuded root surfaces in the advBMP-2 group, whereas incomplete healing with localized root surface resorption was noted in the control group. Eight weeks post implantation, the advBMP-2 group showed significant increase in the bone regeneration than the control one.
Conclusion: Thus, the use of ex vivo BMP-2-engineered autologous MSCs boosted bone and periodontal apparatus regeneration in mandibular periodontal defects. This de novo approach might be suitable for clinical mandibular bone repair and periodontal apparatus repair.

Mandibular alveolar bony defect repair using *BMP-2*-expressing autologous mesenchymal stem cells

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Running Title: Alveolar bony defect repair by autologous mesenchymal stem cells

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ABSTRACT

Background: Mandibular bone regeneration is stepped up by human recombinant bone morphogenetic protein-2 (BMP-2) whose application is also related to limited cementum and periodontal ligament regeneration, local root resorption, and ankylosis. The alveolar bone grafting without traditional autologous bone grafts, remains a challenge for plastic surgeons.

Method: Bilateral mandibular alveolar and periodontal defects were created over the premolar areas in nine mature male beagles. The defects were randomly assigned for either the *adv*BMP-2 group with BMP-2-expressing mesenchymal stem cells (MSCs) or the control with MSCs alone. The regenerated periodontal attachment apparatus was evaluated histologically and the whole regenerated bone volume was scrutinized from three-dimensional computed tomography analysis.

Results: Periodontal apparatus regeneration was significantly better in the *adv*BMP-2 group. New cementum and Sharpey's fibers were observed on the denuded root surfaces in the *adv*BMP-2 group, whereas incomplete healing with localized root surface resorption was noted in the control group. Eight weeks post implantation, the *adv*BMP-2 group showed significant increase in the bone regeneration than the control one.

Conclusion: Thus, the use of *ex vivo BMP-2*-engineered autologous MSCs boosted bone and periodontal apparatus regeneration in mandibular periodontal defects. This *de novo* approach might be suitable for clinical mandibular bone repair and periodontal apparatus repair.

KEY WORDS: Periodontal regeneration; tissue engineering; *ex vivo* gene therapy; bone

morphogenetic protein; mesenchymal stem cells; root ankylosis; root reporption, Sharpey's fiber; bone marrow stem cells; regional cell therapy.

INTRODUCTION

The human recombinant bone morphogenetic protein-2 (*rh*BMP-2) in resorbable collagen sponge (Medtronic Inc.) has been used in an off-label fashion in human alveolar and craniofacial repair for some years. The short term results seem encouraging. However the definite conclusion for the possibility to replace the tradition alveolar bone grafting has not been established at this period of time. In order to restore the tooth-bearing mandibular bone and healthy periodontal apparatus, new strategy is attempted in this study.

A major goal of periodontal therapy is predictable regeneration of the periodontal apparatus. Periodontal apparatus regeneration is more complicated than the simple bone regeneration due to the participation of different cell populations; such as cementogenesis is crucial to periodontal regeneration and requires the action of both cementoblasts and epithelial cells. The appliance of bone morphogenetic proteins (BMPs; members of the TGFβ superfamily) to periodontal defects were demonstrated to boost the regeneration of osseous and periodontal tissue, including cementum and periodontal ligament fibers.[1] Two studies revealed the limitations of this approach by pointing out the abnormal periodontal relationship and risks of complications such as ankylosis and root resorption.[2, 3] The extent of apparatus regeneration and the occurrence of root resorption were stated to positively correlate with higher dosages of recombinant human BMP-2 (rhBMP-2).[4] Slower release and more prolonged exposure to rhBMP-2 increased cementogenesis,[5] but supraphysiologic concentrations were required to surmount the short half-life of the protein.[6]

A gene therapy approach allows for the prolonged delivery of desired signals.

