# Comparative histomorphologic study of regenerated bone for dental implant placement in the atrophied posterior maxilla

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**Abstract**

The purpose of this study is to evaluate the regenerative capacity of reconstruction in the atrophied posterior maxilla by comparing bone graft procedures and alveolar distraction osteogenesis (ADO) techniques.

We performed the autogenous iliac bone graft (AGB group, 5 specimens in 3 patients), and the combination (Mixed group, 3 specimens in 3 patients) of the autogenous and deproteinized bovine bone (Bio-Oss®, Geistlich Co., Switzerland) as the ratio of 2:1 in the sinus floor elevation procedures. ADO procedures using TRACK® (KLS Martin Co., Germany) were also performed to augment vertical alveolar height in atrophied posterior maxilla (ADO group, 5 specimens in 4 patients). Newly generated bone tissues were obtained with the 2.0mm diameter trephine bur (3i Co., USA) during implant fixture installation after 5-7 months. Routine histolomorphological observation, immunodot blot assay for quantitative evaluation, and immunohistochemical staining with antibodies to MMP-1, -9, -10, TIMP-1, -2, and BMP-2, -4 were all carried out.

Lamellar bone formation was well shown in all specimens and new bone formations of ADO group increased than those of other procedures. In immunohistochemical staining, the strong expression of BMP-2 was shown in all specimens, and immunodot blot assay showed that bone formation is accompanied by the good induction of factors associated with angiogenesis and appeared more increased amount of osteogenic and angiogenic factors in ADO group.

ADO is the most effective technique for new bone formation compared to sinus floor elevation with autogenous or mixed bone graft in the atrophied posterior maxilla. In the quantitative immunodot blot assay, the regenerated bone after ADO showed more increased products of VEGF, BMP-2, PCNA and MMP-1 than those after the other procedures, and these findings were able to be confirmed by immunohistochemical stainings.

**Key words**

Alveolar distraction osteogenesis (ADO), Autogenous iliac bone graft, Deproteinized bovine bone, Sinus floor elevation, Immunodot blot assay, Immunohistochemical staining

## INTRODUCTION

Rehabilitation of partially or totally edentulous patients with dental implants has become common practice in the last decades with reliable long-term results. But insufficient bone height in the posterior maxillary region is one of the most frequent problems in dental implantation procedure. To solve this problem, various surgical procedures have been proposed, such as the use of pterygoid implants, inlay bone graft in the maxillary sinus, onlay bone graft, vertical guided bone regeneration, and alveolar distraction osteogenesis (ADO)[1,2]. Among these procedures, the inlay bone grafting in the maxillary sinus known as sinus lifting or sinus floor elevation has been the most widely used technique, recently[3,4].

Grafting materials are known to encourage new bone formation by many osteogenic processes. Autogenous bone is known to induce new bone through osteogenesis, while allogenic bone is thought to be osteoinductive due to the presence of growth factors. Autogenous iliac bone graft has been used as augmentation material with excellent result[5-9]. However, there are several shortcomings,

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such as donor site morbidity, prolonged healing period, and unpredictable resorption of the graft[10,11]. Xenografts, such as bovine bone mineral (BBM), and alloplastic substitutes encourage the apposition of new bone by osteoconduction, and Bio-Oss® (Geistlich Co., Switzerland) has been widely reported to be effective in producing an effective bone regenerative matrix[12,13].

ADO is a popular technique in the anterior and posterior mandible but is not a routine procedure in the atrophied posterior maxilla due to the difficulty in the vector control, inaccessibility, severe pneumatization, and very few documented known researches, etc[14,15]. But if several requirements are met, i.e. over 7 mm remaining alveolar bone height, no severe sinus disease, sufficient bone width and good accessibility, it seems that ADO can also be an acceptable bone augmentation technique in the atrophied posterior maxilla[15,16].

The purpose of this study is to evaluate the regenerative capacity of the augmentation procedure, comparing with sinus lifting procedure and ADO in the atrophied posterior maxilla, by the histomorphological and quantitative immunodot blot assay methods.

## MATERIALS AND METHODS

### 1. Patients and reconstructive procedures

Total 10 specimens from 10 patients aged between 34 and 68 years (mean 55.3 years) were used in this study. Three patients were reconstructed by sinus floor elevation with autogenous iliac bone graft (AGB [autogenous bone] group), another three patients were reconstructed by sinus floor elevation with mixed bone graft (Mixed [autogenous & deproteinized bovine bone] group), and remaining four patients were reconstructed by ADO procedure (ADO group) (Table 1).

The sinus floor elevation was carried out by the lateral window approach technique. Autogenous bone was harvested from anterior iliac crest, the mixed bone was made of mandibular symphyseal particularized bone and deproteinized bovine bone (Bio-Oss®, Geistlich Co., Switzerland) as a volume ratio of 6 to 4 or 7 to 3. Primary wound closure without using any membrane was performed with 4-0 Vicryl (Polyglactin 910, Johnson & Johnson Co., USA). Patients were examined next day and one week later, and stiches were removed in two weeks The grafted sinus was left to heal during 6 months.

ADO was performed with an intraoral incision on the alveolar crest with lateral releasing incisions. Careful subperiosteal buccal dissection was performed to obtain adequate visibility of the underlying bone, but mucoperiosteum of the palate was not dissected to preserve adequate blood supply to the osteotomized segment. After molding of the intraoral distractor Track® (KLS Martin Co., Germany) device, the bone segment was completely separated from the basal bone with reciprocating and oscillating saw. After completing osteotomy, distractor was adapted and fixated to both the basal bone and the osteotomized segment with microscrews. The osteotomized segment was immediately mobilized by activating the distractor to check the direction of distraction and freedom in movements. Finally, the

**Table 1.** Patient's data.

| | Patient | Age | Sex | Location | Specimen acquisition | Sinus disease |
|---|---|---|---|---|---|---|
| AGB | 1 | 57 | M | Both | 6M2W | Rt. Mucosal thickening |
| | 2 | 61 | M | Both | 6M | N/S |
| | 3 | 55 | M | Rt. | 6M2W | N/S |
| Mixed | 1 | 34 | M | Lt. | 7M2W | N/S |
| | 2 | 60 | M | Lt. | 7M | N/S |
| | 3 | 55 | M | Rt. | 6M3W | N/S |
| ADO | 1 | 68 | M | Lt. | 5M | N/S |
| | 2 | 49 | M | Lt. | 5M3W | Mucosal thickening |
| | 3 | 53 | M | Lt. | 5M | N/S |
| | 4 | 61 | M | Both | 5M2W | N/S |

