



Effect of fibrin glue on the healing efficacy of deproteinized bovine bone and autologous bone in critical-sized calvarial defects in rats

Chengwei Tu^{1,4} · Aisha Bajwa² · Andi Shi^{2,4,5} · Gang Wu^{2,3} · Jingxiao Wang¹

Received: 5 March 2021 / Accepted: 30 September 2021 / Published online: 29 January 2022
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2021

Abstract

Objectives In order to verify the hypothesis that fibrin glue (FG) is able to seal the area of bone grafting and facilitate bone regeneration.

Materials and methods Twenty-one Sprague–Dawley rats with critical-sized calvarial bone defects were randomly assigned to three groups: (A) co-administrated deproteinized bovine bone (DBB) and autologous bone grafts with FG [fibrin (+)], (B) co-administrated DBB and autologous bone grafts without FG [fibrin (–)], and (C) no graft as control. Four weeks and 8 weeks later, micro-CT analysis and histomorphometric analysis were carried out to evaluate following parameters: bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp), percentage of new bone area (Pe.NB), average thickness of bone defect (Th.BD), average thickness of basal bone (Th.BB), and percentage of new bone in center of the skull defect (Pe.NBc).

Results BV/TV, Tb.Th, and Tb.N in fibrin (–) group were significantly higher than that of fibrin (+) group ($p=0.008$, 0.000 , 0.007 , respectively) and control group ($p=0.004$, 0.001 , and 0.007 , respectively) at 8 weeks. Pe.NB in fibrin (–) group ($33.67 \pm 11.72\%$) was significantly higher than that of fibrin (+) group ($12.33 \pm 3.21\%$) ($p=0.038$) and control group ($9.66 \pm 8.50\%$) ($p=0.045$) at 8 weeks. Pe.NBc in fibrin (–) group ($12.05 \pm 3.91\%$) was significantly higher than that of fibrin (+) group ($4.79 \pm 1.21\%$) ($p=0.005$) and control group ($0.00 \pm 0.00\%$) ($p=0.000$) at 4 weeks.

Conclusions Administration of both DBB and autograft stimulates calvarial bone defect regeneration, while combination of FG does not additionally accelerate new bone formation.

Clinical relevance The use of fibrin to cement traditional bone graft materials in oral clinical practice requires caution.

Keywords Fibrin glue · Bone defect · Deproteinized bovine bone · Bone regeneration · Micro-CT · Rats

Chengwei Tu, Aisha Bajwa, and Andi Shi contributed equally to this work.

Gang Wu and Jingxiao Wang shared the last authorship.

✉ Gang Wu
g.wu@acta.nl

✉ Jingxiao Wang
wangjingxiao@zju.edu.cn

¹ Department of Stomatology, the First Affiliated Hospital, Wenzhou Medical University, Wenzhou, Zhejiang 325006, People's Republic of China

² Department of Oral and Maxillofacial Surgery/Pathology, Amsterdam UMC and Academic Center for Dentistry Amsterdam (ACTA), Vrije Universiteit Amsterdam (VU), Amsterdam Movement Science (AMS), Amsterdam, the Netherlands

³ Department of Oral Cell Biology, Academic Center for Dentistry Amsterdam (ACTA), University of Amsterdam

(UvA) and Vrije Universiteit Amsterdam (VU), Amsterdam, Netherlands

⁴ Laboratory for Myology, Department of Human Movement Sciences, Faculty of Behavioural and Movement Sciences, Vrije Universiteit Amsterdam (VU), Amsterdam Movement Sciences (AMS), Amsterdam, the Netherlands

⁵ Department of Prosthodontics, Affiliated Stomatology Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangdong Engineering Research Center of Oral Restoration and Reconstruction, Guangzhou Key Laboratory of Basic and Applied Research of Oral Regenerative Medicine, Guangzhou, Guangdong 510182, People's Republic of China

Introduction

Critical and irregular bony defects sometimes may exceed the healing capability of osteogenic tissue resulting in delayed re-ossification or non-unions [1, 2]. In such clinical challenge, additional bone grafting techniques, bone substitutes and materials are required to promote bone healing. Autologous bone has commonly been referred as the gold standard for bone grafting procedures [3]. However, it has some disadvantages associated with complications regarding harvesting procedures, such as donor site morbidity, infections, chronic pains, limited graft availability, and unpredicted graft resorption rate [4]. A xenograft such as a deproteinized bovine bone (DBB) mineral can provide a scaffold and have osteoconductive properties, leading to new bone formation [5]. The bone grafting procedure most commonly used in clinical alveolar bone augmentation surgery is the usage of mixed DBB powder and autologous bone debris, in which the proportion of autologous bone debris is usually 50–60% [6, 7]. The combination of autologous bone and xenograft takes advantage of each graft, reducing the amount of autologous bone as well as decreasing the healing time compared with using bone graft substitutes alone [8].

Mechanical instability and unfavorable displacement of the graft are two major concerns when grafting in the oral environment. Since particulated autologous bone chips and xenograft granules can hardly be immobilized in the bone defect, the displacement or deformation of the graft is often found in clinical practice, which may lead to poor clinical outcomes such as delayed osteogenesis, even infection [9]. Fibrin glue (FG) is a natural sealant used to create a fibrin clot for hemostasis and/or wound healing [1, 10, 11]. It has been widely used in many surgical disciplines including cranioplasty [12], spine surgery [13, 14], and maxillofacial surgery [10, 15, 16]. Beside its hemostatic capabilities, FG has been employed as scaffolds for bone and cartilage tissue engineering [17–19]. It has been studied in vitro studies that the addition of fibrin network to collagen sponge increased the osteoblast differentiation in a dose-dependent way, suggesting that these materials may favor bone repair [20]. Several in vivo and clinical studies have evaluated the influence of FG on bone repair, with contradictory results [12, 21].

There are various types of commercial fibrin glues in clinic, and the composition of them is different [22, 23]. In our study, a Chinese brand fibrin glue (FIBINGLURAAS®) was adopted because it contains only fibrin and thrombin with no other additives (such as aprotinin, coagulation factor, BMP, etc.), which can eliminate the interference of other factors and is more suitable for experimental research. The effect of fibrin glue on bone regeneration in the defect region repaired with autologous bone, deproteinized bovine bone, and FG is unknown. The aim of this study was to primarily

testify the feasibility of using fibrin glue to stabilize graft particulates in bone defects without compromising bone regeneration. Hence, the present pilot study was designed to test the hypothesis that fibrin glue improves the bone regeneration of the calvarial defects in rats when treated with autologous bone and deproteinized bovine bone at a ratio of 1:1.

Material and methods

Preparation for DBB and fibrin glue

Deproteinized bovine bone granules (Bio-Oss®, 0.25–1.0 mm, Geistlich Biomaterials, Wolhusen, LU, Switzerland) were filtered by a filter screen with a hole diameter of 0.75 mm then separated into single packages, each of which containing about 0.02 g Bio-Oss® in the diameter of 0.25–0.75 mm, which was confirmed under an optical microscope. This preparation was carried out in a laminar flow cabinet to keep the material sterile.

Fibrin glue (FIBINGLUEAAS, RAAS, Shanghai, China), a combination of human fibrinogen and thrombin, was prepared immediately before using according to the manufacturer's instructions. In brief, lyophilized fibrinogen (50 mg/mL) was dissolved in 2 mL sterilized water using a syringe, gently agitated the vial until the fibrinogen was completely reconstituted that the solution should be clear and have no undissolved particles. In another flask, a solution of thrombin (500 IU/mL) was combined with calcium chloride and kept at 37 °C. Fibrinogen and thrombin with calcium chloride were placed in separate syringes connected by a Y-piece that allowed them to mix in 1:1 (v/v) immediately before implantation in bone defect.

Animal experiment and surgical procedure

The research protocol was submitted to and approved by the Ethical Committee for Animal Research at the Faculty of Wenzhou Medical University, Zhejiang, China (catalogue number: wyd2017-0021).

