Bone augmentation around a dental implant using demineralized bone sheet containing biologically active substances

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This study was designed to evaluate the volume of alveolar bone augmentation after immediate implant placement using demineralized bone. We examined the collagen matrix of demineralized bone and biologically active substances contained therein. Rat maxillary first molars were extracted, and the animals were divided into five groups as follows: tooth extraction only, implant into the mesial root socket, implant and other root sockets covered with demineralized bone sheet, implant and other root sockets filled with demineralized bone powder under the sheet, and implant and other root sockets covered with demineralized bone sheet from which proteins were extracted. We ascertained whether biologically active substances are contained in extracted proteins. Biologically active substances were detected in extracted proteins. Conditions using demineralized bone sheet with biologically active substances significantly augmented the height of the alveolar bone. Such resorbable membranes containing biologically active substances hold promise as clinical agents for bone augmentation upon implantation.

Keywords: Immediate implant placement, Resorbable membrane, Socket preservation

INTRODUCTION

Due to active resorption, the height of the bone that remains at the incision site following tooth extraction is generally insufficient for optimal implant placement. Indeed, there is a remarkable reduction in the bone height and width of the alveolar ridge after single or multiple tooth extractions. This frequently leads to an inadequate crown-implant ratio and is associated with poor aesthetic restorations and functional problems. To overcome this problem in the clinic, guided bone regeneration (GBR) methods using membranes and bone graft materials that inhibit or compensate for bone resorption have been applied. During GBR procedures that use membranes, mechanical barriers are used to isolate the bone defect from the surrounding connective tissue; this provides bone-forming cells with access to a secluded space intended for bone regeneration. Although both resorbable and non-resorbable membranes are available, the former are preferred as they are resorbed by the host, which eliminates the need for second-stage removal surgery. For this reason, commercially available resorbable membranes such as resorbable collagen and resorbable synthetic materials are usually used for GBR surgery. Many graft materials have also been developed and tested for efficacy in the clinic. Such materials include autografts, allografts, and xenografts of biological origin; hydroxyapatite; β-tricalcium phosphate; calcium sulfate; and bioactive glass. Although the structure and ability to absorb compounds are well documented for both commercial membranes and bone graft materials, there are few examples of materials that actively stimulate tissue regeneration. In this regard, Tuli and Singh reported clear-cut radiological, macroscopic, and histological evidence of new bone formation in a demineralized bone-matrix implant. Biologically active substances such as BMP, TGF-β, FGF, and IGF are present in significant quantities in the bone matrix and have various effects on the growth properties and functions of bone cells in vitro. Harada et al. showed that biologically active bone morphogenesis substances in bone extracts could induce ectopic bone formation and stimulated bone marrow stromal cells to differentiate into bone and cartilage tissues.

The aim of this study was to investigate the effect of applying the demineralized bone as a biologically active substance on bone augmentation immediately after tooth extraction. For tooth socket preservation and bone induction around the implant, demineralized bone was used as a grafting material and membrane, and its efficacy for bone augmentation was evaluated.

MATERIALS AND METHODS

Male Sprague-Dawley (SD) rats (Japan SLC, Hamamatsu, Japan) were used in this study. All animal experiments were approved by the Institutional Animal Care Committee of Tsurumi University School of Dental Medicine (Certification Number: 26A031).
Preparation of demineralized bone

Tibiae and femora were taken from male SD rats 6–10 weeks old in order to obtain demineralized bone powder and demineralized bone sheets, respectively. The bone marrow was immediately removed from the collected bone, and then cancellous bone and periosteum were mechanically removed and washed with PBS(−) (Phosphate buffered saline) using an ultrasonic cleaner. To prepare the demineralized bone powder, the tibiae were frozen in liquid nitrogen and ground using a stainless steel mortar and pestle. The powdered bone was 100–500 µm in diameter, and the fine bone powders were demineralized with 0.1 M HCl for 7 h. Subsequently, femurs were demineralized by treating with 0.5 M HCl for 5 h, and part of the articulation was removed and trimmed into 1.5×3.0×0.25 mm fragments in order to derive demineralized bone sheet (DBS°). Following extraction of a portion of DBS° with 4 M guanidine hydrochloride (Gdn•HCl) at 4°C, a derivative demineralized bone sheet (DBS°) was obtained17. As compared to non-demineralized bone, these demineralized bone materials (Demineralized bone powder, DBS° and DBS ) had a high radiolucenty. Supernatant from the extract was dialyzed, and solutes were lyophilized; this preparation is referred to as demineralized bone extract (DBE).