AGB : Autogenous bone graft, Mixed : Autogenous & Deproteinized bovine bone, ADO : Alveolar Distraction Osteogenesis

osteotomized segment was repositioned at its initial position and the surgical access was sutured with 4-0 Vicryl. Antibiotics, non-steroidal analgesics, soft diet, and oral hygiene regimens were followed as the same protocol used in sinus floor elevation procedure. The activation of the distraction device was started after 5-7 days of the latency period and stiches were removed in 2 weeks. The distraction rate of 1mm per day was performed until the desired amount of distraction was obtained. The distractor was then maintained in position for 8 weeks to obtain the maturation of the neocallus formed between the basal bone and the distracted segment. After this consolidation period, the distractor was removed. After 4 weeks since the distractor was removed, the implant placement was performed.

Routine radiographic documentations such as panorama, CT, tomographs and intraoral radiographs, were obtained, immediately after the operation, at the end of the distraction procedure, at the time of the implant placement, at the time of prosthetic rehabilitation, and annually thereafter.

**2. Harvesting of the specimens**

All 10 specimens were harvested at the time of implantation about 5 to 7 months after reconstructive procedure. All patients consented these procedures with high interests to the bone regenerative capacity of their own, and these all clinical studies were also approved by Institutional Review Board (IRB) of Kangnung National University Dental Hospital. Under local anesthesia, adequate incisions were made, a full thickness flap was raised and mobilized for tension-free closure. Bone was inspected, and a trephine bur of 2mm diameter (3i Co., USA) was used to take the specimen from the reconstructed bone (Fig. 1-a, b).

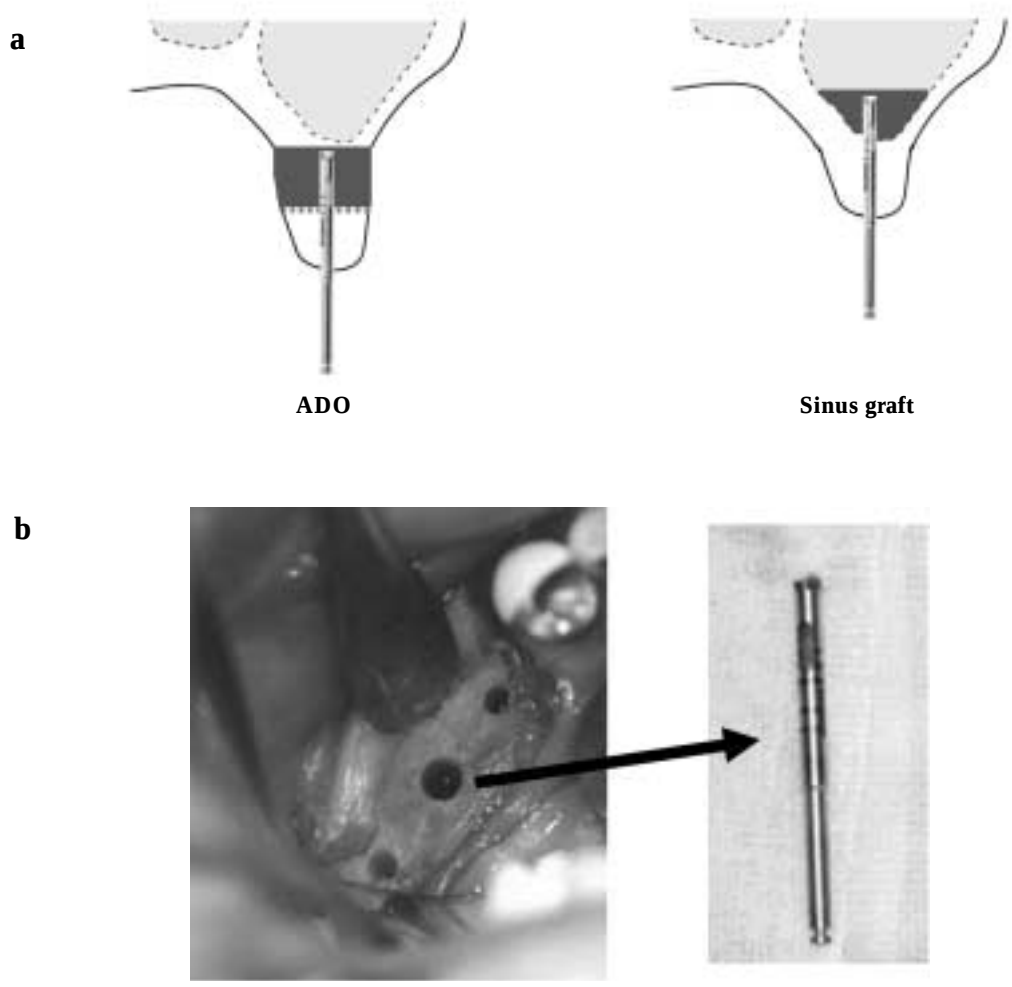



**Fig. 1**. a. Drawing of harvesting of specimen through crestal approach., b. Intraoperative figure of harvesting of specimen using trephine bur.

### 3. Histological procedures and assays

Specimens were immediately fixed in 10% NBF (Neutral Buffered Formalin), pH 7.4 and stored for 24 hour at 4℃. Specimens were then decalcified in 9% formic acid/formalin solution and dehydrated through graded ethanols, cleared in xylene, embedded in paraffin, cut into slices of about 4㎛ thickness and stained with hematoxylin and eosin (H&E), Masson Trichrome (MT), and immunohistochemical stainings of MMP-1, -9, -10, TIMP-1, -2, and BMP-2, -4. And the immunodot blot assay was performed for the quantification of the target mRNA especially by computer-assisted analysis of images formed by a microscope.