Twenty-one male Sprague–Dawley rats weighting 250–300 g were obtained from the Wenzhou Medical University Animal Research Unit. The rats were housed in plastic cages at the temperature of 29 ± 3 °C, under natural day/night cycle. They were allowed to adapt to the new environment for 1 week prior to the study. For this animal model, a sample size of $n = 3–4$ animals per group per time point is commonly adopted [24, 25]. According to the results of sample power analysis, the sample size was set to be 3–4 per group of each observation point. The follow-up times of 4 weeks and 8 weeks, respectively representing the early and

later period of osteogenesis, were reasonable to be the observation time for rat calvarial defect models [12]. Hence, the rats were checked for general health, weighed, numbered, and randomized into three groups:

- A Group with fibrin [fibrin (+)] ($n=7$): DBB, autologous bone, mixed in 1:1 (w/w), and fibrin glue [0.1 mL fibrinogen (50 mg/mL) mixed with 0.1 mL thrombin (500 IU/mL)]
- B Group without fibrin [fibrin (-)] ($n=7$): DBB, autologous bone, mixed in 1:1 (w/w)
- C Group control ($n=7$): without bone graft

For the random allocation of the rats to three groups at two times, we first numbered all the rats and then used the formula “=rand()” to generate a random number list. Briefly, we first numbered all the rats and put the numbers in a row in Excel. In the right adjacent row, we used the formula “=rand()” to generate a row of random numbers between 0 and 1, and then we sorted both row in ascending order in the random number row so that the rats were also randomly aligned.

10% chloral hydrate solution (0.3 mL/100 g body weight) (Solarbio, Beijing, China) was injected for rats to achieve appropriately anesthesia before shaving the calvaria and disinfected with 5% povidone-iodine alcoholic solution (MINSHENG, Hangzhou, China). An incision was made along the sagittal suture to expose the parietal bones. An 8-mm-in-diameter skull defect was created in the middle of skull bone with an 8 mm diameter trephine by removing both external and internal cortical and preserving the dura mater. The dental drill was set at 10,000 revolutions per minute (RPM), and the trephine tip was cooled with normal saline flushes during the burring process. The bone defects were filled with different materials according to the grouping

mentioned above. The cranial bone flap was served as the source of autologous bone, which was fully crushed with a tiny needle holder to bone mortars and then mixed with DBB granules of equal quality (about 0.02 g for each defect). After the suture of the periosteum with 4–0 VICRYL rapide (Ethicon, the USA), the skin was sutured with 3–0 MER-SILK (Ethicon, the USA) (Fig. 1). Following skin closure, the animals received a single dose of long-acting benzathine benzylpenicillin solution (150,000 IU/100 g body weight) (NCPC, Heibei province, China). After 4 and 8 weeks, the rats were euthanized and the calvaria were harvested and processed for micro-CT scanning and histomorphometric analyses.

Micro-CT scanning and morphometric analysis

Samples that have been fixed in 10% buffered formalin for 36 h were submitted to micro-computed tomography (micro-CT) for morphometric analysis using the SCANCO MicroCT μ 100 system (SCANCO Medical, Switzerland). Images were acquired at a resolution of 10 μ m, 80 kV and 100 μ A and reconstructed using the imaging software (SCANCO MicroCT μ 100 evaluation, SCANCO Medical, Switzerland). To clearly show native bone, including autogenous bone and new bone, the gray value was set at 0–0.075. The smoothing was set at 1, ring artifact correction was set at 5, and beam hardening correction was set at 20%. We reconstructed three-dimensional images of all the hard tissues in the defect area of the rat skull. On each micro-CT images, we manually delineated the DBB according to the morphology (DBB was a polygonal particles with pores) and much higher density of DBB than newly formed bone tissue, thereby removing the DBB from the images [26, 27], and the bone tissue (including autogenous bone and new bone) was evaluated for morphometric parameters, including bone

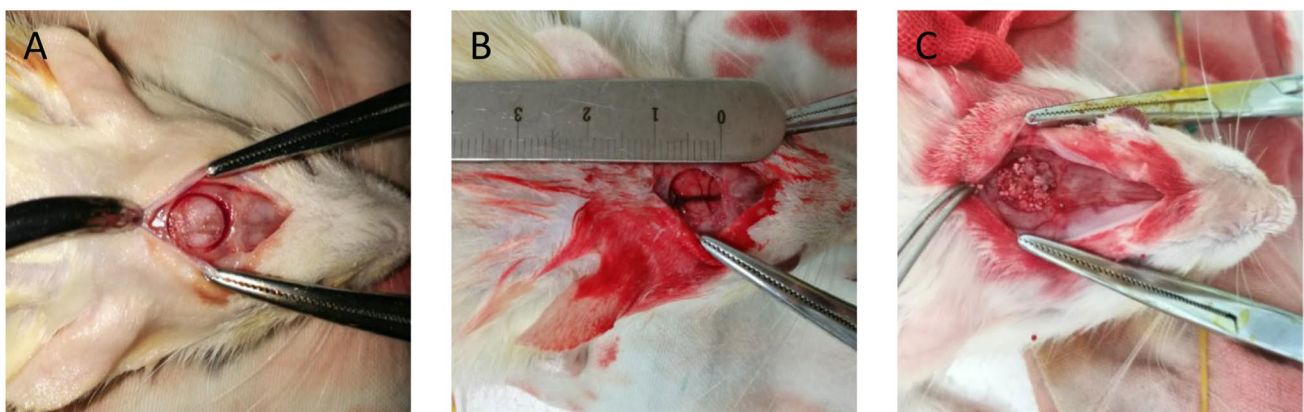


Fig. 1 Operation of creating calvarial defect and implanting repair materials. **A** 8 mm-diameter circular bi-cortical cut was made by trephine burr in the parietal bone. **B** 8 mm-diameter bi-cortical parietal

bone defect came out after removing the segment. **C** The bone defects were filled with 50% DBB and 50% autologous bone in the presence or absence of fibrin

