Article

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Complex Tissue Alterations After Socket Preservation With Demineralized Bone Matrix Binding With Growth Factors: A Study in the Beagle Dogs

Bi-he Zhang[1,†], Jian-hua Zhu[1,†], Tian-cheng Qiu[1], Jian-min Han[2,*], Chuan-bin Guo[1,*]

[1]Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, 100081 Beijing, China
[2]National Engineering Laboratory for Digital and Material Technology of Stomatology, Department of Dental Materials, Peking University School and Hospital of Stomatology, 100081 Beijing, China
*Correspondence: hanjianmin@bjmu.edu.cn (Jian-min Han); guodazuo@sina.com (Chuan-bin Guo)
†These authors contributed equally.
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Background: To study the hard tissue change after tooth extraction filled with demineralized bone matrix (DBM) collagen scaffold bound with growth factors.

Methods: In 15 Beagle dogs, 90 socket sites were prepared by bilateral extraction of the mandibular premolars. Sockets were divided into six groups as follows: Group A: Blank (without any material); Group B: filled with HealiAid® Collagen Wound Dressing (SA001020, Maxigen Biotech Inc.); Group C: DBM; Group D: DBM-collagen-binding domain (CBD)-basic fibroblast growth factor (bFGF) (0.05 µg/mL); Group E: DBM-CBD-bFGF (5 µg/mL); Group F: DBM-CBD-bFGF (5 µg/mL) + bone morphogenetic protein 2 (BMP_2) (300 µg/mL). The changes in width and height of the buccal and lingual bone plates were determined by Cone-Beam Computed Tomography (CBCT) scan. At the 1st, 2nd, 3rd months after surgery, a total of 15 dogs were euthanized for Micro-computed tomography (Micro-CT) scan and histological examinations.

Result: At 1 month postoperatively, the differences in buccal bone height (BBH) of Group D [0.34 (0.18, 1.02) mm] and Group F [0.12 (0.04, 1.38) mm] were significantly lower than that of Group E [2.07 (1.03, 2.46) mm] (Group D vs. Group E, p = 0.032; Group F vs. Group E, $p = 0.047$). In the horizontal direction, from 1 to 3 months postoperatively, the median horizontal width changes at 1 mm, 3 mm and 5 mm below the alveolar ridge of both Group D and Group F were generally smaller than those of Group E, but there was no significant difference between the groups ($p > 0.05$). No significant differences were found among the indices of bone micro-structure measured by Micro-CT scan ($p > 0.05$). However, the fibers in the new bone zone of Groups E and F exhibited a higher level of maturity, as observed by Masson's trichrome stain.

Conclusion: Compared with the blank group and the control group, the DBM group with the addition of growth factors showed good alveolar ridge preservation, reduced bone resorption, and promoted the bone maturation of the extraction socket.

Keywords: collagen sponge; growth factor; socket preservation; tooth extractions

Introduction

Implant therapy has been widely accepted and confirmed to successfully and efficiently replace missing teeth [1]. But after the tooth is extracted, alveolar bone resorption is a common problem; ridge dimension change brings not only the challenge of placing a restoration-driven implant, but also threatens the stability of the implant in the long term. In the 12 months after the excision of premolars and molars, ridge width may decrease by approximately 50%, with two-thirds of the loss occurring in the initial 3 months of recovery [2]. Based on a systematic review, the average clinical width and mid-buccal bone height (BBH) loss of humans after tooth extraction were 3.87 mm and 1.67 mm, respectively [3,4].

It has been demonstrated that socket filling efficiently reduces the amount of alveolar change after tooth extraction, a technique called alveolar ridge preservation (ARP) [5]. A systematic review of randomised clinical trials determined that ARP was an efficacious treatment for preventing the average reduction in alveolar ridge dimensions following tooth extraction [6]. There has been no consensus regarding the optimal material and method for ARP, leading to a substantial amount of new bone formation and perfect dimensional stability after the specific healing period.

In clinics, absorbable collagen sponge has been extensively used to fill sockets following tooth extraction; this method has been shown to effectively improve the initial healing of soft tissues and periodontal defects, and to reduce early-stage post-operative complications [7,8] due to a series of advantages: low antigenicity, excellent biocompatibility, and biodegradability [9,10]. However, its mechanical strength is poor, which is unsuitable for maintaining alveolar shape.

A demineralized bone matrix (DBM) collagen scaffold is obtained from cancellous bone tissues. DBMs are appropriate for bone tissue repair due to their composition and structural similarity to human bone. Previously, DBM has displayed increased mechanical strength after being modified by cross-linked heparin [11].