### 3-1. Immunohistochemical assay

The endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 minutes and washed 3 times with PBS (phosphate buffered saline). Antibodies diluted in DW (distilled water) 1:50 were added to the sections overnight at 4℃ and washed 3 times with PBS (Table 2). The secondary antibody solution was placed on the sections for 30 minutes and washed 3 times with PBS. Then, horseradish peroxidase-conjugated streptavidin-biotin complex was placed on the sections for 30 minutes and washed 3 times with PBS, followed by a peroxidase reaction using 3,3'-diaminobenzidine (DAB) as a chromogen. Finally, the sections were washed 3 times with PBS, dehydrated through graded ethanols, and mounted. Negative control of immunohistochemical study was also performed (Fig. 2-a to 2-c). Each slide was evaluated according to the intensity of positive immunostaining, which were graded as +++, ++, +, +/-, - , which mean strong, moderate, slight, rare and negative respectively. A 'rare' grade of +/- was defined to represent a focal or questionable weakly positive signal in the tissue sections.

**Table 2.** Antibodies used in this study.

| Name | Animal | Dilution | Type | Company |
|---|---|---|---|---|
| MMP-1 | Rabbit | 1:50 | Polyclonal | Neomarkers |
| MMP-9 | Rabbit | 1:50 | Monoclonal | Santa Cruz |
| MMP-10 | Mouse | 1:50 | Monoclonal | Neomarkers |
| TIMP-1 | Mouse | 1:50 | Monoclonal | Neomarkers |
| TIMP-2 | Mouse | 1:50 | Monoclonal | Neomarkers |
| BMP-2 | Goat | 1:50 | Monoclonal | Santa Cruz |
| BMP-4 | Mouse | 1:50 | Monoclonal | Santa Cruz |
| VEGF | Mouse | 1:50 | Monoclonal | R&D systems |
| PCNA | Mouse | 1:50 | Monoclonal | Dako |

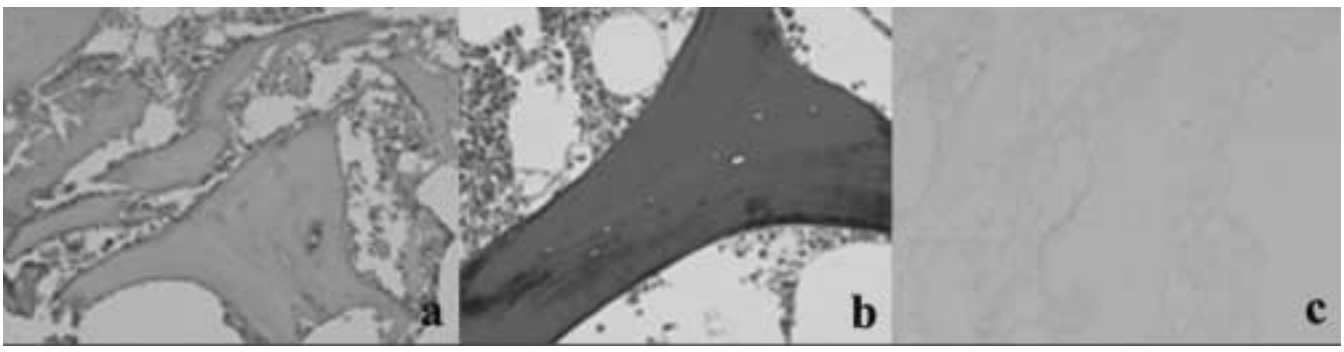



**Fig. 2.** Histologic assessement of negative control group (magnification, ×200).
a. H&E staining, b. MT staining, c. Immunohistochemical stainig

### 3-2. Immunodot blot assay

#### 3-2-1. mRNA extraction

Sections of the bony tissues with 10㎛ thickness were obtained and deparaffinized with xylene, followed by hydration with graded alcohols. After centrifugation, the tissue precipitates were lysed with lysis buffer (300㎕ SDS [sodium dodecyl sulfate] + 6M urea with PMSF [phenylmethylsulfonyl fluoride] and 0.1M DTT [dithiothreitol]). These mixtures were boiled for 3 minutes in 100℃, and then centrifuged (12,000 rpm, 20 minutes). Supernatants were kept in the - 80℃ freezer until use.

#### 3-2-2. Procedures of immunodot blot

Nitrocellulose transfer membrane (PROTRAN®, Schleicher&Schuell BioScience Co., Germany) was placed on the Bio-Dot® apparatus (Bio-Rad Co., USA) using vacuum suction. The experiment was performed, using 6 antibodies such as VEGF, BMP-2, PCNA, MMP-1, MMP-9, and TIMP-1. A small iliac bone fragment from one of the patients was used as the control group. About 1㎍ of the extracted protein was diluted with 100㎕ PBS and transferred on the membrane. The blotted membrane was cut in groups and fixed on the labeled slide glass. After 1 hour's blocking with skim milk solution in 1× TBST (Tris-buffered saline with 0.1% Tween 20), the membrane was incubated with each antibody for 1 hour using cover glass apparatus. And then membranes were washed with the 1x TBST and incubated in secondary antibody solution. Biotinylated link, with membranes for 30 minutes was used. 1x TBST incubation wass performed for 30 minutes, and membranes were incubated with Streptavidine-HRP for 30 minutes, and 1x TBST incubation was done for 30 minutes. The color developer, DAB solution activated by $H_2O_2$, was applied to the membrane, and then the membrane was washed and dried out after the color appearance.

#### 3-2-3. Quantification and statistics

Membranes were scanned in 300 dpi (dots per inch) and saved in TIF (tagged image file) after changing to gray shade using Adobe photoshop program (Version 7.0, Adobe Co., USA). Images were analyzed using ImageQuant® (Version 5.2, Molecular Dynamics Co., USA) and Microsoft Excel (2002, Microsoft Co., USA). Comparisons in each group were done with ANOVA using SPSS (Version 12.0, SPSS Inc., USA) for Windows and mean values were followed by 95% confidence intervals. Correlation of each factors was analyzed by Pearson's correlation test ($p < 0.05$).

## RESULTS

### 1. Histomorphologic findings

#### 1-1. Sinus floor elevation with autogenous bone graft (AGB group)

In H&E staining, the new regenerated bone is observed and can not be easily distinguished from the natural bone in all cases (Fig. 3-a). In MT staining, the new bone is found on the periphery of old trabecular bone in most cases (Fig. 3-b).

#### 1-2. Sinus floor elevation with mixed bone graft (Mixed group)

In H&E and MT staining, newly formed trabecular bone is predominant as the form of lamellar type with some portions of Haversian canals, but is inferior to the autogenous bone group in the both bony quality and quantity aspects (Fig. 4-a, b).