Contrary to protein therapy (rhBMP-2) whose effect was limited by protein degradation and inactivation, *ex vivo* BMP-2 gene transfer to cells used for implantation can achieve sustained release of BMP-2 and promote more complete repair of bony lesions.[7] The use of viral vectors to introduce the BMP-2 gene into bone marrow mesenchymal stem cells (MSCs) provides an efficient way to attain prolonged delivery of BMP-2 *in vivo*.[8, 9] Indeed, the continuous release of BMP-2 attained by the gene therapy approach decreased the necessitated *in vivo* BMP-2 concentration by approximately 1000-fold.[10]

The application of MSCs to periodontal regeneration was attested to boost cementum, periodontal ligament, and alveolar bone regeneration.[11] In previous work, we demonstrated bone formation in critical-size craniofacial bone defects followed the application of MSCs modified by adenovirus BMP-2 gene transfer.[12, 13] Continuous secretion of BMP-2 protein over several weeks granted more physiologically relevant dosage and accomplished more comprehensive bone regeneration than MSCs alone throughout the same healing period.

Pluronic F127 (PF127) is a polyethylene oxide and polypropylene oxide copolymer that presents in a liquid state at cold temperatures and solidifies into gel at physiologic temperatures. PF127 breaks up slowly and is cleared by renal and biliary excretion. It has been used as a delivery material for graft as well as for antibiotic, anti-inflammatory, and antineoplastic agents. PF127 significantly increases the rate of wound and burn healing and has been utilized in other tissue engineering applications.[14] In an autologous swine model comparing a variety of biodegradable biomaterials, PF127 motivated the least reactive inflammatory reaction.[15] PF127 was established beneficial in early postsurgical wound healing by facilitating early attachment and enhancing the growth rate of human

gingival fibroblasts.[16] It also enabled the tissue engineering of autologous chondrocytes and formed elastic cartilage of useful quality.[15, 17] From our previous experiments, PF127 was an ideal carrier for engineered mesenchymal stem cells in maxillary defects in rabbits.[18]

Based on the properties of PF127 and those of BMP-2-expressing MSCs, the purpose is to evaluate the regeneration of osseous tissue and periodontal apparatus in surgically created mandibular transgingival periodontal defects after applying *ex vivo* BMP-2-engineered autologous MSCs with PF127 as a scaffold.

MATERIALS AND METHODS

Nine healthy male adult beagles (9.8–13 kg body weight), obtained from a breeder, were used in this study. All animal procedures were conducted in accordance with the guidelines of the Animal Care and Use Committee of Chang Gung Memorial Hospital, Taipei, Taiwan.

Construction of recombinant adenovirus

The E1-deleted and replication-defective adenovirus BMP-2 (*adv*BMP-2) vector used in this study has been described elsewhere.[8]

Scaffold preparation

A 25% (w/v) solution of PF127 (BASF, Mt. Olive, NJ, USA) powder in Dulbecco's Modified Eagle Medium (DMEM) was mixed overnight at 4℃. After filtering, this solution was kept in a liquid state at 4℃ before mixing with cells.

Bone marrow aspiration and adenoviral infection

For bone marrow aspiration, all dogs were anesthetized by intramuscular injection of 2% Rompum (1 ml/10 kg, Bayer, USA) and ketamine (4 ml/10 kg of 50 mg/ml solution, Yung Shin Pharmaceutical Industrial Co., Taiwan). MSCs were cultured *in vitro* as previously described.[13] MSCs were grown in DMEM with 10% FBS to 90% confluence in a roller bottle. MSCs were infected with *adv*BMP-2 seven days before creation of the periodontal alveolar defects. MSCs were infected at 37℃ overnight and cultured in complete medium for another six days. Before implantation, cell density was determined

using a hemocytometer. MSCs were mixed with PF127 at a concentration of 50×10^6 cells/ml; the mixture took 10 minutes to solidify into gel form in an incubator.