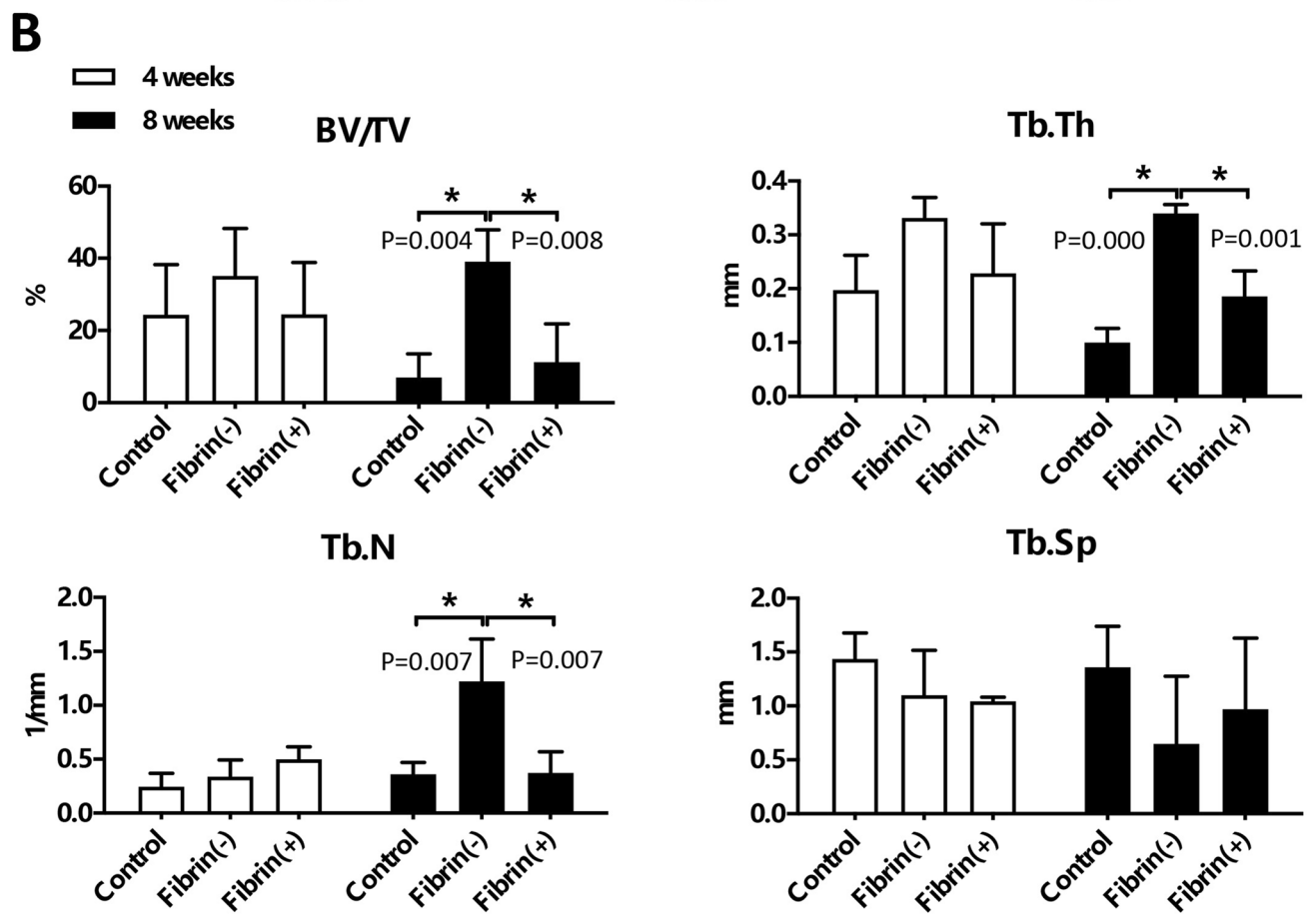
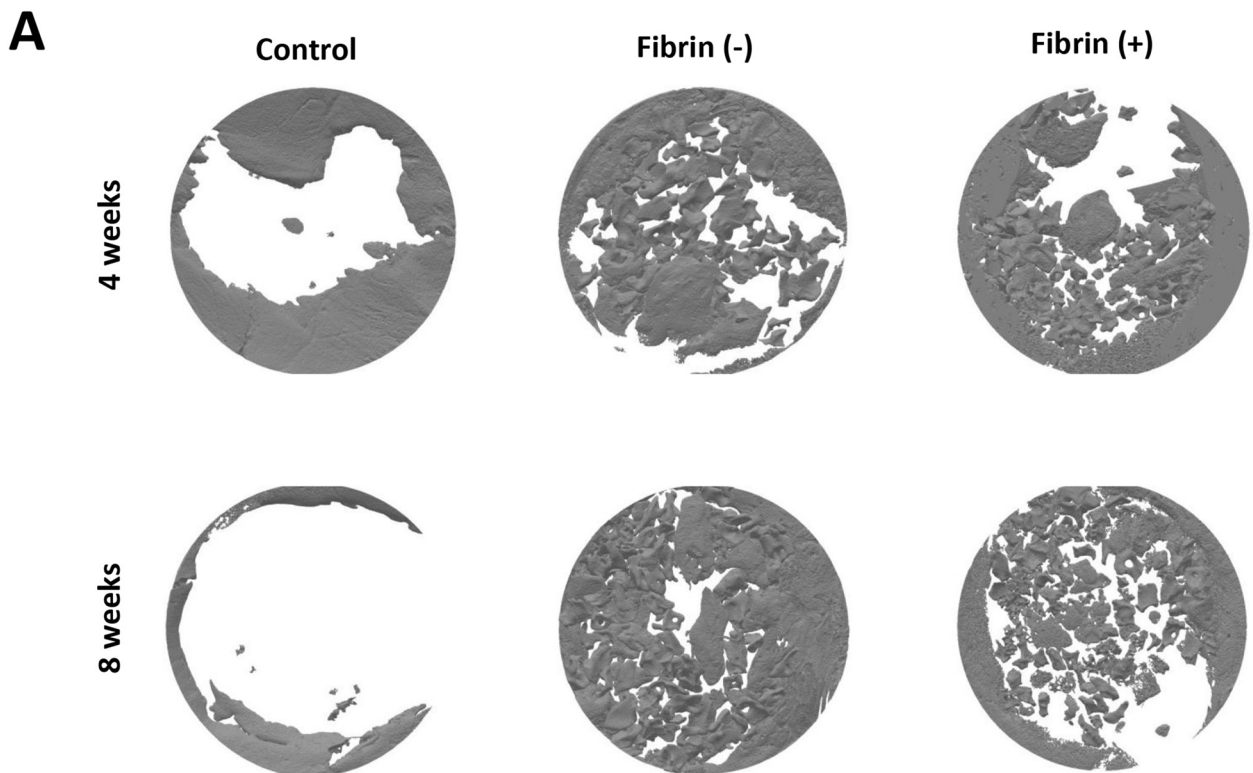


Fig. 2 Deproteinized bovine bone and autograft increased bone formation of rat calvarial bone defect in the absence of fibrin. **A** 3D reconstruction images from micro-computed tomography (micro-CT). **B** Morphometric parameters, including percentage of new bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) were evaluated. * $p < 0.05$ indicates significant difference

volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Th.N), and trabecular separation (Tb.Sp).

Histomorphometric analyses

Undecalcified specimens were fixed in 4% formaldehyde solution. Afterwards, the samples were dehydrated in a series of graded ethanol solutions and embedded in methyl methacrylate. The samples were cut along their vertical axis into 10 slices of 500- μm thickness [28] at an interval of 1 mm using a precise microtome (Leica SP 1600®, Leica Microsystems B.V, Germany). Five alternate sections from each sample were mounted separately on plexiglass holders and marked with numbers. Samples' surfaces were smoothed using polishing disks (micro-Cut Discs, Beuhler, USA) with different paper roughness from course to fine grit, 600p, 1200p, 2500p, and 4000p respectively. The slices were mounted on plexiglass holders, polished, and surface-stained with McNeal's tetrachrome [29], basic fuchsin, and toluidine blue O [30]. Using this protocol, newly formed bone stains deep red, cell nuclei blue, collagen fibers pink, and the calcium-phosphate coating pale red. The histological sections were photographed with light microscopy (Axioskop 40, Zeiss, Germany) at a final magnification of 200 \times and microscopy (AxioCamHR, Zeiss, Germany) at a final magnification of 1.6 \times for stereological estimation. Blinded, the percentage of new bone area (Pe.NB), DBB particles (Pe.DBB), and autologous bone (Pe.AB) were measured for each group using point counting technique [31]. Average thickness of bone defect (Th.BD) was calculated by divided total points in the defect by length of defect (total points horizontally). Average thickness of bilateral basal bone (Th.BB) was measured. Percentage of new bone formation in the center of the defect (Pe.NBc) was determined by selecting one section with the widest defect. The defect was then divided into three parts and the center was marked to count the new bone ($n = 3\text{--}4$).

Statistical analysis

The results of micro-CT and histomorphometric analysis are presented as mean values and standard deviation (mean \pm SD). Statistical analysis was performed with GraphPad Prism 8, USA). If the difference between groups was significant (one-way ANOVA), a post hoc

(Student–Newman–Keuls) was performed. Statistical significance was defined as $p < 0.05$.

Results

Micro-CT scanning

Skull defects were filled with mineralized tissue (including the new bone, the slow resorbable DBB, and residual autologous bone) when both DBB and autologous bone were given regardless of fibrin glue at 4 and 8 weeks. In contrast, in control groups, new bone formation was only observed at the defect's margins but not in the center of defects at 4 and 8 weeks. Fibrin (–) group had less distinct defect boundary in comparison to fibrin (+) group at the same time points. After 8 weeks, the amount of hard tissue was more prominent in fibrin (–) group than in fibrin (+) group (Fig. 2A).