Characterization of demineralized bone sheet

1. SEM

To compare DBS° and DBS , both sheets were rinsed with PBS, dehydrated through a graded series of ethanol baths, and then freeze-dried with tert-butyl alcohol (ID-2, Eiko, Tokyo, Japan). Cross sections of DBS° and DB were observed using a scanning electron microscope (SEM) (JSM-5600LV, JEOL, Tokyo, Japan).

2. SDS-PAGE

SDS-PAGE was performed with e-PAGEL 5–20% Tris-glycine (ATTO, Tokyo, Japan) using DBE19. The gels were stained with Coomassie Brilliant Blue (CBB) (SimplyBlue SafeStain; Life Technologies/Invitrogen) and Stains-All (SA) (Sigma-Aldrich).

3. Detection of biological activity

C2C12 mouse myoblast cells (C2C12 cells) and human periodontal ligament (hPDL) cells purchased from RIKEN (Tokyo, Japan) and stromal cell basal medium (SCBM, Lonza, USA) were returned to consciousness by an intraperitoneal injection of atipamezole hydrochloride (1.0 mg/kg) as a preventive medicine and fed a powdered diet and tap water ad libitum during the experimental period. To monitor new bone formation around the implants, the rats were anesthetized with ether and injected subcutaneously with 0.1% calcein solution at 14 days and with 0.3% alizarin red S solution 21 days after implantation.

Evaluation of bone augmentation

1. Micro-computed tomography

At 28 days post-implantation, all rats were sacrificed by excessive inhalation under ether anesthesia. After each maxilla containing the implant was dissected and

Animal experiments for bone augmentation

1. Design of screw-type titanium implants

Screw-type implants were manufactured according to a previous study with slight modifications (Nishimura Metals, Sabae, Japan)19. The implant size (1.37 mm in diameter and 2.0 mm in length) was designed to approximate the length of mesial roots and the diameter of rat maxillary first molars19. Implants were milled with pure titanium grade 2 and had a machined surface.

2. Male implant placement

Male SD rats 39 days old were used in this study. All rats were injected intraperitoneally with ketamine hydrochloride (40 mg/kg) and medetomidine hydrochloride (0.4 mg/kg). The implant size (1.37 mm in diameter and 2.0 mm in length) was designed to approximate the length of mesial roots and the diameter of rat maxillary first molars19. Implants were milled with pure titanium grade 2 and had a machined surface.

3. Placement of demineralized bone materials

Other sockets were treated using one of the following three methods (Fig. 1e–g). The IS group had sockets covered with DBS°; the IS-P group had sockets filled with demineralized bone powder and then covered with DBS°; the IS’ group had sockets covered with DBS° (n=5). All sheets were placed adjacent to the implant. After incisions of the periodontal tissue were sutured, the rats were injected subcutaneously with benzyl penicillin procaine (~2×10⁶ units/kg) as a preventive medicine and returned to consciousness by an intraperitoneal injection of atipamezole hydrochloride (1.0 mg/kg). Rats were housed at 20–25°C on a 12-h circadian light rhythm and fed a powdered diet and tap water ad libitum during the experimental period. To monitor new bone formation around the implants, the rats were anesthetized with ether and injected subcutaneously with 0.1% calcein solution at 14 days and with 0.3% alizarin red S solution 21 days after implantation.
placed in 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde at 4°C, they were horizontally scanned at 26 µm intervals with a micro-computed tomography (micro-CT) system (MCB-CB100MFZ, Hitachi Medical, Tokyo, Japan). The micro-CT images were obtained and analyzed by reconstruction of the two-dimensional image in any plane using three-dimensional reconstruction software (ExaVision LITE, Ziosoft). A cross-sectional area was measured at the frontal plane of 1.5 mm mesially from the maxillary left second molar at the mesial maximum convexity. In this section, the bone area including the marrow (BAM) was defined as the area delineated by the lines tangential to the most buccal, upper, lower, and inner sides of the alveolar bone. Bone height was measured from highest side of the alveolar bone to the bottom, and bone width was measured from the sutura palatina mediana to the most buccal side of the alveolar bone. These measurements were further analyzed using ImageJ 1.46r (National Institutes of Health, USA).