Western blot detection of adenoviral BMP-2 expression in MSCs

The cells were infected with 20 ml of *adv*BMP-2 working stock (10^7 PFU/ml) at a multiplicity of infection of 50 for 12 hours. The infected cells were cultured in DMEM with 10% FBS. The medium (25 ml) was collected six days later and rhBMP2 was immuno- precipitated with 10 μl of goat anti-human BMP-2 antibody (#AF355; R&D System, Inc., Minneapolis, MN, USA) at 4℃ for 2-3 hr. Subsequently, 50 μl of protein A Sepharose (Amersham Pharmacia Biotech., Piscataway, NJ, USA) was included and the mixture was rotated overnight at 4℃. The medium was then centrifuged at 1500 rpm for 5 min and the pellet (precipitated immune complexes) was assembled.

The precipitated immune complexes (antigen-Ab-Sepharose complex) were resuspended in 50 μl of 2× sample buffer, boiled for 5 min, resolved by 12.5% sodium dodecyl sulfate -polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride transfer membrane (Millipore Corporation, Bedford, MA, USA). The membrane was blocked with 5% BSA solution at room temperature for 1 hr and then exposed to goat anti-human BMP-2 in 2% BSA at 4℃ overnight. Blots were reacted with horseradish peroxidase-conjugated donkey anti-goat IgG (#HAF109; R&D System, Inc.) in 2 % BSA for 1 hr and then developed with enhanced chemiluminescence (Amersham Pharmacia Biotech).

The animal model

Surgery took place under general anesthesia. An incision was made along the palatal gingival sulcus and a full thickness gingival periosteal flap was reflected. Bilateral mandibular transgingival periodontal defects were created over the premolar area and the roots were denuded 4 mm below the cementoenamel junction (CEJ) in nine adult male beagles (Fig. 1A-C). The eighteen periodontal defects were randomly assigned into two groups. Group 1 was treated with *adv*BMP-2-infected MSC/PF127 (*adv*BMP-2 group) and Group 2 was the control group treated with MSC/PF127 (MSC group). Each defect was filled with 3 ml of cell/PF127 construct and parental antibiotic injections were performed for 3 days to prevent wound infection. The surgical wounds were closed in a watertight manner (Fig. 1D). All dogs were sacrificed 8 weeks after surgical implantation of the regenerative materials.

Histological examination:

Harvested samples were divided into two pieces. Both samples were fixed in buffered 10% formalin for 72 hours. One of the two fixed specimens was decalcified in Decalcifier I solution (Surgipath, Northbrook, IL, USA) for 48 hours. After cutting through the middle of the incisor in the sagittal direction mesiodistally, the specimens were embedded, sliced, and stained with hematoxylin and eosin (HE) and trichrome.

Three-dimensional computer tomography

Three-dimensional helical scanning computerized tomography (3D-CT) of the dogs' heads was performed immediately after surgery and after sacrifice. Primary data manipulation at 1-mm thickness was performed in the Radiology Department and the raw data were transferred to an IBM-compatible personal computer in the Medical Imaging

Laboratory. Reformation of the CT data was performed with the voxel unit set equal to 0.27 mm^3.[19] The volume of regenerated bone was measured. The CT data reformation, image display, and volume measurements were performed using Analyze 5.0 software (Biomedical Imaging Resource, Mayo Foundation, Rochester, Minnesota, USA).

RESULTS

Human recombinant BMP-2 expression

Western immunoanalysis showed that MSCs in the *adv*BMP2 group expressed and processed hrBMP-2 gene as expected. The molecular weight of the secreted BMP-2 indicated a mature protein with no pre-peptide forms (Fig. 2).

Gross observations

There were no adverse reactions or infections during the 8-week healing interval. All defects healed uneventfully with limited signs of inflammation. Eight weeks after surgery, different periodontal apparatus regeneration patterns were observed between the two groups. No evidence of residual PF127 or ankylosis was observed in any specimens.