The bone quality was estimated using the following parameters: BV/TV (bone volume/tissue volume, %), Tb.N (trabecular number, 1/mm), Tb.Th (trabecular thickness, mm), and Tb.Sp (trabecular separation, mm). After 4 weeks, there was no difference in BV/TV among fibrin (+) group ($24.5 \pm 14.4\%$), fibrin (–) group ($35.1 \pm 13.2\%$), and control group ($24.3 \pm 13.9\%$). At 8 weeks, the percentage of BV/TV in fibrin (–) group ($39.1 \pm 8.8\%$) was significantly higher than those of fibrin (+) group ($11.3 \pm 10.6\%$) ($p = 0.008$) as well as control group ($7.0 \pm 6.5\%$) ($p = 0.004$). At each time point, no significant difference was detected between fibrin (+) and control group. At 4 weeks, no significant difference was seen in Tb.Th among the three groups. At 8 weeks, Tb.Th significantly increased in fibrin (–) group (0.34 ± 0.02 mm) when compared to control group (0.10 ± 0.03 mm) ($p = 0.000$) and fibrin (+) group (0.19 ± 0.05 mm) ($p = 0.001$). After 4 weeks, Tb.N showed no significant difference among fibrin (+) (0.50 ± 0.12 1/mm), fibrin (–) (0.34 ± 0.15 1/mm), and control group. At 8 weeks, Tb.N significantly increased in fibrin (–) group (1.22 ± 0.39 1/mm) than fibrin (+) group (0.37 ± 0.20 1/mm) ($p = 0.007$) and control group (0.36 ± 0.11 1/mm) ($p = 0.007$). There was no significant difference among the three groups in Tb.Sp. A decrease tendency of Tb.Sp was found for each group at 8 weeks (Fig. 2B, Table 1).

Histological analysis

Although BV/TV, Tb.Th, and Tb.N increased in defect filled with DBB and autograft in absence of fibrin, it was not clear the shard tissue was the new formed bone or the autograft particles. McNeal staining has been used to stain different tissues. DBB was referred to light pink particles with sharp

Table 1 Micro-CT evaluation of bone regeneration induced by DBB and autologous bone without or with fibrin in calvarial bone defects of rats at 4 and 8 weeks post-operation. The bone quality was estimated using the following parameters: BV/TV (bone volume/tissue volume, %), Tb.N (trabecular number, 1/mm), Tb.Th (trabecular thickness, mm), and Tb.Sp (trabecular separation, mm). All data are presented as mean values together with the standard deviation (mean \pm SD)

Group	BV/TV (%)	Tb.Th (mm)	Tb.N (1/mm)	Tb.Sp (mm)
4 weeks				
Control	24.3 \pm 13.9	0.20 \pm 0.06	0.24 \pm 0.12	1.44 \pm 0.24
Fibrin -	35.1 \pm 13.2	0.33 \pm 0.04	0.34 \pm 0.15	1.10 \pm 0.42
Fibrin +	24.5 \pm 14.4	0.23 \pm 0.09	0.50 \pm 0.12	1.04 \pm 0.04
8 weeks				
Control	7.0 \pm 6.5	0.10 \pm 0.03	0.36 \pm 0.11	1.36 \pm 0.38
Fibrin -	39.1 \pm 8.8 ^{*#}	0.34 \pm 0.02 ^{*#}	1.22 \pm 0.39 ^{*#}	0.65 \pm 0.63
Fibrin +	11.3 \pm 10.6 [#]	0.18 \pm 0.05 [#]	0.37 \pm 0.20 [#]	0.97 \pm 0.66

^{*}Significant difference ($p < 0.05$) between control group and grafting-treated group (with or without fibrin) at the same time point

[#]Significant difference ($p < 0.05$) between fibrin (-) group and fibrin (+) group at the same time point

margin, autograft was stained in red with aligned cancellous bone lacuna inside, and new formed bone was stained in darker red wrapping bone grafts.

After 4 weeks of healing, bone repair is visible in the central portion of the defects in fibrin (+) and fibrin (-) group (Fig. 3A, C). Control group showed empty defect with mild new bone formation at the defect margins. At higher magnification, fibrin (+) and fibrin (-) group augmented defect sites frequently showed the presence of a thin bridge of newly formed bone (woven bone) which is profoundly visible at the margins extending from basal bone as well as from the autologous bone particles. This bone was continuous with the “old bone” tissue of the defect margins as well as inside the defect and contained several DBB particles. The particles appeared to be encapsulated by connective tissue, harboring large numbers of graft particles in fibrin (+) group, while there was less connective tissue encapsulation in fibrin (-) group (Fig. 3, 4 weeks).

After 8 weeks, all the groups tend to have reduced fibrous scar tissue and bone remodeling from the periphery of the defects. In fibrin (+) group, minimum new bone formation in comparison to fibrin (-) group is observed, where ossification was more prominent, although connective tissue was also observed in the interface between basal bone and the grafted bone. At higher magnification, autologous bone particles could not be clearly distinguished anymore, which is a sign of remodeling (Fig. 3, 8 weeks).

Histomorphometric analysis

The histological morphology of new bone was estimated using the following parameters: Pe.NB (the percentage of new bone area, %), Pe.DBB (the percentage of DBB, %), Pe.AB (the percentage of autologous bone, %), Th.BD (the average thickness of bone defect, mm), Th.BB (the average thickness of bilateral basal bone, mm), and Pe.NBc (the percentage of new bone formation in the center of the defect, %) (Table 2).

There was no significant difference in percentage of new bone area (Pe.NB) after 4 weeks among fibrin (+) group (23.75 \pm 2.87%), fibrin (-) group (29.00 \pm 6.33%), and control group (23.67 \pm 7.23%). After 8 weeks, Pe.NB in fibrin (-) group (33.67 \pm 11.72%) was significantly higher than that of fibrin (+) group (12.33 \pm 3.21%) ($p = 0.038$) and control group (9.66 \pm 8.50%) ($p = 0.045$). There was no significant difference between fibrin (+) and control group ($p = 0.638$) (Fig. 4A).

There was no significant difference in the percentage of DBB area (Pe.DBB) between fibrin (+) group (8.50 \pm 2.65%) and fibrin (-) group (7.00 \pm 2.45%) either after 4 weeks ($p = 0.043$) or after 8 weeks between fibrin (+) group (13.67 \pm 5.69%) and fibrin (-) group (9.33 \pm 7.51%) ($p = 0.047$) (Fig. 4B). There was also no significant difference in the percentage of autologous bone (Pe.AB) between fibrin (+) group (10.50 \pm 4.36%) and fibrin (-) group (10.25 \pm 5.19%) either after 4 weeks ($p = 0.943$) or after 8 weeks between fibrin (+) group (3.33 \pm 2.08%) and fibrin (-) group (8.00 \pm 5.00%) ($p = 0.209$). At both time points, no autograft was found in control groups (Fig. 4C).

Average thickness of bone defect (Th.BD) significantly increased in fibrin (+) (1.28 \pm 0.45 mm) ($p = 0.043$) after 4 weeks. After 8 weeks, Th.BD in fibrin (+) group (1.45 \pm 0.37 mm) ($p = 0.033$) and fibrin (-) group (1.53 \pm 0.05 mm) significantly increased ($p = 0.000$) as well. Difference of Th.BD between fibrin (+) and fibrin (-) was not significant at both time points (Fig. 4D).

After 4 weeks, average thickness of bilateral basal bone (Th.BB) was not significantly different in each group, whereas after 8 weeks bilateral Th.BB was significantly thicker in group fibrin (+) (1.44 \pm 0.05 mm) ($p = 0.001$) and group fibrin (-) (1.57 \pm 0.13 mm) ($p = 0.0004$) when compared with control group (Fig. 4E).