2. Histological analysis
The maxillae were dehydrated through a graded series of alcohol baths and embedded undecalcified in methyl methacrylate21). Sagittal sections were cut with a low speed diamond wheel saw (MODEL 650, Meiwafosis, Tokyo, Japan) and were ground down to approximately 130 µm thick. Fluorochrome labels of the sections were observed using a fluorescence microscope (EZ-800, KEYENCE). Thereafter, the sections were surface stained with basic fuchsin and methylene blue after treatment with 0.1% formic acid22). In each image of the longitudinal sections of implants, bone-to-implant contact (BIC)23 was calculated as the percentage of the implant perimeter that was occupied by direct new bone-to-implant contact. Bone area (BA) was also measured as the percentage of the area of new bone in relation to the pitch of the implant and the surrounding area. In each case, ImageJ 1.46r was used for morphometric calculations.

3. Statistical analysis
The results of the ALP assay, BIC, and BA were compared using the Mann-Whitney U test. The results from micro-CT image measurements were evaluated using a one-way ANOVA and the Bonferroni test for multiple comparisons at significance level of α=0.05.

RESULTS
Analysis of demineralized bone sheets
Figure 2a shows SEM sections of sheets selected from the cross sections of long bones. High-magnification imaging revealed that the dense structure of DBS+ was preserved following the extraction and subsequent formation of the DBS−. Large numbers of proteins stained with CBB were observed in the DBE, which were extracted from the DBS+. Some of the acidic proteins that were stained by SA were also detected in the DBS+ (Fig. 2b).

To assess their biological activities, DBE was added to the cell cultures. This led to increased ALP activity in the C2C12 cells, which was inhibited by simultaneous addition of the BMP-specific inhibitor LDN-193189 (Fig. 2c). Similarly, DBE increased ALP activity in hPDL.
Bioactivity of the bone sheets and extracted proteins.

a) SEM analysis of cross sections of DBS+ and DBS− (magnification×200, scale bar=100 µm) (Magnification×1,000, scale bar=10 µm). b) SDS-PAGE (5–20% gradient gel) stained with Coomassie Brilliant Blue (Left, CBB) or Stains-All Stain (Right, SA). c) ALP-inducing activity of C2C12 cells exposed in the extracted sample by 4 M Gdn•HCl (DBE) and rhBMP-2 without (−) or with (+) LDN193189. d) ALP-inducing activity of hPDL cells exposed by DBE and rhTGF-β1 without (−) or with (+) SB431542. Data are means±SD of 3 culture wells. Means±SD are shown (c,d). (*: Mann-Whitney U test: p<0.05)

Bone augmentation in tooth sockets

Most of the rats could be used as experimental models because they had no inflammation or residual tooth root (25/32, n=5). Micro-CT images of the left maxilla after implantation are shown in Fig. 3. Examination of the sagittal sections confirmed that the implants were...
Bone area including the marrow (BAM) was measured from the bone outlines in the range demarcated from line A to line D. The lengths of lines E and F reflect the bone height and width, respectively (Fig. 4a). There was no significant difference in the BAM between control animals (3.17±0.12 mm$^2$) and those in the I group (3.55±0.42 mm$^2$) (p>0.05). There was also no significant difference in the BAM between the IS$^-$ group (4.33±0.18 mm$^2$) and the IS$^+$ group (4.57±0.29 mm$^2$) (p>0.05). However, the size of the bone formation was significantly higher in these groups than in other treatment groups (p<0.05). The BAM in animals of the IS$^-$ group (3.53±0.26 mm$^2$) was also significantly less than that in the IS$^+$ group (p<0.05) (Fig. 4b). The bone height of each group followed the same trend as the BAM (Fig. 4c). However, there were no significant differences in bone width among all groups (p>0.05) (Fig. 4d).