#### 1-3. Alveolar distraction osteogenesis (ADO group)

In H&E and MT staining, well-formed new trabecular bone is seen in the distraction gap. Osteophytes, which are layers of osteoid tissue covered with active osteoblasts and typical forms of bony regeneration in ADO, are observed at the outer surface of the trabecular bone (Fig. 5-a, b).

### 2. Immunohistochemical staining

#### 2-1. Sinus floor elevation with autogenous bone graft (Table 3, Fig. 3-c to 3-i)

Expressions of MMP-1 and MMP-10 are unreliable and invalid, which means that the expression is positive or negative (Fig. 3-c, e), and the positive reaction of MMP-9 is not observed either (Fig. 3-d). Both TIMP-1 and TIMP-2 are not expressed in immunohistochemical staining, which is negative reaction (Fig. 3-f, g). The expression of BMP-2 is moderately positive, which were observed at the outer surface of the trabecular bone (Fig. 3-h). But the expression of BMP-4 is negative (Fig. 3-i).

#### 2-2. Sinus floor elevation with mixed bone graft (Fig. 4-c to 4-i)

The expression of MMP-1 is unreliable and invalid,

**Table 3.** Expressions of antibodies in immunohistochemical staining.

| | MMP-1 | MMP-9 | MMP-10 | TIMP-1 | TIMP-2 | BMP-2 | BMP-4 | Negative Control |
|---|---|---|---|---|---|---|---|---|
| AGB | +/- | - | +/- | - | - | ++ | - | - |
| Mixed | +/- | - | - | - | - | + | - | - |
| ADO | +/- | - | +/- | - | - | ++ | - | - |

- : negative, +/- : rare, + : slight, ++ : moderate, +++ : strong

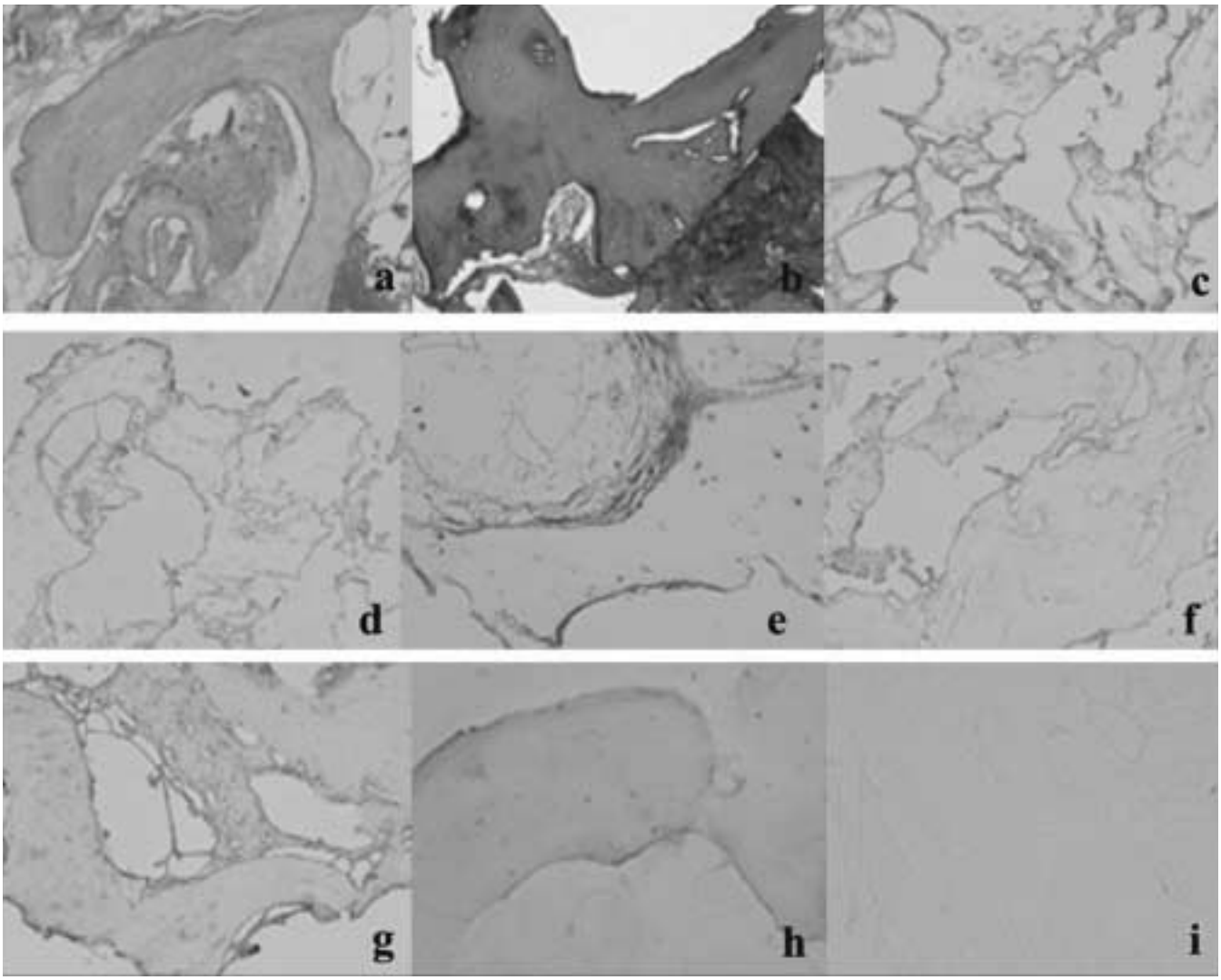



**Fig. 3.** Histologic assessement of autogenous bone graft group (magnification, ×200).
a. H&E staining
b. MT staining
c. Immunohistochemical stainig, MMP-1 (1:50, polyclonal Ab, Rabbit)
d. Immunohistochemical stainig, MMP-9 (1:50, monoclonal Ab, Rabbit)
e. Immunohistochemical stainig, MMP-10 (1:50, monoclonal Ab, Mouse)
f. Immunohistochemical stainig, TIMP-1 (1:50, monoclonal Ab, Mouse)
g. Immunohistochemical stainig, TIMP-2 (1:50, monoclonal Ab, Mouse)
h. Immunohistochemical stainig, BMP-2 (1:50, monoclonal Ab, Goat)
i. Immunohistochemical stainig, BMP-4 (1:50, monoclonal Ab, Mouse)

which means that the expression is positive or negative (Fig. 4-c), and the positive reactions of MMP-9 and MMP-10 are not observed (Fig. 4-d, e). Both TIMP-1 and TIMP-2 are not expressed in immunohistochemical staining, which is negative reaction (Fig. 4-f, g). The expression of BMP-2 is slightly positive, which were observed at the outer surface of the trabecular bone (Fig. 4-h). But the expression of BMP-4 is negative (Fig. 4-i).