Combining bio-materials and growth factors has drawn much attention in orthopaedic and craniofacial surgery [12,13]; it can avoid short half-life and rapid local clearance of growth factors after administration [14]. Bone morphogenetic protein 2 (BMP_2) is well-known for promoting osteogenesis [15,16], and basic fibroblast growth factor (bFGF) has been recently discovered to be highly expressed in osteoblasts, which modulate the proliferation and differentiation of mesenchymal stem cells [17]. These factors have a stronger effect when they work synergistically to repair bone defects [18–20]. Previous studies incorporated a particular collagen-binding domain (CBD), a protein with the sequence "TKKTLRT", at the N-terminal of native bFGF or BMP_2. This alteration demonstrated significant collagen-binding capability while preserving the cytokine activity of this protein [11,21,22]. Modifying the collagen scaffold (DBM) with CBD-growth factors provides an efficacious therapeutic tool for tissue repair [21]. In this study, we prepared three kinds of CBD-growth factor protocols loaded on DBM, including DBM-CBD-bFGF (0.05 μg/mL), DBM-CBD-bFGF (5 μg/mL), DBM-CBD-bFGF (5 μg/mL) + BMP_2 (300 μg/mL). We filled the sockets of Beagle dogs with these materials after teeth were extracted to compare the effect on the preservation of alveolar bone.

Materials and Methods

Materials Preparation

The DBM scaffold (2019111401), formed from bovine spongy bones, was supplied by Xi-Ling Biotechnology Inc. (Jiangsu, China). The procedures for preparing DBM were similar to those described previously [22].

The Chinese Academy of Sciences' Institute of Genetics and Developmental Biology (Beijing, China) supplied CBD-bFGF and CBD-bFGF + BMP_2 in powder form. CBD-growth factors were previously evaluated for their expression (Fig. 1), purification, biological activities, and collagen-binding capacity [21]. Consistent with the previous method, DBM scaffolds were saturated with CBD-bFGF and CBD-BMP_2 solutions according to the reference standards [22].

Animals

In this study, the experimental protocol was followed under the ethical guidelines of Peking University's Institutional Animal Care and Use Committee, Beijing, China (approved IACUC No.LA2020514). Fifteen young Beagle dogs (aged 1 to 1.5 years, weighing 10 to 12.5 kg) were used as animal models, purchased from the Laboratory of Peking University School and Hospital of Stomatology. Animals were kept in controlled laboratory conditions at 15 to 21 °C with 30% relative humidity. An automatic timer was used to regulate the light cycle (12 h of light/dark).

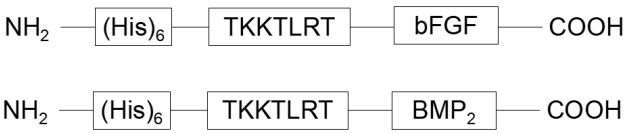

Fig. 1. The expression vector for CBD-based growth factors is illustrated in the diagram. A mature bFGF or BMP_2 encoding frame and $(His)_6$ affinity tag were encoded in the vector. TKKTLRT is a heptapeptide that is composed of CBDs. BMP_2, bone morphogenetic protein 2; bFGF, basic fibroblast growth factor; CBD, collagen-binding domain.

Surgical Procedure

Anesthesia was induced by intravenous injection of 3% pentobarbital sodium (30 mg/kg intravenous injection). After the anesthetic effect, animals were put on a 37 °C thermostatic operating table. A combined solution of articain in 4% epinephrine (1:100,000) was injected subcutaneously to induce local anesthesia.

The sockets on both sides of the mandible that contained the mesial roots of the second, third, and fourth premolars (P2, P3, P4) served as experimental sites (Fig. 2). For each animal, six sites were randomly inserted with different material as designed: Group A: Blank (without any material); Group B: filled with HealiAid® Collagen Wound Dressing (SA001020, Maxigen Biotech Inc.), as control group, which is made from natural collagen extracted from bovine tendon tissue and have been used in clinic; Group C: DBM; Group D: DBM-CBD-bFGF (0.05 μg/mL); Group E: DBM-CBD-bFGF (5 μg/mL); Group F: DBM-CBD-bFGF (5 μg/mL) + BMP_2 (300 μg/mL). The alveolar wound was sutured, and penicillin (20 U/kg/d, Intramuscular injection) was administered for 3 days post-operation.

The animals were divided into three groups, and each group received an intravenous injection of 200 mg/kg sodium pentobarbital at 1, 2, and 3 months postoperatively. After cessation of breathing and heart function, their mandible was dissected and fixed immediately by immersion in a 4% formaldehyde solution.

Cone-Beam Computed Tomography (CBCT) Analysis

Immediately after surgery, the mandible region of all the animals was scanned using a Cone-Beam Computed Tomography (CBCT) machine (NewTom 5G, Verona, Italy). Before sacrifice, subsequent CBCT scans for each group were performed at the 1st, 2nd, and 3rd months after surgery. The exposure parameters were voltage (110 KV), current (0.55 mA), and exposure time (3.6 sec), with a voxel size of 0.300 mm. In DICOM 3.0 format, axially sliced im-