**Bone regeneration around the implants**

During 14–28 days after surgery, bone metabolism was observed by fluorescence imaging around the implant in the sagittal sections (Fig. 5). Active bone formation was observed around the implants, especially in the pitch of the implant, in all groups. No difference was observed in bone neoplasia among all groups. Histologic staining also revealed that bone induction occurred in the inside pitches of the implant (Fig. 6a). Bone tissue had penetrated to the position where the DBS$^-$ was placed, suggesting that the DBS$^-$ itself in the IS$^+$ group had replaced to the bone. In contrast, bone only reached the lower surface of the DBS$^+$ in animals of the IS$^+$ group, as there was no replacement of the host bone tissue with material from the DBS$^+$. Examination of the IS$^+$ group revealed that demineralized bone powder was not able to stimulate bone replacement, since only trace amounts were visible even at 28 days post-surgery (Fig. 6a, arrow). BIC and BA were evaluated by histological analysis on tissue sections that were in the areas surrounding the implant. BIC was measured as the range covered along the mesial and distal sides of the long axis, excluding the bottom of the implant (Fig. 6b, green line). The BA was measured inside and surrounding two pitches of the upper part of the implant (Fig. 6b, red and blue areas). Both the IS$^-$ group (38.03±10.13%) and the IS$^+$ group (34.87±6.75%) exhibited significantly greater BIC values than did the I group (16.46±10.35%) (p<0.05). There were no significant differences in the BIC values between the I group and the IS$^+$ group (27.01±13.74%) and between the IS$^-$ and IS$^+$ groups (Fig. 6c). BA values of the implant inside the pitch (red area) were significantly greater in the IS$^+$ (62.13±21.53%) and IS$^+$ groups (47.42±8.25%) than in the I group (26.57±17.93%) (Fig. 6d). Samples from the IS$^+$ group (65.56±15.48%) had the highest BA values around the implant screws (blue area) among all groups (Fig. 6e). A significant difference was observed between the IS$^-$ and I groups (31.72±23.63%) (p<0.05) and between the IS$^+$ and IS$^+$ groups (38.84±14.90%) (p<0.05). There were no significant differences between the IS$^-$ and IS$^+$ groups (55.52±15.29%) (p<0.05), although there was a tendency for samples in the IS$^+$ group to have smaller BA values.

**DISCUSSION**

In this study, we were able to examine the properties of demineralized bone when used as membrane and bone graft material, as well as the effect of biologically active substances derived from the demineralized bone. Indeed, many proteins were observed in the DBE, and we were able to detect activity of the cytokines, TGF-$\beta$ and BMP. Since the structures of DBS$^-$ and DBS$^+$ were similar, we therefore suggest that the ability of DBS$^+$ (but not DBS$^-$) to promote bone regeneration was due to the biological...
Fig. 4  Morphometry of bone growth at the implant sites.
Cross sections were measured at the frontal plane 1.5 mm mesially to the maxillary left second molar buccal height of the contour. a) Bone area including the marrow (BAM) was measured by the outline of the bones in the range demarcated from line A to line D (blue line). Bone height and bone width were measured to line E (yellow line) and to line F (red line), respectively. b) BAM. c) Bone height. d) Bone width (Bonferroni test: \( p<0.05 \)). Means±SD are shown c, d).

Fig. 5  De novo bone formation at implant sites.
Fluorescence images of new bone formation around screw-type titanium implants. Rats were injected subcutaneously with calcein at 14 days and with alizarin red at 21 days after implantation, respectively. Imp: screw-type implant, ms: mesial side. Scale bar=0.5 mm. Arrow: Autofluorescence.
Fig. 6  Quantitative analysis of bone augmentation at implant sites.
Basic fuchsin and methylene blue staining of sections prepared from undecalcified maxillae tissue around the implant a). Scale bar=0.5 mm, Imp: screw-type implant, ms: mesial side. Arrow: demineralized bone powder. b) BIC was measured as the range covered along the mesial and distal sides of the long axis, excluding the bottom of the implant (green line). BA was measured inside two pitches of the upper part of the implant (red area) and around the implants (blue area). c–e) Graphical representation of BIC values, BA values of the implant inside the two pitches (red area), and BA values around the implant screw (blue area). Means±SD are shown. (*: Mann-Whitney U test: p<0.05)

activities of the extracted proteins.