**2-3. Alveolar distraction osteogenesis (Fig. 5-c to 5-i)**

The expressions of MMP-1 and MMP-10 are vague, which means that the expression is positive or negative (Fig. 5-c, e), and the positive reaction of MMP-9 is not observed (Fig. 5-d). Both TIMP-1 and TIMP-2 are not expressed in immunohistochemical staining, which is negative reaction (Fig. 5-f, g). The expression of BMP-2 is moderately positive, which were observed at the outer surface of the trabecular bone (Fig. 5-h). But the expression of BMP-4 is negative (Fig. 5-i).

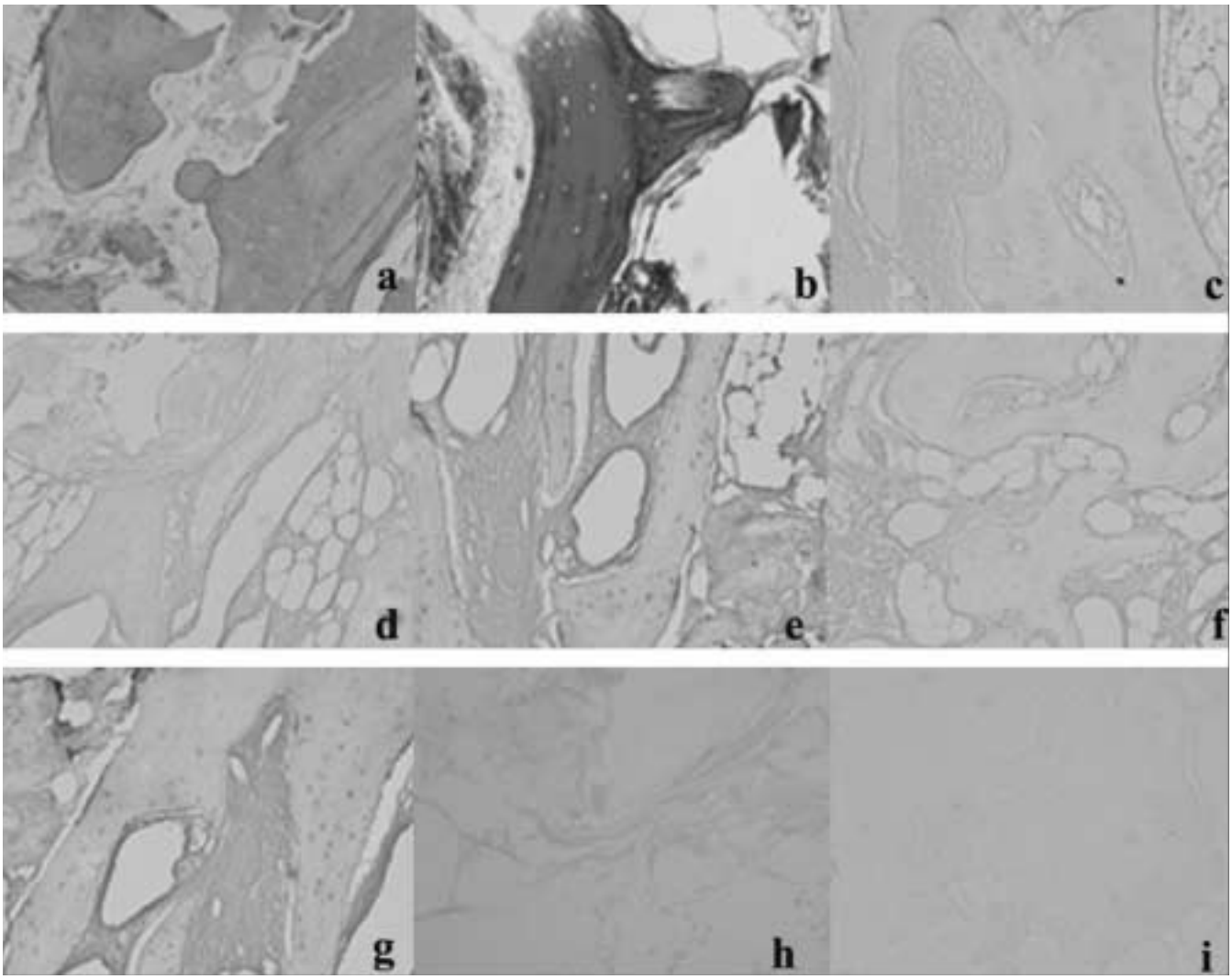



**Fig. 4.** Histologic assessement of mixed bone graft group (magnification, ×200).
a. H&E staining
b. MT staining
c. Immunohistochemical stainig, MMP-1 (1:50, polyclonal Ab, Rabbit)
d. Immunohistochemical stainig, MMP-9 (1:50, monoclonal Ab, Rabbit)
e. Immunohistochemical stainig, MMP-10 (1:50, monoclonal Ab, Mouse)
f. Immunohistochemical stainig, TIMP-1 (1:50, monoclonal Ab, Mouse)
g. Immunohistochemical stainig, TIMP-2 (1:50, monoclonal Ab, Mouse)
h. Immunohistochemical stainig, BMP-2 (1:50, monoclonal Ab, Goat)
i. Immunohistochemical stainig, BMP-4 (1:50, monoclonal Ab, Mouse)

**3. Immunodot blot assay (Fig. 6, 7)**

**3-1. Comparative quantification of VEGF**

ADO group showed high density and followed by AGB group, and then Mixed group. The difference of each group is all statistically significant ($p < 0.05$).

**3-2. Comparative quantification of BMP-2**

ADO group showed high density and followed by AGB group, and then Mixed group. The difference between ADO group and AGB group is statistically significant ($p < 0.05$), but the difference between AGB group and Mixed group is not significant ($p > 0.05$).