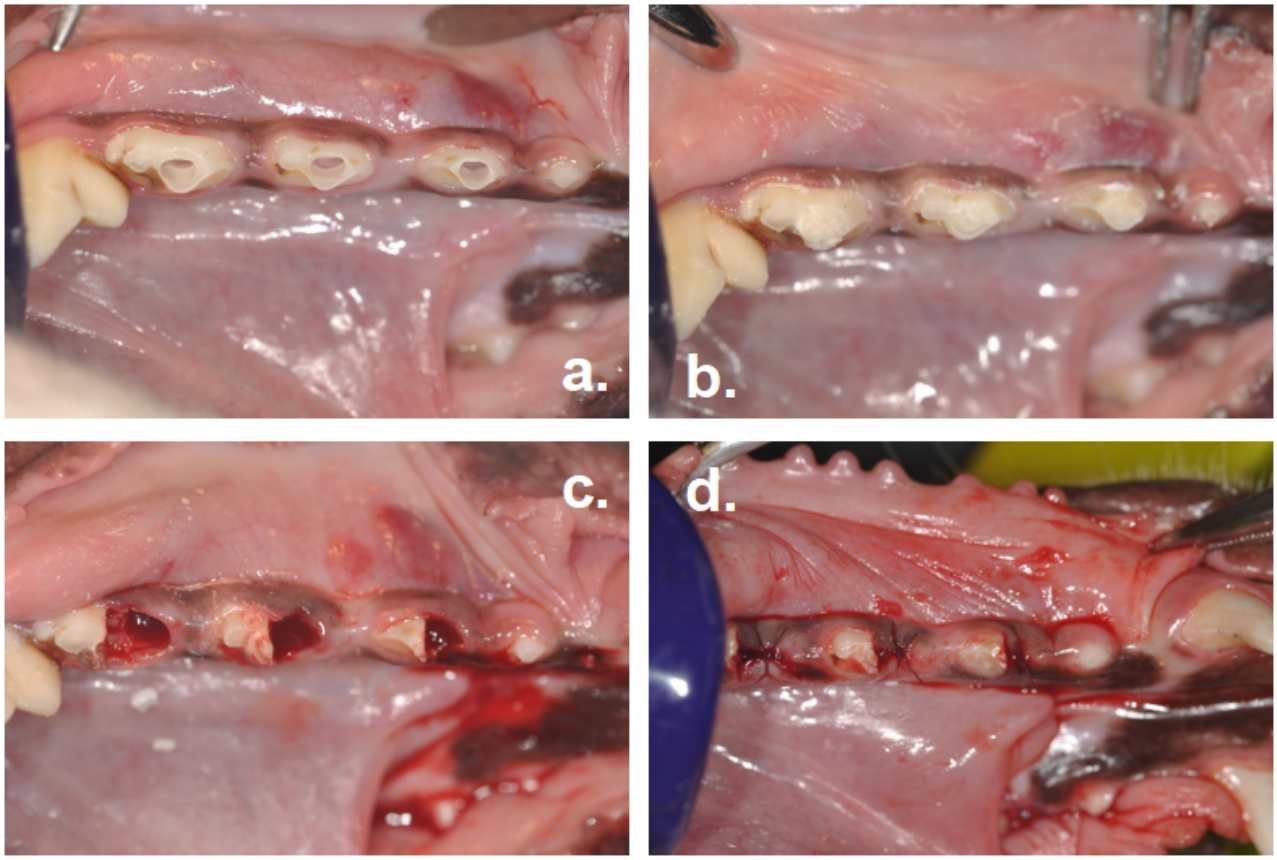

Fig. 2. Tooth hemisectioning by surgical procedures. (a) The pulp was opened, and the root canal was prepared. (b) The root canals were filled with Vitapax. (c) In advance, the teeth underwent hemissection, and the mesial root was extracted. (d) Following the filling of the material, the sockets were sutured.

age data were transferred to a personal computer and assembled using Simplant's interactive software 3.0 (Materialise, Brussels, Belgium).

The CBCT radiograph taken immediately after surgery was compared to each subsequent image (1st, 2nd, and 3rd months) taken before animals were sacrificed. Vertical and horizontal difference values, and Micro-computed tomography (Micro-CT) measurements, were calculated between each pair of images.

Vertical Measurements

The determination of the vertical change in the lingual and buccal bone walls was carried out as follows: The distance (height) from the top of the lingual crest to a line passing through the root point, which is also perpendicular to the long axis of the tooth, was measured along a line parallel to the tooth's long axis. The difference value between the image immediately after surgery and at each scan before sacrifice was the height change in the lingual bone wall. The BBH change was assessed in the same way (Fig. 3a)

Horizontal Measurements

The total bone widths between the buccal and lingual alveolar ridge (W1, W3, and W5) were measured as the horizontal distances. The distances were measured perpendicular to the line at 1, 3, and 5 mm below the top of the buccal crest, respectively. The difference value between the image immediately after surgery and at each scan before sacrifice indicated the width change in the alveolar ridge (Fig. 3b).