Histological evaluation

Considerably more bone was formed in the *adv*BMP-2 group than in the MSC group, extending from the apical aspect of the defect through the coronal extension (Fig. 3A-C). Connective attachments were also present (Fig. 3A), and bone and cementum had regenerated in the furcation defect. Sharpey's fiber was regenerated and local root surface resorption was apparent (Fig. 3B). Newly formed periodontal ligament fibers were functionally oriented and new connective tissue fibers inserted into both the new cementum and the new bone (Fig. 3B). The sparse stroma and fibrovascular space was dense with cells and consisted of scattered fine bundles of collagen fibrils (Fig. 3B).The newly formed trabecular bone was covered with a thick layer of osteoid and osteoblasts, with evidence of continual bone-forming activity (Fig. 3C). Narrow marrow space was

evenly dispersed through the bone trabeculae (Fig. 3B). The blend of woven and lamellar bone in the central part of the new bone appeared physiologically mature (Fig. 3C).

In the MSC group, only small amounts of immature bone were formed. The new bone contained some fatty marrow space and extended from the apical aspect of the defect to the middle of the root (Fig. 3D-F). No cementum and limited bone regeneration were visible in the furcation defect (Fig. 3D, F). Moderate downgrowth of junction epithelium and connective fibrous tissue repair were also observed; long junctional epithelium attachments were formed in some specimens (Fig. 3E).

Three dimensional tomographic image

Figure 4 displays 3D CT images of the transgingival periodontal alveolar bony defects before and after implantation and healing. A greater degree of bone regeneration was observed in the *adv*BMP-2 group after eight weeks *in vivo* (Fig. 4D). The total volume of bone regenerated in the defects was significantly greater in the *adv*BMP-2 group than in the MSC group: 578.69 ± 68.13 vs. 282.73 ± 36.68 mm^3, respectively ($p<0.01$).

DISCUSSION

Using an *ex vivo* approach, adenovirus-mediated BMP-2 infection of MSCs has been used to successfully regenerate bone in different bony defects with different scaffolds.[12, 13] Space provision has been suggested to be an essential factor for regenerating alveolar bone and cementum in periodontal defects.[20] Because of the reverse thermo-sensitive properties of PF127, the mixture and delivery of the MSC/PF127 gel can be performed easily using an 18-gauge needle,[15, 17] making it a very convenient method for implantation into periodontal defects. PF127 has been suggested to improve early wound healing in third-degree burns and osteogenesis[21] and has been considered as a carrier for osseous graft materials and growth factors.[22] PF127 may benefit early wound healing by facilitating the spread, attachment, and growth of human gingival fibroblasts.[16] This biomaterial has already proved to enhance transgene expression and to be a suitable carrier for regional gene therapy.[14] In our study, PF127 proved to be suitable for use as a periodontal regeneration scaffold.

The *adv*BMP-2-infected autologous MSC/PF127 mixture offers an alternative and superior approach for the delivery of growth factors for periodontal apparatus regeneration. The release of BMP-2 protein can stimulate transgingival periodontal defect regeneration, including the regeneration of bone, periodontal ligaments, and cementum. Larger volumes of bone were regenerated in the *adv*BMP-2 group than in the MSC group without the complications of root ankylosis and resorption. New cementum and functional periodontal fibers (Sharpey's fibers) were also formed. Our results support the application of this approach for periodontal regeneration and achieving healthy periodontal relationship. Additional studies are required to optimize the properties of the

3D scaffold and to determine the feasibility of applying BMP-2-engineered autologous MSCs in a more relevant, larger animal model with kinetic healing profiles more similar to those of human beings.