Using two-dimensional images of the defined plane in the micro-CT images, restoration of the alveolar bone was evaluated by measuring the BAM, bone height, and bone width. There were no significant differences between the control and the I group; this is consistent with other studies in which placement of the implant did not inhibit alveolar bone resorption\textsuperscript{24,25}. Although the BAM and bone heights were significantly higher in the IS\textsuperscript{+} and IS\textsuperscript{+P} groups as compared to the other groups, there were no significant differences in bone widths among the five groups. The surgical procedures followed in this study effectively control the direction of the bone augmentation. Since there were no significant
differences in the BAM and bone heights between the IS' and IS'-P groups, we infer that the use of DBS' alone may be sufficient to induce the desired biological effect. On the other hand, there was a significant difference between the IS' and IS groups, which is likely explained by the loss of biologically active substances, such as TGF-β and BMP, following extraction from the DBS' (IS' group). In the IS' and IS'-P groups, bone formation extended to a clearly higher level than the alveolar bone level immediately after tooth extraction.

In histologic staining, while DBS' was able to replenish bone tissue at the position of the membrane in the IS' group and IS'-P group, this was not the case for DBS' in the IS' group. This is most likely due to the biologically active proteins that are present in DBS'. Therefore, the height of the alveolar bone was augmented in the IS' and IS'-P groups.

BIC was measured in a limited range that did not include the bottom of the implant. Because we observed the adhesion of some bone to the tip of the mesial root when the first molar was extracted, it is possible that an individual maxillary sinus was slightly perforated. In our study, we chose to include the implant body above the bone level in the measurement range of BIC, as we assumed that the effects of the sheet and the powder would effectively shift the point of contact between the implant and the bone to a higher position. As a result, the BIC percentage we observed (I group; 16.46±10.35%) was lower than that found with a titanium-machined implant surface in a previous study (approximately 25%)

Absorbed demineralized bone powders often remained inside the alveolar bone and could, thus, adversely affect the initiation of BIC (Fig. 6a, arrow). BIC in the IS' and IS' groups was significantly greater than that of the I group; we also observed a similar tendency in bone area, due to the fact that the connective tissue cells entered the implant site and, thus, precluded the invasion of osteogenic cells. The effectiveness of the barrier membrane was demonstrated.

Following implant placement surgery, some threads of the implant do not make physical contact with alveolar bone due to its scarcity. GBR methods using autografts, xenografts, and barrier membranes have been applied to solve this problem; however, the results obtained from such approaches are insufficient. Our current results suggest that placement of DBS' would fulfill an unmet need, as exposed threads would be rapidly covered by new bone growth. In the future, the development of artificial resorbable membrane with recombinant biologically active substances is expected for the clinic.

Appropriate bone formation in tooth extraction sockets and around the implant was obtained in the IS' group. Based on these results, we suggest that demineralized bone sheets with biologically active substances (DBS') could facilitate socket preservation and augment alveolar bone growth around dental implants. Consequently, membrane with biologically active substances should be applied for bone augmentation, as it has the potential to significantly improve prospective patients' quality of life.

CONCLUSIONS

Within the limitations of this study, we have concluded that demineralized bone sheets have significant potential clinical utility for augmenting bone growth around dental implants when used immediately after tooth extraction. The salient findings of our study are as follows:

1. Use of demineralized bone sheets increases the alveolar bone height around the implant.
2. Biologically active substances within the demineralized bone sheets contribute to bone replacement at the implant site.
3. Whether with or without biologically active substances, bone regeneration under demineralized bone sheet was sufficiently obtained as compared with other groups.
4. The immediate use of membranes with biologically active substances is recommended for socket preservation and bone augmentation.

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