Micro-CT

The samples were scanned using an INVEON MM GANTRY STD CT Micro-CT device (serial No.3121, Siemens Medical Solutions USA, Inc., Knoxville, TN, USA) operating at 70 kVp and 114 mA following one week of fixation at 25 °C. A slice-based method was used to identify the volume of interest (VOI) region in the CBCT image immediately following the operation, based on the shape of the mesial roots, after the acquisition of three-dimensional (3D) reconstructions. Initially, a circular area with a diameter of 1 mm was selected as the region of interest (ROI) at the centre of each specimen section. To generate the VOI, the ROI was interpolated from the centre of the core outward toward the surfaces at 30 sections. A grayscale threshold of 55 (maximum) and 30 (minimum) was used for the evaluation of bone tissue, and a threshold of 80 (maximum) and 55 (minimum) was applied for the evaluation of biomaterials. The dimension analysis of trabecular bone

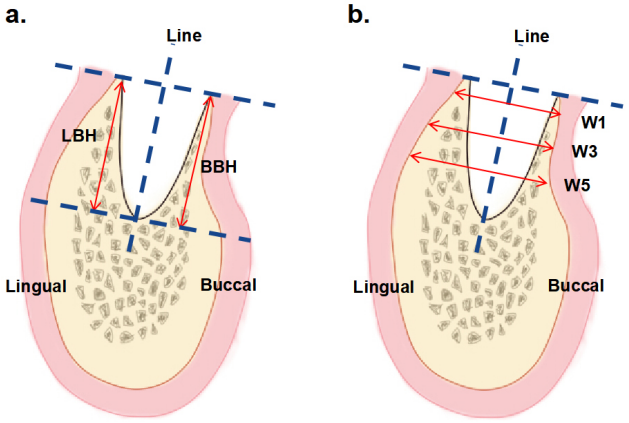

Fig. 3. A schematic representation of the vertical (a) and horizontal (b) CBCT results obtained from the tooth extraction socket (drawn by Adobe Photoshop CS7 (Adobe Systrems Inc., San Jose, CA, USA). CBCT, Cone-Beam Computed Tomography; LBH, lingual bone height; BBH, buccal bone height; W1, widths between the buccal and lingual alveolar ridge 1 mm m below the top of the buccal crest; W3, widths between the buccal and lingual alveolar ridge 3 mm m below the top of the buccal crest; W5, widths between the buccal and lingual alveolar ridge 5 mm m below the top of the buccal crest.

micro-architecture was conducted using the subsequent parameters: trabecular number (Tb. Nb), trabecular thickness (Tb. Th), and bone volume/tissue volume (BV/TV).

Hematoxylin and Eosin (H&E) Staining

Following Micro-CT evaluation, the samples of the mandible were cut into blocks and decalcified by 10% EDTA at 37 °C for 4 weeks. Following a series of ethanol dehydration, the specimens were fixed in paraffin. These paraffin blocks were cut into mesial-distal sections (4-µm thick) parallel to the socket axis and stained using Hematoxylin and Eosin (H&E). The specimens were examined under the Omlipase-telescope around the region of the extraction site. All specimens were evaluated at the same magnification.

Masson's Trichrome Staining

To evaluate the mineralized bone matrix and osteoid, sections (4-µm) were stained with Masson's trichrome (Solarbio, Beijing, China) per the manufacturer's instructions.

Statistical Analysis

The findings were statistically analyzed using SPSS 21.0 (IBM, Armonk, NY, USA). Measurement data that do not follow a normal distribution were compared between groups using the Mann-Whitney rank-sum test. A threshold of $p < 0.05$ was selected for statistical significance.

Result

Clinical Observation

All animals were euthanized at the end of the study, and healing proceeded uneventfully in all of the sockets. None of the treatment groups displayed clinical indications of infection, necrosis, or substantial osteolysis throughout the 3-months healing period.

CBCT Evaluation

At 1 month postoperatively, the ΔBBH values of Group D [0.34 (0.18, 1.02) mm] and Group F [0.12 (0.04, 1.38) mm] were relatively low, indicating the least bone resorption. The bone resorption values of Group C [1.54 (0.56, 2.08) mm] and Group E [2.07 (1.03, 2.46) mm] were relatively high. Among them, the bone resorption values of Group D and Group F were significantly lower than those of Group E (Group D vs. Group E, $p = 0.032$; Group F vs Group E, $p = 0.047$) (Fig. 4a). In the second and third months, the median ΔBBH values of Group E were still relatively high, and the median ΔBBH values of Group D and Group F were consistently lower than those of Group E, but the differences between the groups were not significant ($p > 0.05$, Fig. 4a).

Compared to BBH, there were smaller changes in lingual bone height (LBH) during the post-operative period. In the 1st month, the median ΔLBH in all groups was less than 1 mm. In the 3rd month, the ΔLBH values of all the groups filled with collagen-binding growth factors (Group D, Group E, Group F) were lower than those of Group A [1.61 (0.81, 1.65) mm] and Group B [1.11 (0.76, 1.79) mm]. Among them, the ΔLBH values of Group E [1.07 (0.74, 1.14) mm] and Group F [1.06 (0.23, 1.42) mm] were the lowest, but there was no significant difference among the groups (Fig. 4b, $p > 0.05$).

There was a notable change in the horizontal width of the alveolar ridge at 1 mm below the alveolar ridge. Group E [1.94 (1.31, 2.35) nm] showed the most apparent change at the 2nd month, with a change value greater than that of Group D [0.83 (0.58, 1.99) nm] and Group F [1.39 (0.79, 1.53) nm], but no significant difference was found between the groups ($p > 0.05$) (Fig. 4c). On the other hand, during the three months, the width at 3 mm and 5 mm below the alveolar ridge was relatively more stable than the width at 1 mm, and the reduction in most groups was less than 0.5 mm, especially the groups filled with CBD-growth factors (Group D, Group E, Group F). There was no significant difference among the groups at 3 mm or 5 mm ($p > 0.05$, Fig. 4d,e).