Four weeks after the grafting, new bone formation in the center of the skull defect (Pe.NBc) significantly increased in fibrin (-) (12.05 \pm 3.91%) when compared to control (0.00 \pm 0.00%) ($p = 0.000$) and fibrin (+) (4.79 \pm 1.21%) ($p = 0.005$). On the contrary, there was no significant difference of Pe.NBc among the three groups after 8 weeks (Fig. 4F).

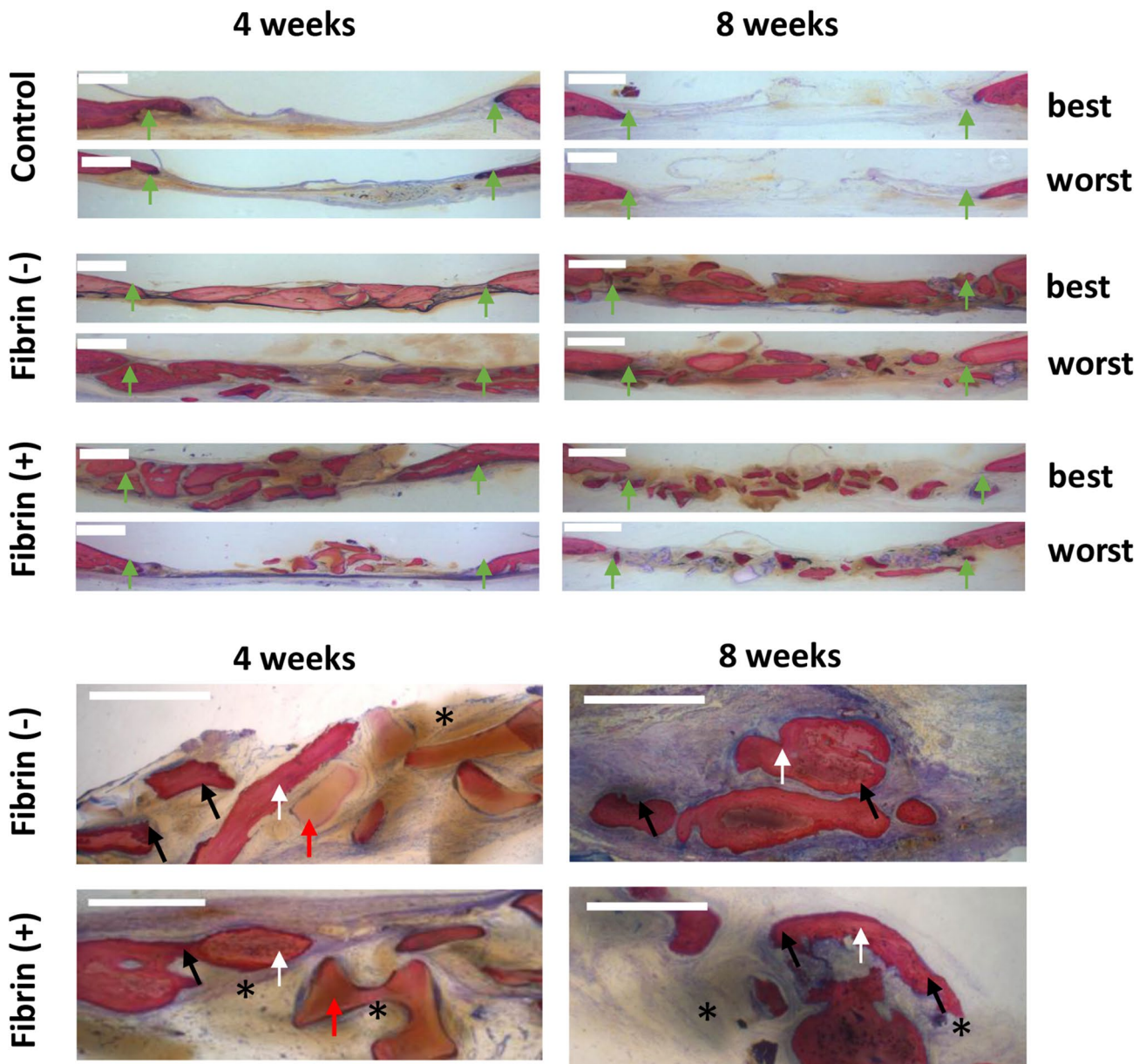


Fig. 3 Light micrographs of the bone grafting sections in the presence or absence of fibrin at time of 4 weeks and 8 weeks post-operation at low and high magnifications. Green arrow, edge of bone

defect; black arrow, new bone; red arrow, DBB; white arrow, autologous bone; asterisk (*), connective tissue wrap. Magnification bars: 1 mm

Discussion

Xenograft such as deproteinized bovine bone (DBB) and autologous bone grafts are co-administrated for grafting critical-sized bone defects that exceed the self-healing capacity. However, mechanical forces generated by oral muscle movements and chewing result in a mechanically instable microenvironment, which may lead to dispersion of bone graft particles and impairment of bone regeneration. This pilot study was designed to test the hypothesis that fibrin glue could be used as an adhesive for bone graft particles

without negative effect on bone regeneration. The rat calvarial defect model (8 mm in diameter) adopted in our experiment is economical, practical, and reproducible, and can fully simulate the pathological state of cranio-maxillofacial bone defects [32, 33]. Micro-CT-based morphometric and histology-based histomorphometric analyses were carried out using rat calvarial 8 mm critical-sized defect model. At 4 weeks and 8 weeks post-surgery, fibrin (-) group showed more bone formation when compared to fibrin (+) group indicating that fibrin glue may inhibit osteogenesis within the first 8 weeks of bone growth.