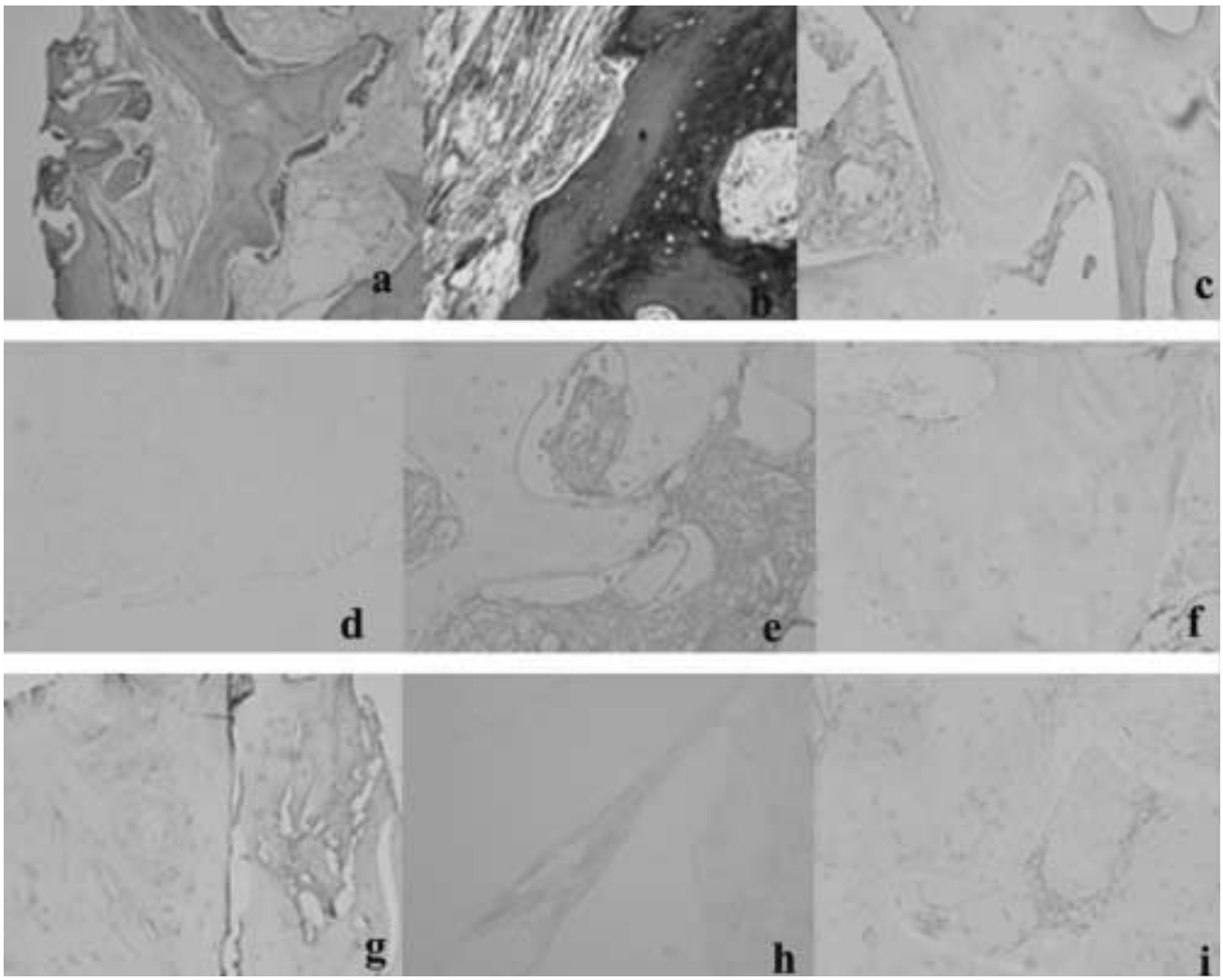



**Fig. 5.** Histologic assessement of ADO group (magnification, ×200).
a. H&E staining
b. MT staining
c. Immunohistochemical stainig, MMP-1 (1:50, polyclonal Ab, Rabbit)
d. Immunohistochemical stainig, MMP-9 (1:50, monoclonal Ab, Rabbit)
e. Immunohistochemical stainig, MMP-10 (1:50, monoclonal Ab, Mouse)
f. Immunohistochemical stainig, TIMP-1 (1:50, monoclonal Ab, Mouse)
g. Immunohistochemical stainig, TIMP-2 (1:50, monoclonal Ab, Mouse)
h. Immunohistochemical stainig, BMP-2 (1:50, monoclonal Ab, Goat)
i. Immunohistochemical stainig, BMP-4 (1:50, monoclonal Ab, Mouse)