Osteogenesis and cementogenesis are the main concerns of periodontal regeneration. The success of periodontal regeneration is based on the early induction of cementogenesis with rapid colonization and extracellular matrix synthesis by cementoblasts along the root surface, followed by the morphogenesis of Sharpey's fibers inserting into the newly formed cementum.[4] In our study, histological analysis showed that extensive root resorption occurred in the absence of cementum coverage, whereas limited root resorption occurred when new cementum and fibers were present. It has been reported that root resorption occurs prior to the formation of new cementum, indicating an interaction between cells and dental substrate.[23] These observations suggest that the rate of cementogenesis is the most important aspect of effective periodontal regeneration. This hypothesis is supported by the observation that dental tissue (dentin, cementum) can promote differentiation of cementoblast-precursors within the bone cell population.[24]

The similar staining patterns of osteoblasts and cementoblasts associated with the cementum indicate that they are phenotypically similar and may derive from a common osteoprogenitor cell.[25-27] It was hypothesized that reparative cementum is derived from osteoblasts and that both these osteoblasts and their precursors might be targets for BMP-2.[28] Indeed, evidence suggests that BMP-2 may induce new cementum formation in addition to new bone formation.[26] A recent study showed that early treatment with BMP-2 promoted cell recruitment and resulted in a nearly three-fold increase in cementogenesis as compared with controls.[29] It has been suggested that prolonged

exposure and slower release of BMP-2 can increase cementogenesis;[30, 31] as shown here, *ex vivo* gene transfer of BMP-s to MSCs can achieve sustained release of BMP-2 and promote more effective cementogenesis.

Wound healing during periodontal regenerative therapy has been conceptualized as a race between migrating epithelium and connective tissue. It has been suggested that TFG-β can inhibit epithelial downgrowth.[30] The marked effect of BMP-2 is its ability to induce differentiation of mesenchymal cells. Connective tissue formation may play a significant role in the early stage of periodontal repair, and these processes were suggested to inhibit epithelial downgrowth.[31] Each of these factors may have contributed to the more limited epithelial downgrowth observed in the *adv*BMP-2 group as compared to the MSC group.

The presence of the MSCs in the defect is a crucial aspect of the gene therapy approach to periodontal regeneration. The implantation of purified MSCs has been proved effective in promoting healing of a critical-sized defect in the canine femur[32] and enhancing periodontal regeneration.[11] Our *ex vivo* approach provided an osteoinductive gene to the desired site and supplied MSCs that were capable of participating in the osteoinduction. MSCs have inherent osteogenic capacity and can respond to the BMP they have been engineered to secrete by differentiating into osteoprogenitor cells for osteogenesis and cementogenesis.

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ACKNOWLEDGMENTS

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FIGURE LEGENDS

FIGURE 1. Bilateral mandibular transgingival periodontal defects were created over the premolar area. (A) Before implantation. (B) Full thickness gingival periosteal flap was reflected with an incision along the palatal gingival sulcus. (C) Root denuded at 4 mm below cementoenamel junction. (D) The surgical wounds were closed in a watertight manner.

FIGURE 2. Western blot analysis of adenovirus BMP-2 expression by MSCs *in vitro*. Note the 18 kDa band in the *adv*BMP-2 group.

FIGURE 3. Photomicrographs comparing healing in the periodontal bony defect between the *adv*BMP-2 group (A, HE 40×; B, HE 100×; C, Trichrome 100×) and the MSC group (D, HE 40×; E, HE 100×; F, Trichrome 100×). d, Dentin; b, bone; e, epithelium. (A, B) Bone and cementum regenerated in the furcation defects in the *adv*BMP-2 group. (C) Sharpey's fiber was regenerated in the presence of local root surface resorption. (D, F) No cementum and limited immature bone regeneration in the furcation defect; the defect was filled only with PF127. (E) Epithelial downgrowth and connective fibrous tissue repair were the major findings.

FIGURE 4. The 3D CT image demonstrating the transgingival periodontal alveolar bony defects in the advBMP-2 group (B, D) and the MSC group (A, C). (A, B) The surfaces of the mandibular incisor roots were denuded. (C, D) There were different degrees of bone regeneration in after 8 weeks *in vivo*.