Micro-CT

From the 1st month to the 3rd month post-operation, the BV/TV of each group gradually increased, showing new bone filling the sockets (Fig. 5a). The Tb. Nb of each group had reduced with healing, due to the new bone trabeculae

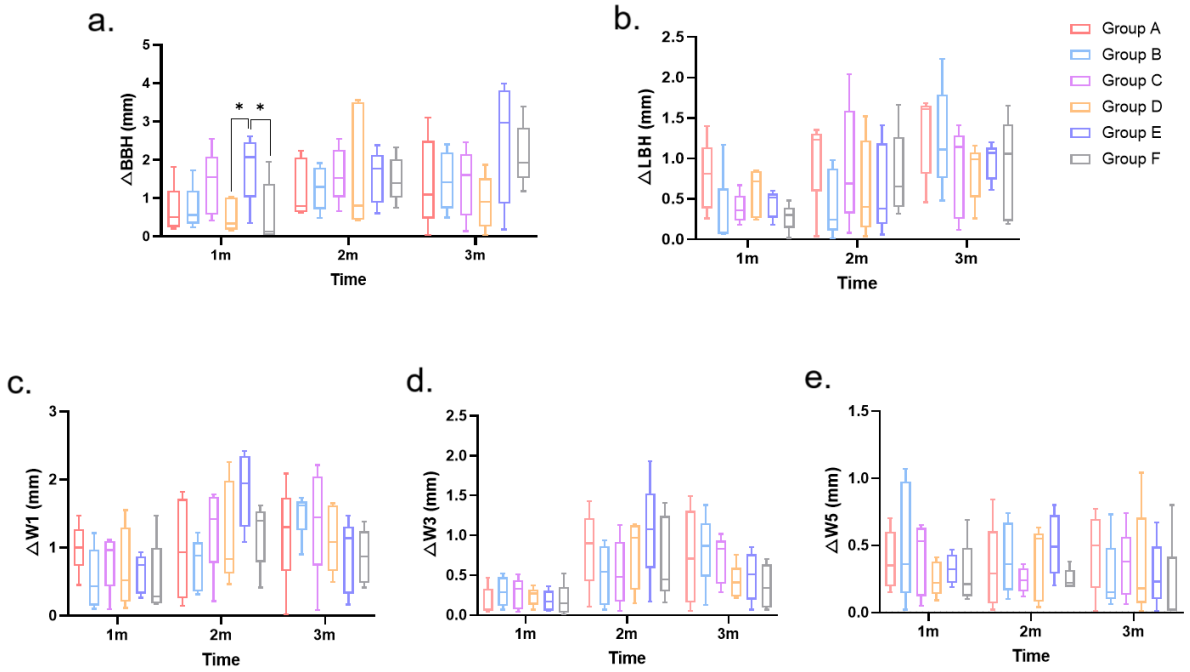

Fig. 4. Extraction socket CBCT measurements at multiple time points following surgery, like ΔBBH (a), ΔLBH (b), ΔW1 (c), ΔW3 (d), and ΔW5 (e). *$p < 0.05$. LBH, lingual bone height; BBH, buccal bone height; W1, widths between the buccal and lingual alveolar ridge 1 mm m below the top of the buccal crest; W3, widths between the buccal and lingual alveolar ridge 3 mm m below the top of the buccal crest; W5, widths between the buccal and lingual alveolar ridge 5 mm m below the top of the buccal crest. n = 5.

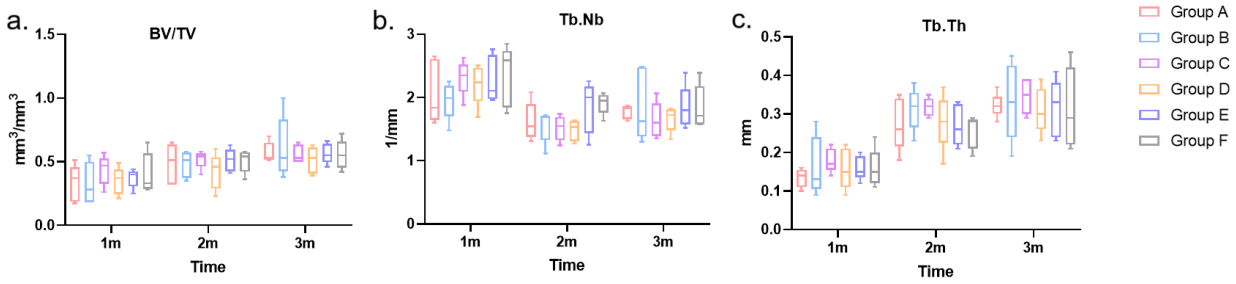

Fig. 5. Micro-CT analysis of bone morphological parameters. BV/TV (a), bone volume/tissue volume; Tb. Nb (b), trabecular number; Tb. Th (c), trabecular thickness. Micro-CT, Micro-computed tomography. n = 5.

merging together (Fig. 5b). At the same time, bone trabeculae had thickened while the new bone matured (Fig. 5c). All of the parameters evaluating trabecular bone microarchitecture were similar, no matter which material was inserted in the socket sites and there were no significant differences between groups ($p > 0.05$, Fig. 5).