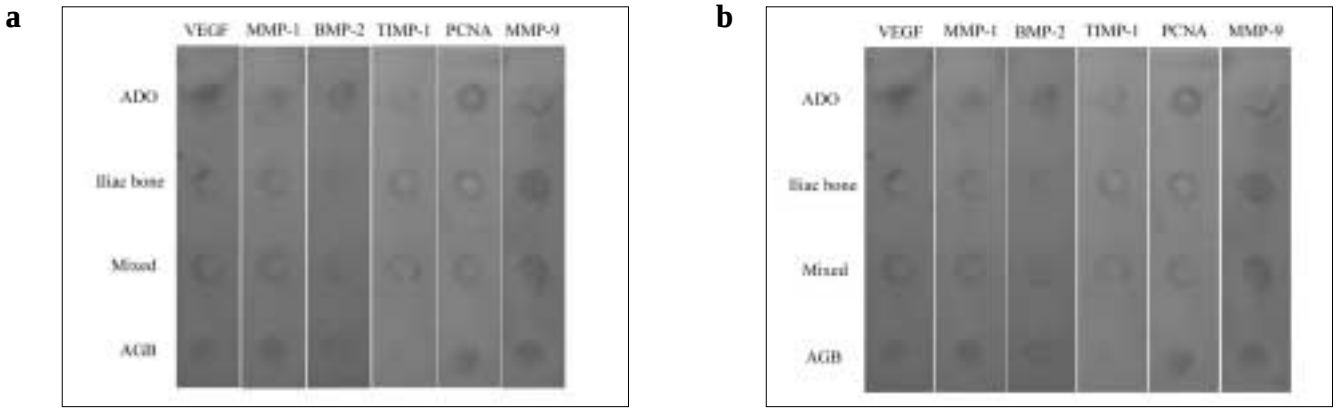
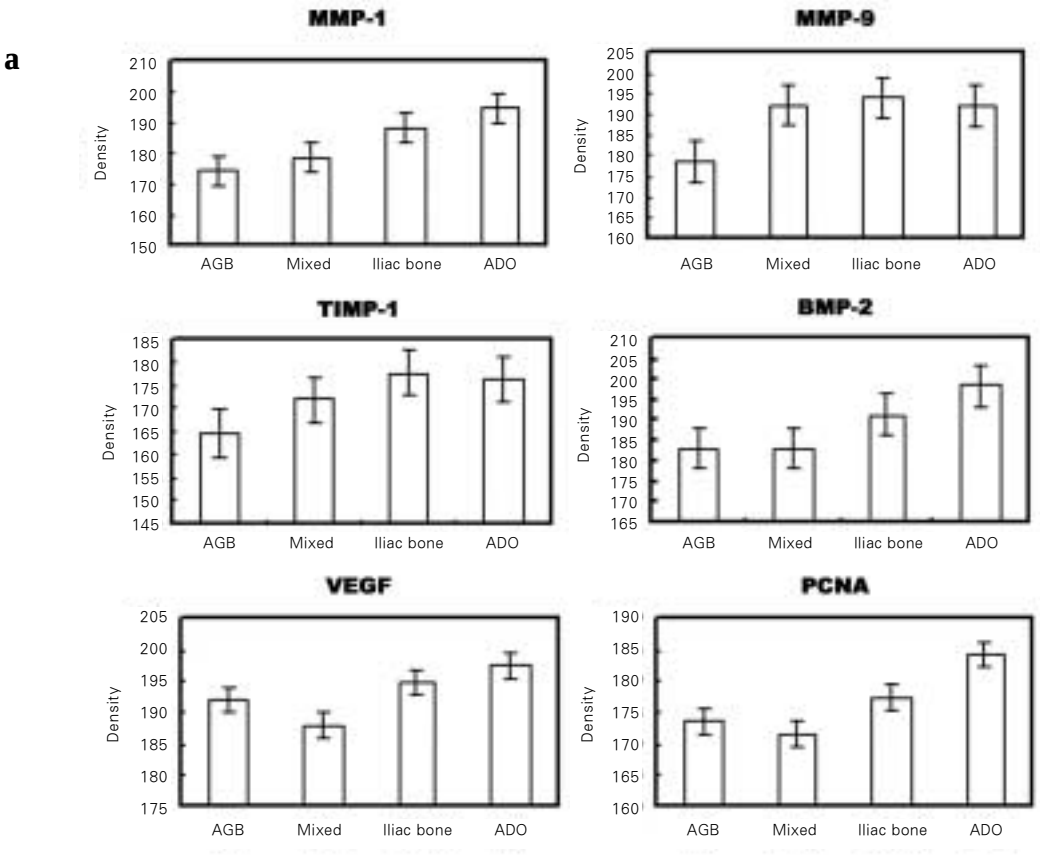



**Fig. 6.** a. Natural figure obtained after immunodot blot assay, b. Grayish figure for immunodot blot analysis.



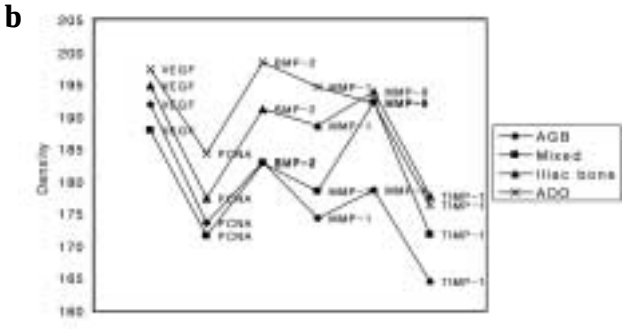




**Fig. 7.** a. Histogram of immunodot blot assessment.
b. Scatter diagram of correlations.

**3-3. Comparative quantification of MMP-1**

ADO group showed high density and followed by Mixed group, and then AGB group. The difference of each group is all statistically significant ($p < 0.05$).

**3-4. Comparative quantification of MMP-9**

Mixed group showed high density and followed by ADO group, and then AGB group. The difference between ADO group and AGB group is statistically significant ($p < 0.05$), but the difference between Mixed group and ADO group is not significant ($p > 0.05$).

**3-5. Comparative quantification of TIMP-1**

ADO group showed high density and followed by Mixed group, and then AGB group. The difference of each group is all statistically significant ($p < 0.05$).

**3-6. Comparative quantification of PCNA**

ADO group showed high density and followed by AGB group, and then Mixed group. The difference between ADO group and AGB group is statistically significant ($p < 0.05$), but the difference between AGB group and Mixed group is not significant ($p > 0.05$).

**3-7. Correlations of each group (Fig. 7-b)**

There are statistically significant correlations between all groups except for MMP-9 factor ($p < 0.05$).

## DISCUSSION

ADO is not a routine or promising reconstructive procedure in the atrophied posterior maxilla, compared with the sinus floor elevation with bone graft procedures. ADO on the edentulous posterior maxilla of Baboon was introduced in the Boyne' s article to know about the possibility to increase the height of atrophic posterior maxillary alveolar ridges and to determine if it is possible to distract the bony antral floor itself, producing new bone between the sinus membrane and the alveolar crest. Complete osseous regeneration of the nasal floor and alveolar ridge greater than 10mm in height was produced, and it was concluded that very small segments of bone of the posterior maxilla can be distracted to produce significant increases in the alveolar bone height and that a new osseous nasal-antral floor enhancement can be produced[17].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases capable of degrading connective tissues, and are involved in bony matrix degradation during osteogenesis and remodeling. Matrix metalloproteinases, together with their tissue inhibitors, are responsible for the controlled degradation of collagen and other matrix substrates in bone and other tissues and, in part, reflect the state of bony remodelling following mandibular lengthening by distraction osteogenesis. There are three groups of MMPs; Group I are the collagenases (MMP-1, 8, 13), which degrade interstitial collagens (types I-III); Group II are the gelatinases (MMP-2, 9), which degrade collagen type IV and gelatin; Group III are the stromelysins (MMP-3, 7, 10) which are more general proteinases and degrade proteoglycan, fibronectin, laminin, gelatin, casein and some collagens. The activity of MMPs is regulated by tissue inhibitors of matrix metalloproteinases (TIMPs). TIMPs inactivate MMPs by forming non-covalent bimolecular complexes and prevent proenzyme activation. There are four members of the TIMP family (TIMP-1, 2, 3, 4), of which TIMP-1 has been the most extensively studied. TIMPs and MMPs have been known to play important roles in bone formation and remodeling procedures, and have been studied in the embryonic rabbit mandible, human osteophytic bone, neonatal rib and heterotopic bone. And MMPs and TIMPs can also reflect the state of bony remodelling following mandibular lengthening by distraction osteogenesis[18-21].