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Figure 01

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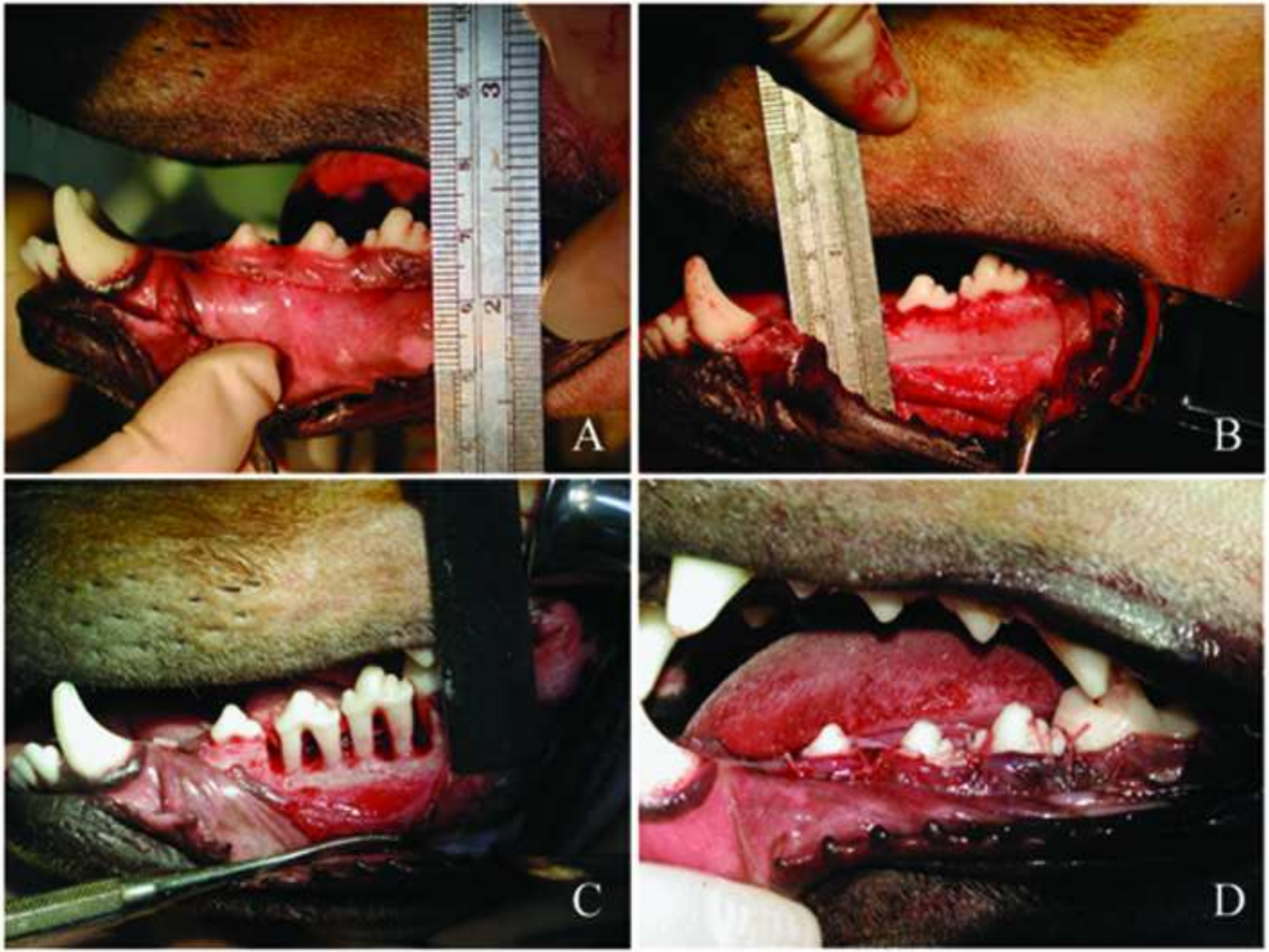