Histological Observations

A proportion of sites exhibiting growth factor collagen incorporation during the initial month presented a more moderate infiltration of macrophages and neutrophils by histological analysis (Fig. 6). The density of bone trabecula was higher in the groups with DBM than in Group A and Group B, the blank and control groups. The new vessels could be observed among bone trabeculae in each group, but vessel density was not greater in Groups D, E and F, all of which were filled with material that included bFGF. In the 2nd-month post-operation, some of the woven bone was gradually replaced by lamellar bone, especially in Groups E and F, where mature fiber stained red by Masson's trichrome stain also started appearing. At three months, more lamellar bone was present in the groups filled with DBM material, whereas there was still a certain proportion of woven bone in Group A, without any material filled. Moreover, trabecular bone in Group E and Group F was more mature than that of other groups, with more red regions indicated by Masson's trichrome stain (Fig. 7).

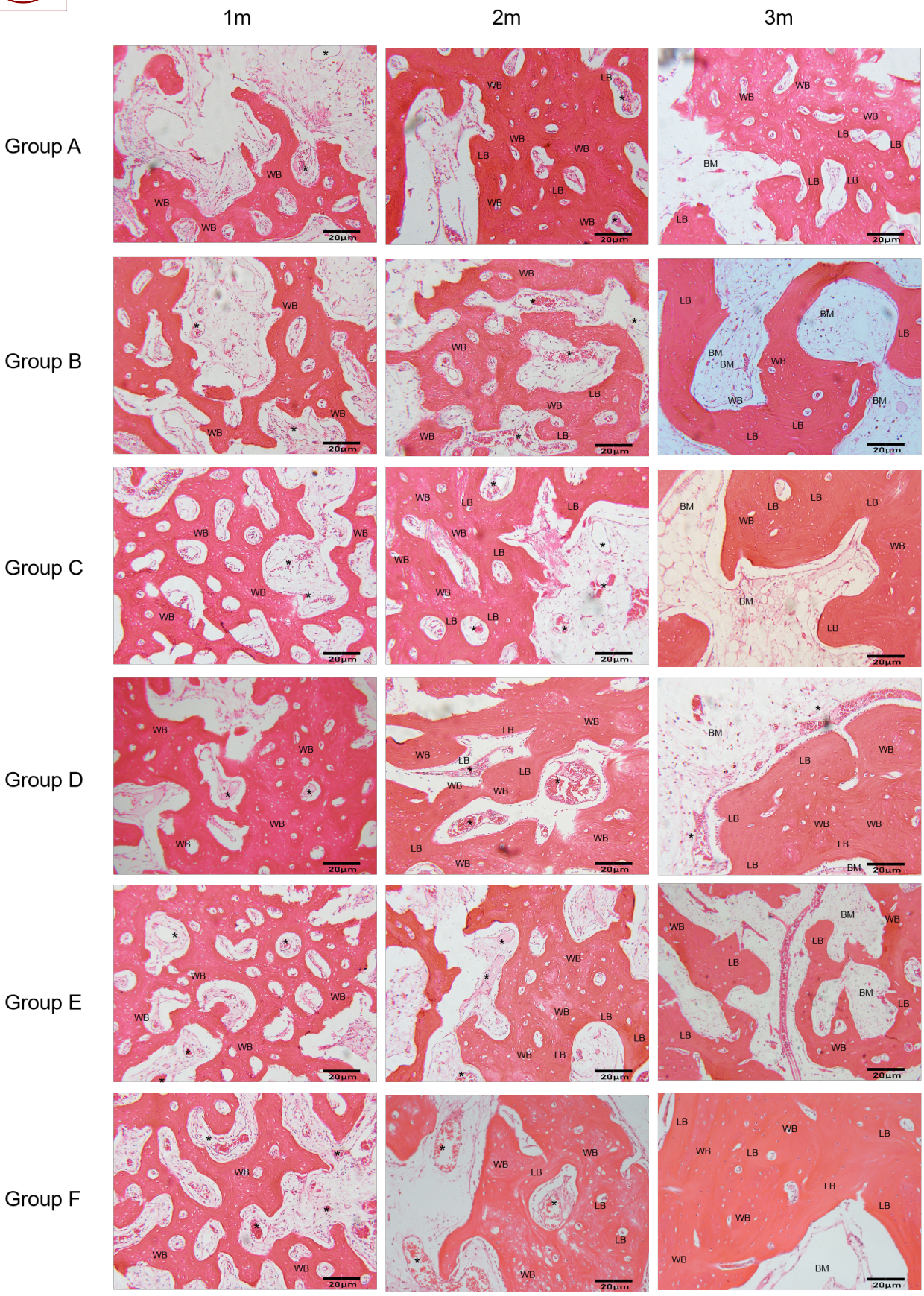

Fig. 6. Images (high magnification) of Hematoxylin and Eosin (H&E) staining of regenerated bone in the extraction socket after surgery. LB, lamellar bone; WB, woven bone; BM, bone marrow; black star, blood vessel. Original magnification, ×100.