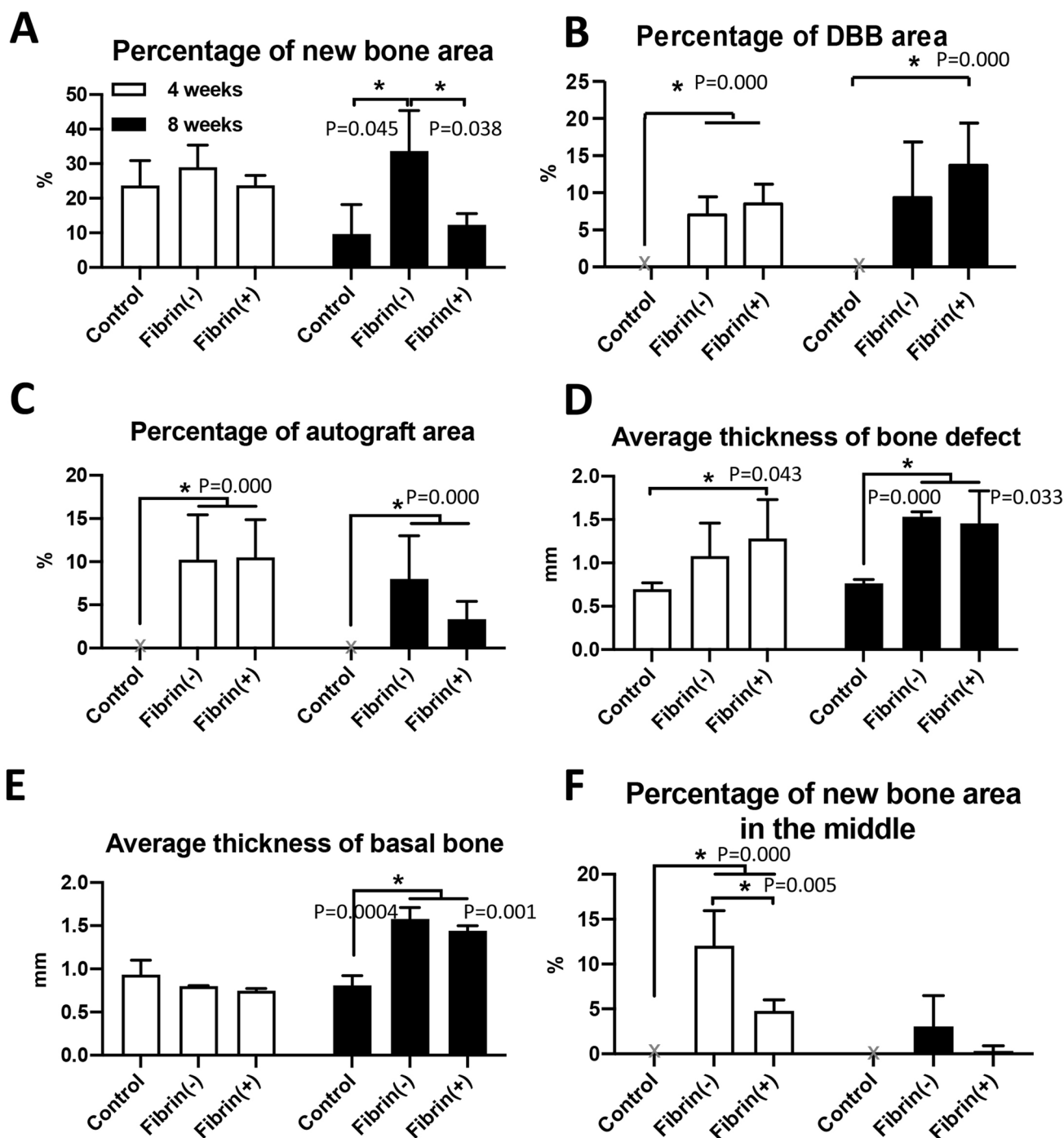


Fig. 4 Relative total new bone area increased in fibrin (-) group. Percentage of area of new bone area (A), DBB area (B), autograft area (C), average thickness of bone defect (D), average thickness of basal bone (E), and percentage of new bone area in the middle of

bone defect (F) were analyzed. All data are presented as mean values together with the standard deviation (mean \pm SD). Asterisks indicate statistically significant differences between the groups, * $p < 0.05$ ($n = 4$ for 4 weeks group, $n = 3$ for 8 weeks group)

We evaluated the effect of the clinically available fibrin glue on the efficacy of bone grafts (mixture of DBB and autologous bone) in healing the critical-size bone defects. For the rat calvarial defects, the 8-mm-in-diameter bone defects are generally accepted to be of critical size, which

cannot heal spontaneously [34]. Typically, only one defect can be created per rat due to the limited area of rat calvaria. Another frequently adopted model is 5-mm-in-diameter bone defects. In comparison with the 8-mm-in-diameter bone defects, the 5-mm-in-diameter bone defect can enable

Table 2 Histomorphometric analysis of bone regeneration induced by DBB and autologous bone without or with fibrin in calvarial bone defects of rats at 4 and 8 weeks post-operation. The histological morphology of new bone was estimated using the following parameters: Pe.NB (the percentage of new bone area, %), Pe.DBB (the percentage

of DBB, %), Pe.AB (the percentage of autologous bone, %), Th.BD (the average thickness of bone defect, mm), Th.BB (the average thickness of bilateral basal bone, mm), and Pe.NBc (the percentage of new bone formation in the center of the defect, %). All data are presented as mean values together with the standard deviation (mean \pm SD)

Group	Pe.NB (%)	Pe.DBB (%)	Pe.AB (%)	Th.BD (mm)	Th.BB (mm)	Pe.NBc (%)
4 weeks						
Control	23.67 \pm 7.23	0.00 \pm 0.00	0.00 \pm 0.00	0.70 \pm 0.07	0.93 \pm 0.17	0.00 \pm 0.00
Fibrin –	29.00 \pm 6.32	7.00 \pm 2.45	10.25 \pm 5.19	1.08 \pm 0.38	0.80 \pm 0.01	12.05 \pm 3.91 ^{##}
Fibrin +	23.75 \pm 2.87	8.50 \pm 2.65	10.50 \pm 4.36	1.28 \pm 0.45 [*]	0.75 \pm 0.03	4.79 \pm 1.21
8 weeks						
Control	9.67 \pm 8.51	0.00 \pm 0.00	0.00 \pm 0.00	0.76 \pm 0.05	0.81 \pm 0.12	0.00 \pm 0.00
Fibrin –	33.67 \pm 11.72 ^{##}	9.33 \pm 7.51	8.00 \pm 5.00	1.53 \pm 0.05 [*]	1.58 \pm 0.13 [*]	3.03 \pm 3.45
Fibrin +	12.33 \pm 3.22 [#]	13.67 \pm 5.69	3.33 \pm 2.08	1.46 \pm 0.37 [*]	1.44 \pm 0.06 [*]	0.33 \pm 0.57

^{*}Significant difference ($p < 0.05$) between control group and grafting-treated group (with or without fibrin) at the same time point

[#]Significant difference ($p < 0.05$) between fibrin (–) group and fibrin (+) group at the same time point

the creation of two bone defects in one animal, which dramatically reduce the needed number of animals [35]. On the other hand, the 5-mm-in-diameter bone defects are of subcritical size and thus may heal without intervention [36]. Studies whose primary goal is the regeneration of bone in a defect where natural regenerative capacity no longer suffices should avoid such designs. Additionally, the potential for interactions between these two adjacent defects should be considered. Specifically for our current study, we adopted the clinically available particulate DBB and on-the-spot harvested autologous bone chips as bone-defect-healing materials and their size ranged from 0.25 to 1 mm. In our preliminary study, we adopted the 5-mm-in-diameter bone defects and found that only few materials might be placed fit into the defects, which resulted in a large heterogeneity among different defects. Therefore, we determined to adopt the 8-mm-in-diameter bone defects.

In our study, fibrin (–) group had dominance in BV/TV, Tb.Th, and Tb.N values in comparison to fibrin (+) and control group during the whole observation period. This suggests that fibrin glue compromises bone formation by decreasing trabecular thickness and trabecular number during the bone production phase. Furthermore, Tb.Sp showed a decrease tendency after 8 weeks of healing in all the three groups, while no significant difference of Tb.Sp among the groups was found. Our data showed that 8 weeks post-operation, the values of Tb.Th/Tb.N (0.34 mm/1.22 mm⁻¹) in the fibrin (–) group at this time point were significantly higher than those in the control group (0.1 mm/0.36 mm⁻¹) and the fibrin (+) group (0.18 mm/0.37 mm⁻¹). In a similar pattern, Tb.Sp of fibrin (–) group already showed a much lower mean value (0.65 mm) in comparison with those in the control group (1.36 mm) and in the fibrin (+) group (0.97 mm), in which no statistical difference was found. Such a phenomenon might be partially explained by the less

sensitivity of this parameter due to the presence of DBB in the grafts. DBB is well known to have a very low degradation rate [37]. Within the short monitoring span in this study, we assumed that DBB degraded only very mildly. Consequently, the constant presence of DBB resulted in a relatively stable separation of trabecular bone. On the other hand, the significantly higher Tb.N and Tb.Th caused certain decrease of Tb.Sp. Therefore, the joint effect of these two factors made the Tb.Sp less sensitive than Tb.N and Tb.Th, which caused the insignificantly lower level of Tb.Sp in the fibrin (+) group than the other two groups.