Figure 02

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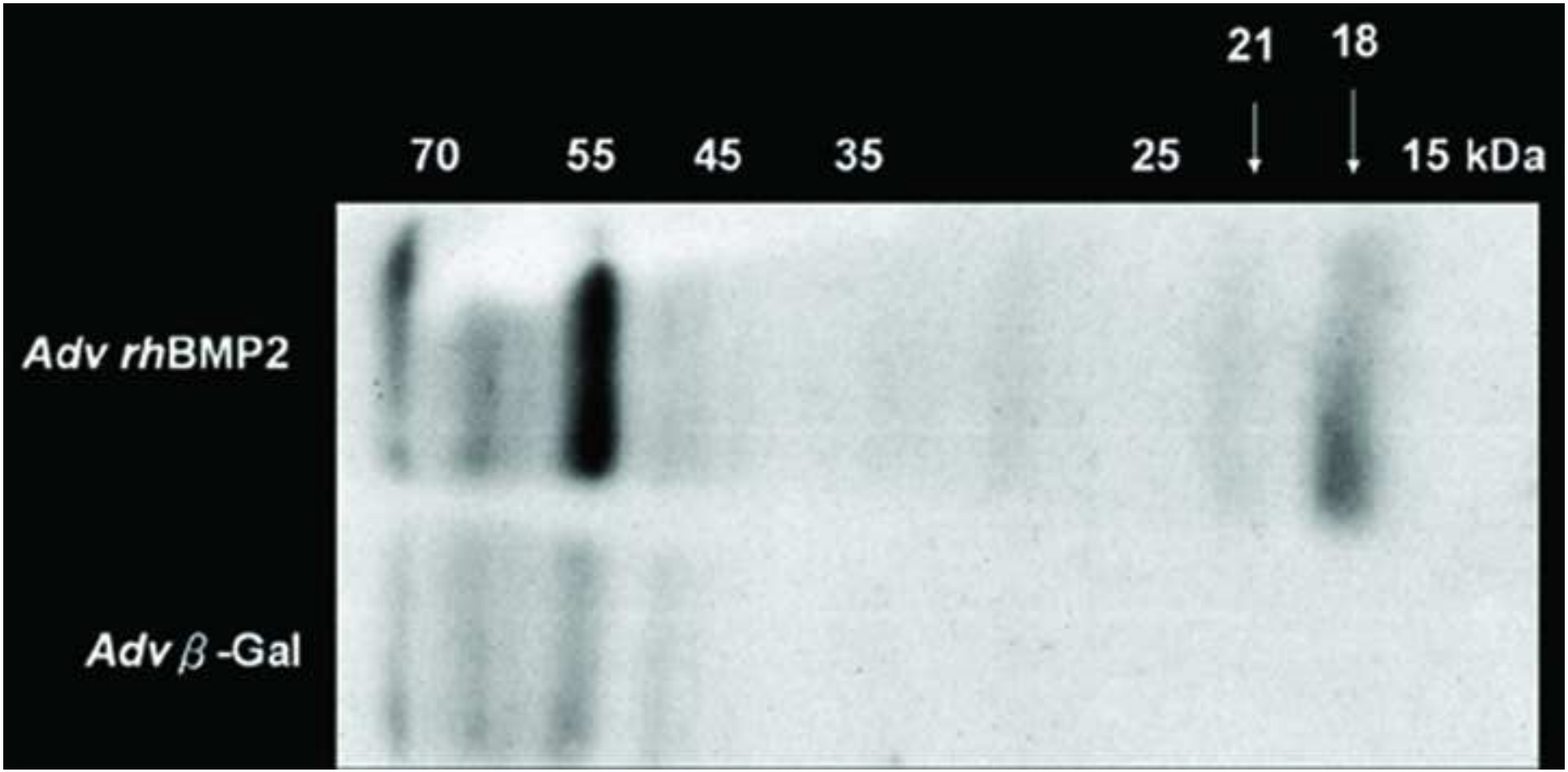