The expression of MMPs and TIMPs in bony remodelling was evaluated in a bilateral sheep mandible model up to 12 months following lengthening by distraction osteogenesis by Marucci et al. Expression levels were marked at 3 months and decreased thereafter becoming similar to undistracted controls by 12 months and the histologic development of mature lamellar cortical bone was similar to undistracted controls by 9 months following distraction. And it was also reported that MMPs and TIMPs may, in part, reflect the state of bony remodelling following mandibular lengthening by distraction osteogenesis and MMPs and TIMPs expression were comparable to undistracted controls by 12 months, suggesting that equilibrium had been achieved and that bony relapse is unlikely[18,22]. It was reported that the mechanical and radiographic properties of distracted bone was equivalent to undistracted bone by 6 months with no relapse in bilateral sheep mandible model[22]. These informations may be useful to determine the time of implant placement.

In this experiment, regenerated tissue was harvested

after 5 to 7 months, because the chance to harvest the regenerated tissue for the histologic assessment in human body could be taken at the time at the time of implantation. It was known that the expression of MMPs and TIMPs in each group is marked at 3 months, but characteristic by 12 months. But from our results, it looks insufficient to explain the state of maturity and remodelling of regenerated bone. The reason of the vague expression can be considered, such as loss of antigen during decalcification of specimens, failure of removal of false positive reaction, and reduced titer of primary antibodies. But, H&E and MT staining showed the maturity and remodelling of ADO and other bone graft group. In the bone maturation process, woven bone precedes lamellar bone. The former is characterized by an unorganized morphology of collagen fibers, whereas concentric layers of collagen in organized parallel strips are characteristic of the latter[5].

Bone morphogenetic proteins (BMPs) are potent inducers of osteogenesis in both embryological bone formation period and fracture repaird period. Among these BMP families, BMP-2, -4, and -7 have been shown to be especially important for osteogenesis, and they have proliferative effects on various cell types, exhibit chemotactic properties, and can induce the differentiation of noncommitted mesenchymal cells into osteoblastic and chondroblastic lineage cells. The temporal pattern of BMPs expression strongly suggests that cellular BMP production is directly or indirectly enhanced by the mechanical stimulus provided by distraction osteogenesis. BMPs are known to stimulate the proliferation of precursor cells and the temporal and spatial expression of BMPs appears to match that of the proliferative activity in the distracted callus. In addition, BMPs can induce the differentiation of mesenchymal cells into both chondrocytes and osteoblast lineages[23]. In our results, ADO group showed high density and followed by AGB group, and then Mixed group in the comparative quantification of BMP-2.

In our experiments, results of H&E and MT staining in three surgical procedures disclosed that disclosed that new bone formation was evident surrounding old bone in direct connection mainly incorporated with newly formed bone and occasionally with soft tissue marrow. Consequently, woven bone with an abundance of osteocytes was established and lamellar bone also appeared. In immunohistochemical staining, the strong expression of BMP-2 and negative expression of BMP-4 means that the ossification mechanism is not endochondral bone formation but intramembranous bone formation. Histologic findings of ADO showed much more new bone formation than that of other procedures in the point of regeneration time.

Bone formation is preceded by vascular invasion and osteogenesis takes place in the vicinity of newly formed vessels, that mediate delivery of osteoprogenitor cells, secrete mitogens for osteoblasts, and transport nutrients and oxygen[24]. Immunohistologic assessment was carried out using vWF, VEGF, and bFGF. Von Willebrand factor was used to identify newly formed blood vessels while staining for VEGF and bFGF was carried out to spatially localize the regions within the distraction zone that were expressing the morphogenic factors that promoted new vessel formation. In our immunostaining, VEGF was expressed primarily in osteoblasts and undifferentiated mesenchymal cells in the distraction zone immediately adjacent to and in front of the leading edge of new columns of bone but not in the fibrous region. Pacicca suggest that angiogenesis occurs first, followed by organized cell growth oriented to the new vessels, once appropriate blood supply is established, the system stops endothelial differentiation and switches to an osteogenic process. And it was reported that angiogenesis appears to set a template for direct osteogenesis without a cartilage precursor[25]. Several authors confirmed sustained cell proliferation during the distraction period by immunohistochemical staining with bromodeoxyuridine or PCNA. PCNA were irregularly scattered throughout the endosteal gap within a fibrovascular non-ossifying matrix, indicative of a relative deficit in endosteal bone formation[26].

Our results of immunodot blot assay suggest that bone formation during distraction osteogenesis is accompanied by the robust induction of growth factors associated with angiogenesis and osteogenesis, and support further investigations to elucidate the mechanisms by which angiogenic events promote bone repair and regeneration in the atrophied posterior maxilla.

## CONCLUSION

1. ADO is the most effective technique for new bone formation as compared to sinus grafting with autogenous bone or mixed bone based on the histomorphologic study and immunodot blot assay. Therefore, it seems that ADO can be used as a new reconstructive

method in the atrophied posterior maxilla.

2. Immunodot blot assay is able to evaluate the regeneration capacity by quantification of the osteogenic, angiogenic and related factors in experimental specimens and supports the result of histomorphologic study, and also compensates for the weak and obscure results in the immunohistochemical staining methods.
3. It is too early to conclude that immunohistochemical staining with MMPs, TIMPs and BMPs is a good indicator for the evaluation of bony regeneration capacities. Instead, it is necessary to obtain many experimental specimens, and to check these capacities for the usefulness as a good bone formation indicator.

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