Figure 2A shows the 3D reconstruction of the mineralized tissues and materials in the rat calvarial bone defects. Different from quantitative analysis, the DBB could not be excluded for 3D reconstruction; therefore, the presence of DBB made the difference of new bone not significant as the quantitative analysis. Our results indeed showed the decreases of BV/TV in the groups of control and fibrin (+), which was not statistically significant. The decreases in both groups might be attributed to different biological mechanisms. In the control group, the skull defect was not filled with any bone-defect-filling materials, leading to a mechanically unstable microenvironment. Mild bone regeneration took place in response to acute injury in the peripheral area of the defects at 4 weeks post-operation. However, the newly formed bone might be thereafter, to some extent, resorbed by osteoclasts in this mechanically unstable microenvironment, which led to a relatively lower BV/TV at 8 weeks post-operation. As for the group of fibrin (+), the degradation might be mediated by the activities of foreign-body giant cells [38] triggered by the presence of fibrin glue. In the fibrin (–) group, it was also reasonable that most bone regeneration happens in the first 4 weeks since the fresh particulated autologous bone graft was the only driving force for bone regeneration. Although no significant changes in

BV/TV from 4 to 8 weeks, we did observe that the trabecular numbers were greatly increased at 8 weeks, which indicated an active remodeling in this period.

The histological results showed new bone formation in grafted groups in contrast with the control group, while there was less new bone formation in fibrin (+) group during the same time interval. Our findings showed no differences between the percentage of Bio-Oss and autograft area which prevent the discriminative evaluation of new formed bone when the amount of bone scaffold was similar regardless of fibrin (Fig. 3B, C). The compromised bone regeneration in fibrin (+) group could be due to fibrin-induced inflammatory response [39], interference of fibrin with natural blood clot formation, foreign body reaction [40, 41], or the bone resorption ability of thrombin by boosting the osteoclastic activity [42]. As it has been observed in this study, fibrous encapsulation of grafted particles could separate the graft particles and hamper the integration of deproteinized bovine mineral particles with autologous bone and newly formed bone [43, 44]. Connective tissue which is rich in fibrinogen may enhance granulation tissue formation and deposition of collagen [45]. Fibrin glue degradation starts as early as 24 h with a half-life of 4–6 days, which means that the fibrin glue in the body will be completely absorbed within 30 days [1]. Therefore, the fibrous capsule that we observed at 8 weeks was not the residual fibrin, but probably foreign body reaction in the body. Detection of macrophages will be carried out in further study.

Fibrin immobilizes bone grafts and maintains space of osteogenesis. Average thickness of skull defect was increased in presence of fibrin since 4 weeks (Fig. 4D). This suggests fibrin glue can act as a scaffold to maintain the thickness of defect and adhere to the bone grafts. Given the high thrombin concentration in fibrin, the sealants provided short fibers and small pores to immobilize the bone graft [46]. The clustered bone grafts encapsulated by fibrin facilitated the bone regeneration as it was convenient to grasp bone graft as a whole. Increased osteogenesis space promoted osteogenic cells migration and increased new bone formation [47]. Despite the positive effect on stabilizing the bone grafts, there was a drawback of fibrin. The increased thickness of basal bone and bone defect after 8 weeks was not significantly different between fibrin (–) and fibrin (+) groups (Fig. 4E). Study in vitro showed that cell migration through the scaffold was increased only if lower concentration of thrombin and fibrinogen was in comparison to high concentrations of fibrinogen and/or thrombin [48, 49]. The increased thickness of basal bone and the percentage of new formed bone in the middle of bone defect were resulted from the application of bone graft rather than application of fibrin which may prevented infiltration of osteoblasts and vessel cells [46] (Fig. 4E, F). Therefore, our results demonstrate that fibrin maintains structure of bone grafts which provide

osteogenic space while it is not an ideal matrix for cell invasion and osteogenesis.

Physiological and mechanical characteristics of the human clinical situation can closely be represented by animal models, but it is essential to consider the anatomic variation between human bone and each animal model when results of such studies are translated into clinical practice. Wide range of fibrinogen concentrations between 50 and 115 mg have been used in different experimental setups. In general, there is a tendency towards fibrinogen concentration between 75 and 110 mg [50]. As we know from the literatures that higher concentrations of fibrinogen could adversely affect the osteogenic potential of fibrin glue, it is obvious that more in vivo studies are needed to find the most optimal treatment regimen for bone regeneration in critical-sized defects when incorporating fibrin glue as an adhesive. Small sample size, shorter follow-up, and higher variation in the data are the limitations of this study. Further studies should be taken to see the effects of fibrin at different dosages/concentrations with larger sample size and longer healing period. Bigger animal models and a bigger sample size would be favorable. Another limitation of this study was the lack of immunohistochemical analysis of inflammatory cells (CD45, CD31, FSP1), osteoclasts (TRAP), and osteogenic markers. The main goal of this study was to primarily testify the feasibility of using clinically available fibrin glue to stabilize graft particulates (mixture of DBB and autologous bone) in bone defects without compromising bone regeneration. However, our results showed the clinically available fibrin glue might compromise the bone healing efficacy of the bone grafts. Therefore, in our subsequent study, we will try to testify other hydrogels, such as diluted fibrin glue, photocrosslinkable GelMA [51–54]. When we find the hydrogels that can both stabilize grafts and also promote their bone healing efficacy, we will further perform the immunohistochemical analysis to systematically assess the performance of the hydrogels. Furthermore, fibrin glue can be used as an injectable delivery system in bone tissue regeneration. This would make its use favorable in the clinical settings.

Conclusion

Combination of fibrin with both DBB and autograft does not additionally accelerate new bone formation. Nevertheless, fibrin is beneficial to bone autograft immobilization and adhesion. Therefore, the clinical application of fibrin to fix bone graft materials should be cautious.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00784-021-04217-8>.

Funding The work was supported by the Foundation of Science and Technology Department Project of Zhejiang Province (2017C33168) as well as the Basic Scientific Research Projects of Wenzhou (2020Y0362, 2021Y0983), China.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Informed consent For this type of study, formal consent is not required.