Figure 03

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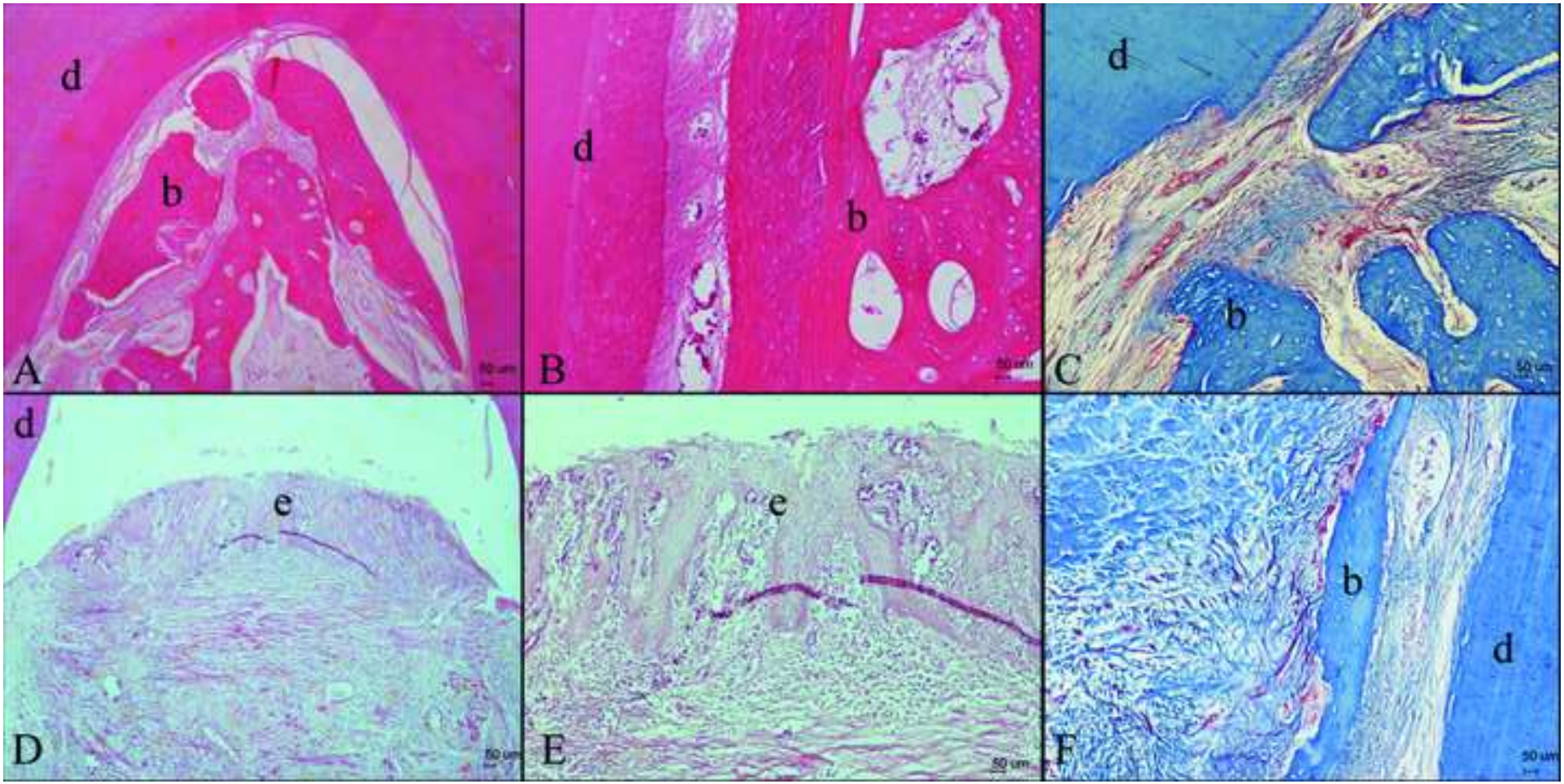

Figure 04

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