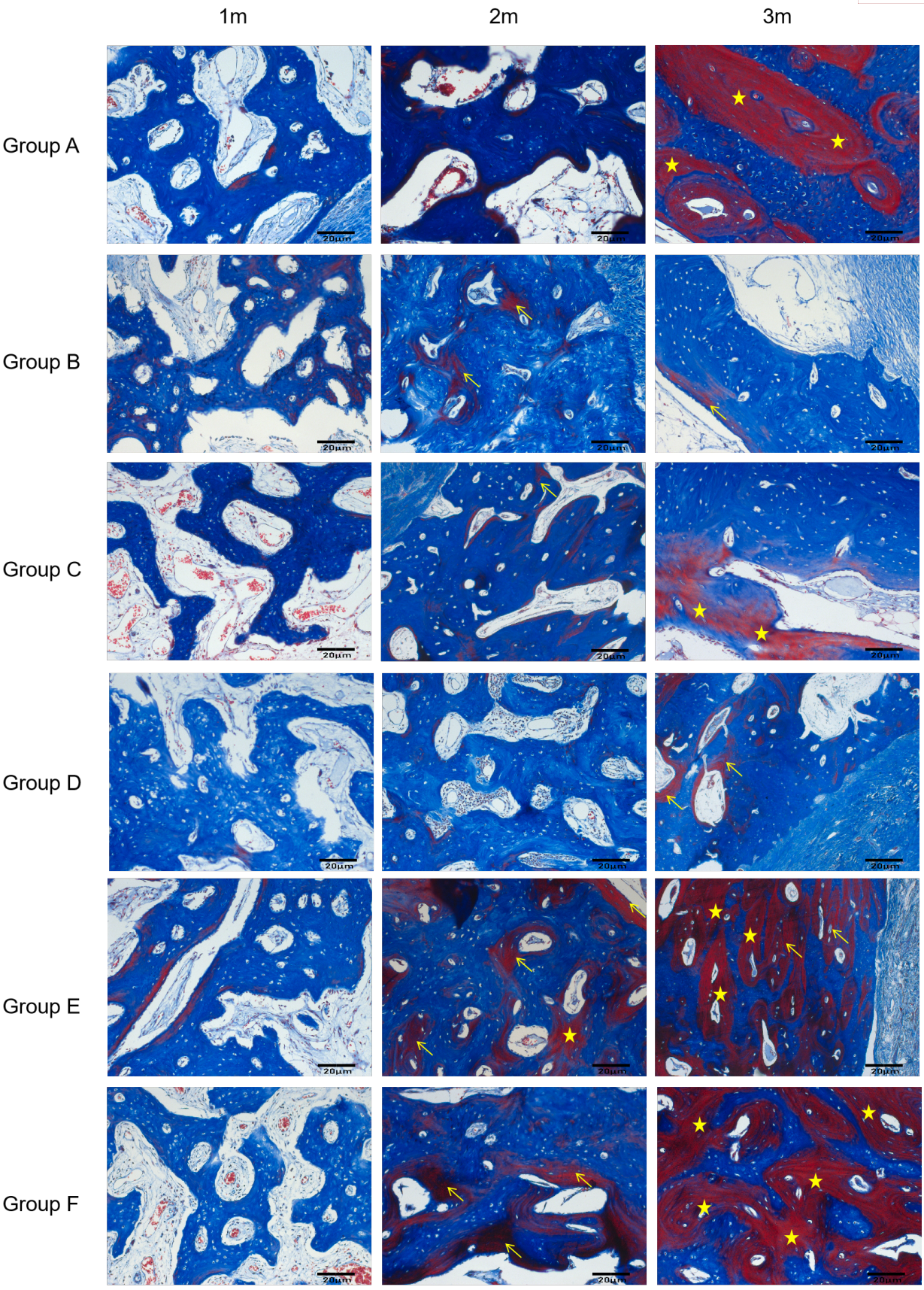

Fig. 7. Images (high magnification) of Masson's trichrome staining of regenerated bone in the extraction socket after surgery. The bone of the red region is mature. Yellow star/arrow, mature bone. Original magnification, ×100.

Discussion

Sufficient amounts of alveolar bone are crucial to attaining satisfactory aesthetic results and promoting the long-term success of dental implants [1,7]. Socket preservation is greatly enhanced by xenogeneic collagen, a high-quality material that offers numerous benefits; it is easily handled, completely absorbable, and forms bone structure naturally [23]. The collagen matrix mechanically protects the grafting material, promoting thrombus formation, wound homeostasis, and wound stabilization [23]. A collagen sponge could be a promising scaffold due to its shapeability [24]. We adapted the DBM collagen scaffold prepared from bovine spongy bones. Compared with the collagen scaffold as the control group (which was extracted from bovine tendon tissue), the DBM collagen scaffold has a structure similar to human bone and maintains stability after modification [22]. Currently, collagen is a critical biomaterial used in delivery systems to protect growth factors against rapid enzymatic degradation and the dispersion of bodily fluids after implantation [25,26]. Thus, growth factors incorporated with collagen scaffold through specific binding have the potential to provide the ideal clinical outcome.