References

- Khodakaram-Tafti A, Mehrabani D, Shaterzadeh-Yazdi H (2017) An overview on autologous fibrin glue in bone tissue engineering of maxillofacial surgery. *Dent Res J (Isfahan)* 14(2):79–86
- Gao R et al (2018) Local application of lactoferrin promotes bone regeneration in a rat critical-sized calvarial defect model as demonstrated by micro-CT and histological analysis. *J Tissue Eng Regen Med* 12(1):e620–e626
- Myeroff C, Archdeacon M (2011) Autogenous bone graft: donor sites and techniques. *J Bone Joint Surg Am* 93(23):2227–2236
- Caubet J et al (2015) Gene expression and morphometric parameters of human bone biopsies after maxillary sinus floor elevation with autologous bone combined with Bio-Oss(R) or BoneCeramic(R). *Clin Oral Implants Res* 26(6):727–735
- Papageorgiou SN et al (2016) Comparative effectiveness of natural and synthetic bone grafts in oral and maxillofacial surgery prior to insertion of dental implants: Systematic review and network meta-analysis of parallel and cluster randomized controlled trials. *J Dent* 48:1–8
- Lohmann P et al (2017) Bone regeneration induced by a 3D architected hydrogel in a rat critical-size calvarial defect. *Biomaterials* 113:158–169
- De Santis E et al (2017) Healing at implants installed concurrently to maxillary sinus floor elevation with Bio-Oss(R) or autologous bone grafts. A histo-morphometric study in rabbits. *Clin Oral Implants Res* 28(5):503–511
- Simunek A et al (2008) Deproteinized bovine bone versus beta-tricalcium phosphate in sinus augmentation surgery: a comparative histologic and histomorphometric study. *Int J Oral Maxillofac Implants* 23(5):935–942
- Myhre AP et al (2006) Postoperative bone graft displacement: an unusual sign of infection following posterior spinal fusion. *Radiol Case Rep* 1(1):21–23
- Kang GC, Sng KW, Tay AG (2009) Modified technique for frontal sinus obliteration using calvarial bone and Tisseel glue. *J Craniofac Surg* 20(2):528–531
- Abiraman S et al (2002) Fibrin glue as an osteoinductive protein in a mouse model. *Biomaterials* 23(14):3023–3031
- Lappalainen OP et al (2015) Healing of rabbit calvarial critical-sized defects using autogenous bone grafts and fibrin glue. *Childs Nerv Syst* 31(4):581–587
- Masuda S et al (2016) The dural repair using the combination of polyglycolic acid mesh and fibrin glue and postoperative management in spine surgery. *J Orthop Sci* 21(5):586–590
- Liu Z et al (2016) Enhancement of posterolateral lumbar spine fusion using recombinant human bone morphogenetic protein-2 and mesenchymal stem cells delivered in fibrin glue. *J Biomater Appl* 31(4):477–487
- Davis BR, Sandor GK (1998) Use of fibrin glue in maxillofacial surgery. *J Otolaryngol* 27(2):107–112
- Cardaropoli D, Gaveglio L, Cardaropoli G (2013) Vertical ridge augmentation with a collagen membrane, bovine bone mineral and fibrin sealer: clinical and histologic findings. *Int J Periodontics Restorative Dent* 33(5):583–589
- Singh K et al (2011) Fibrin glue: a scaffold for cellular-based therapy in a critical-sized defect. *Ann Plast Surg* 66(3):301–305
- Ahmed TA et al (2011) Fibrin glues in combination with mesenchymal stem cells to develop a tissue-engineered cartilage substitute. *Tissue Eng Part A* 17(3–4):323–335
- Wang ZH et al (2010) Cartilage tissue engineering with demineralized bone matrix gelatin and fibrin glue hybrid scaffold: an in vitro study. *Artif Organs* 34(2):161–166
- Kim BS, Kim JS, Lee J (2013) Improvements of osteoblast adhesion, proliferation, and differentiation in vitro via fibrin network formation in collagen sponge scaffold. *J Biomed Mater Res A* 101(9):2661–2666
- Santos Tde S et al (2015) Effect of collagen sponge and fibrin glue on bone repair. *J Appl Oral Sci* 23(6):623–628
- Le Guehennec L et al (2005) MBCP biphasic calcium phosphate granules and tissucol fibrin sealant in rabbit femoral defects: the effect of fibrin on bone ingrowth. *J Mater Sci Mater Med* 16(1):29–35
- Kim SS, Gwak SJ, Kim BS (2008) Orthotopic bone formation by implantation of apatite-coated poly(lactide-co-glycolide)/hydroxyapatite composite particulates and bone morphogenetic protein-2. *J Biomed Mater Res A* 87(1):245–253
- Liu Z et al (2017) The combination of nano-calcium sulfate/platelet rich plasma gel scaffold with BMP2 gene-modified mesenchymal stem cells promotes bone regeneration in rat critical-sized calvarial defects. *Stem Cell Res Ther* 8(1):122
- Song K et al (2011) Enhanced bone regeneration with sequential delivery of basic fibroblast growth factor and sonic hedgehog. *Injury* 42(8):796–802
- Kapogianni E et al (2019) Comparison of material-mediated bone regeneration capacities of sintered and non-sintered xenogeneic bone substitutes via 2D and 3D data. *In Vivo* 33(6):2169–2179
- Aroni MAT et al (2019) Loading deproteinized bovine bone with strontium enhances bone regeneration in rat calvarial critical size defects. *Clin Oral Investig* 23(4):1605–1614
- Spijker HJ (1978) A procedure for obtaining thin sections of undercalcified bone biopsies embedded in methyl methacrylate. *Microsc Acta* 81(1):17–26
- Schenk RK, AJO, Herrmann W (1984) Preparation of calcified tissues for light microscopy. In: GR Dickson (Ed) Elsevier Science Publishers B.V.
- Schenk RK, OA, Herrmann W (1984) Preparation of calcified tissues for light microscopy. *Methods of calcified tissue preparation*. Elsevier Science Publishers, pp 1–56
- Foldager CB et al (2015) A stereological method for the quantitative evaluation of cartilage repair tissue. *Cartilage* 6(2):123–132
- Spicer PP et al (2012) Evaluation of bone regeneration using the rat critical size calvarial defect. *Nat Protoc* 7(10):1918–1929
- Effendy NM, Khamis MF, Shuid AN (2013) Micro-CT assessments of potential anti-osteoporotic agents. *Curr Drug Targets* 14(13):1542–1551
- Liu T et al (2013) Deproteinized bovine bone functionalized with the slow delivery of BMP-2 for the repair of critical-sized bone defects in sheep. *Bone* 56(1):110–118
- Vajgel A et al (2014) A systematic review on the critical size defect model. *Clin Oral Implants Res* 25(8):879–893

36. Kim KS et al (2010) Small intestine submucosa sponge for in vivo support of tissue-engineered bone formation in the presence of rat bone marrow stem cells. *Biomaterials* 31(6):1104–1113
37. Baldini N, De Sanctis M, Ferrari M (2011) Deproteinized bovine bone in periodontal and implant surgery. *Dent Mater* 27(1):61–70
38. Sheikh Z et al (2015) Macrophages, foreign body giant cells and their response to implantable biomaterials. *Materials (Basel)* 8(9):5671–5701
39. Schwarz N et al (1993) Early osteoinduction in rats is not altered by fibrin sealant. *Clin Orthop Relat Res* 293:353–359
40. Gurevich DB et al (2019) Live imaging the foreign body response in zebrafish reveals how dampening inflammation reduces fibrosis. *J Cell Sci* 133(5)
41. Gurevich DB et al (2018) Live imaging of wound angiogenesis reveals macrophage orchestrated vessel sprouting and regression. *Embo J* 37(13)
42. Song SJ et al (2005) Studies on the receptors mediating responses of osteoblasts to thrombin. *Int J Biochem Cell Biol* 37(1):206–213
43. Carmagnola D, Berglundh T, Lindhe J (2002) The effect of a fibrin glue on the integration of Bio-Oss with bone tissue. A experimental study in labrador dogs. *J Clin Periodontol* 29(5):377–83
44. Carmagnola D et al (2000) Bone healing around implants placed in a jaw defect augmented with Bio-Oss. An experimental study in dogs. *J Clin Periodontol* 27(11):799–805
45. Pieters M, Wolberg AS (2019) Fibrinogen and fibrin: an illustrated review. *Res Pract Thromb Haemost* 3(2):161–172
46. Karp JM et al (2004) Fibrin-filled scaffolds for bone-tissue engineering: an in vivo study. *J Biomed Mater Res A* 71(1):162–171
47. Doganay O et al (2019) Guided bone regeneration using BioGlue as a barrier material with and without biphasic calcium phosphate. *J Craniofac Surg* 30(4):1308–1313
48. Laidmae I et al (2012) Salmon fibrin glue in rats: antibody studies. *Biologicals* 40(1):55–60
49. Weisel JW (2004) The mechanical properties of fibrin for basic scientists and clinicians. *Biophys Chem* 112(2–3):267–276
50. Kim SS, Gwak SJ, Kim BS (2008) Orthotopic bone formation by implantation of apatite-coated poly(lactide-co-glycolide)/hydroxyapatite composite particulates and bone morphogenetic protein-2. *J Biomed Mater Res, Part A* 87(1):245–253
51. Catelas I et al (2006) Human mesenchymal stem cell proliferation and osteogenic differentiation in fibrin gels in vitro. *Tissue Eng* 12(8):2385–2396
52. Kim BS et al (2014) Effects of fibrinogen concentration on fibrin glue and bone powder scaffolds in bone regeneration. *J Biosci Bioeng* 118(4):469–475
53. Zheng Y et al (2021) Neuro-regenerative imidazole-functionalized GelMA hydrogel loaded with hAMSC and SDF-1alpha promote stem cell differentiation and repair focal brain injury. *Bioact Mater* 6(3):627–637
54. Wang L et al (2021) Notoginsenoside R1 functionalized gelatin hydrogels to promote reparative dentinogenesis. *Acta Biomater* 122:160–171

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.