This study aimed to assess the efficacy of the delivery matrix proposed by Chen *et al*. [22] in preserving the alveolar ridge using two growth factors, bFGF and BMP_2. Both affect bone repair and produce greater osteogenic effects when combined to repair bone defects in long bones [16,18,19,27]. This study aimed to evaluate the impact of collagen bound with growth factors on alveolar ridge preservation, in order to identify the optimal material to avoid bone volume reduction and maintain a better alveolar condition for implant placement. Accordingly, we prepared 3 protocols to assess the effects on osteogenesis: DBM-CBD-bFGF (0.05 µg/mL), DBM-CBD-bFGF (5 µg/mL), and DBM-CBD-bFGF (5 µg/mL) + BMP_2 (300 µg/mL).

The main focus of this study was to assess the modification of alveolar bone that occurs during the insertion of a collagen scaffold into newly formed extraction sockets. These results were compared to those found in extraction sockets treated with collagen bound with growth factors and to those for an untreated site. The animal group that exhibited a substantial reduction in bone after 12 weeks was Group E, which received a high concentration of DBM-CBD-bFGF (5 µg/mL). From the 1st to the 3rd month after the operation, the median ΔBBH of Group E was the highest. This observation is consistent with the findings of a previous study [28] concerning the long-term effect of a xenogeneic transplant in a newly extracted socket on the remodeling of hard tissue and ridge preservation. On the other hand, from the 1st to the 3rd month after the operation, in the vertical direction, the median ΔBBH values of Group D and Group F remained smaller than that of Group E. Similarly, in the horizontal direction, from the 1st to the 3rd month after the operation, the median horizontal width changes at 1 mm/3 mm/5 mm below the alveolar ridge of Group D and Group F were generally lower than those of Group E. These results suggest that the concentration of bFGF at 0.05 µg/mL and the protocol that combined with bFGF and BMP_2 may be better options to preserve the bone mass of the alveolar ridge.

Due to the fact that collagen could serve as a scaffold for new bone formation, the study's second aim was to assess the quality of bone after bone regeneration induced by collagen binding with growth factors. For that, we compared the tissue at 3 time points of healing by micro CT analysis and histological observation. Bone formed in Groups E and F was significantly more mature after 2 and 3 months of healing, as there were many more red regions as indicated using Masson's trichrome stain. This indicates that growth factors can promote new mineralization of regenerated bone and provide a more reliable quality of alveolar bone for implant treatment. In regenerated socket bone, distinct spongy structures were observed; however, there was no statistically significant variation in the quantity of bone tissue when collagen bound to growth factors was compared to control sites or blank sites. Hence, the use of collagen bound with growth factors did not support the formation of natural bone.

Predictive factors for the efficacy of ARP remain unsupported by evidence [1]. Despite the implementation of standard precautions, surgical outcomes are susceptible to the influence of local and systemic factors. In the present study, DBM bound with growth factors did not efficiently maintain the vertical and horizontal dimensions of the alveolar ridge as we had anticipated. The possible reasons were as follows: firstly, the socket wall, especially the buccal wall, had more extensive bone volume resorption [29,30], and when it was thick, it made the effect of alveolar ridge preservation negligible [31,32]. An animal model with a thinner, even missing buccal wall could be adopted in further study [33]. Secondly, collagen may have a role as a scaffold but lacks excipient properties [34]. In the 1st month, the new bone was still immature, but no material was observed in the sockets, meaning it was absorbed completely. Although DBM has better mechanical strength after modification [11], the support function of the alveolar ridge profile could be weakened when it is gradually absorbed, which may reduce the amount of new bone regeneration 3 months after the surgery. The third reason is that collagen material quickly diminishes [35], and it is difficult for the growth factors to maintain their biotic impact at local regions with specific concentrations. Moreover, although there were data on bFGF and BMP_2 release peaks *in vitro* [19,27], it is still challenging to measure not only the dose but the release rate of growth factors in local circumstances *in vivo*. Therefore, whether and how to maintain a stable concentration of growth factors *in vivo* needs further research.

Conclusion

Two protocols of DBM bound with growth factors, DBM-CBD-bFGF (5 μg/mL) and DBM-CBD-bFGF (5 μg/mL) + BMP_2 (300 μg/mL), promoted new bone development at three months post-operation. Both DBM-CBD-bFGF (0.05 μg/mL) and DBM-CBD-bFGF (5 μg/mL) + BMP_2 (300 μg/mL) are probably beneficial in preserving the vertical dimension of the alveolar ridge, but not for the horizontal dimension. There were slight alterations of the alveolar ridge dimension and shape in the groups filled with collagen bound with growth factors compared to the control and blank groups, but no adverse effects were observed. Additional modified protocols of CBD-growth factors and follow-up studies are required to counteract the contraction of the marginal ridge that occurs after tooth extraction.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Author Contributions

BZ has contributed to validation and investigation. JZ and TQ have contributed to formal analysis and investigation. JH and CG have contributed to concetualization and methodology. All authors were involved in the drafting and critical revision of the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The animal studies were performed after approval from the Institutional Animal Care and Use Committee (IACUC) at Peking University (IACUC approval No.LA2020514).

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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