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Article

# Preparation, characterization and biological properties of a novel bone block composed of platelet rich fibrin and a deproteinized bovine bone mineral

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ABSTRACT

Alveolar bone defects caused by tooth loss often lead to challenges in implant dentistry, with a need for development of optimal bone biomaterials to predictably rebuild these tissues. To address this problem, we fabricated a novel bone block using platelet-rich fibrin (PRF) and Deproteinized Bovine Bone Mineral (DBBM), and characterized their mechanical and biological properties. The bone block was prepared by mixing DBBM, Liquid-PRF, and Solid-PRF fragments in various combinations as follows: (1) BLOCK-1 made with Solid-PRF fragments + DBBM, (2) BLOCK-2 made with Liquid-PRF + DBBM, (3) BLOCK-3 made with Solid-PRF fragments + Liquid-PRF + DBBM. The time for solidification and the degradation properties were subsequently recorded. Scanning electron microscopy (SEM) and tensile tests were carried out to investigate the microstructure and mechanical properties of each block. The bioactivity of the three groups towards osteoblast differentiation was also evaluated by culturing cells with the conditioned medium from each of the three groups including cell proliferation assay, cell migration assay, alkaline phosphatase (ALP) staining, and alizarin red staining (ARS), as well as by real-time PCR for genes encoding runt-related transcription factor 2 (*RUNX2*), *ALP*, collagen type I alpha1(*COL1A1*) and osteocalcin (*OCN*). BLOCK-3 made with Solid-PRF fragments + Liquid-PRF + DBBM had by far the fastest solidification period (over a 10-fold increase) as well as the most resistance to degradation. SEM and tensile tests also revealed that the mechanical properties of BLOCK-3 were superior in strength when compared to all other groups and further induced the highest osteoblast migration and osteogenic differentiation confirmed by ALP, ARS and real-time PCR. PRF bone blocks made through the combination of Solid-PRF fragments + Liquid-PRF + DBBM had the greatest mechanical and biological properties when compared to either used alone. Future clinical studies are warranted to further support the clinical application of PRF bone blocks in bone regeneration procedures.

## 1. Introduction

In the field of oral implantology, horizontal and vertical bone defects often compromise ideal implant placement. For such cases, alveolar ridge augmentation utilizing guided bone regeneration (GBR) provides an evidence-based treatment modality for enabling predictable accommodation of dental implants with excellent long-term survival rates [1-3]. While autologous bone remains the gold standard among the biomaterials used for bone regeneration [4-6], limitations have also been reported including a second surgical site, additional morbidity, chance of complications to the harvested site, and faster than ideal resorption rates [3, 7].

Commercially available bone substitute materials are most frequently available in a particulate form and are easy to disperse, making it difficult to adequately graft the particles in the bone defect and to maintain the desired shape of the rebuilt area. Furthermore, the majority of commercially available bone grafting materials (allografts, xenografts or synthetic alloplasts) contain only scaffolds for osteoconductive appli-

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cation, lacking osteoinductive factors such as living cells and growth factors [7]. A frequently used, thermally regulated, bone grafting material is the Deproteinized Bovine Bone Mineral (DBBM; owing to its lack of proteins/growth factors) or Anorganic Bovine Bone Mineral (ABBM; owing to its lack of organic components) [8, 9].

Recently, platelet-rich fibrin (PRF), a second generation of platelet concentrates, was proposed as a new strategy to offer biological support during bone augmentation procedures [10, 11]. PRF is obtained from the patient's own peripheral blood and centrifuged without any additives such as anti-coagulants [12, 13]. PRF not only acts as a three-dimensional fibrin scaffold, but also contains numerous autologous cells (platelets, leukocytes, macrophages, and neutrophils) [14] and growth factors (such as Platelet Derived Growth Factor, Transforming Growth Factor-$\beta$, Vascular Endothelial Growth Factor, etc.) [15]. In 2019, it was demonstrated that PRF obtained via horizontal centrifugation was more effective at concentrating cells and growth factors in the final PRF matrix when compared to fixed-angle. The horizontal PRF (H-PRF) demonstrated better cell layer separation and minimal cell accumulation on the back-end distal surfaces of centrifugation tubes, and showed up to a four-fold increase in cell numbers when compared to PRF produced by fixed-angle centrifugation [13]. These findings have been also confirmed through histological analysis [16, 17].

As PRF contains a concentration of living cells, autologous growth factors and a dense fibrin structure, its combination with particulate bone grafts hold great promise for enhancing the clinical handling and performance during bone augmentation procedures [18]. It is worth noting that the crosslinking of fibrinogen into fibrin during the formation of PRF provides a network for wrapping cells and growth factors. Thus, combining PRF with DBBM can form a 'sticky' bone block, which facilitates the clinician's handling during a bone augmentation procedure. In a recent study, a combination of Solid-PRF, Liquid-PRF and bone particles was used to prepare a PRF block, which was then found be an effective technique to augment deficient alveolar ridges [12, 19]. However, in these previous studies, PRF has always been prepared by fixed-angle centrifugation with no attempts being made to investigate the mechanical and degradation properties of the graft when fabricated using various methods. Thus, the combination of PRF and bone grafting particles was only evaluated for its clinical impact on bone regeneration without any investigation on its potential influence on the mechanical properties.

Therefore, the aims of the present study were: a) to prepare a novel bone tissue engineered biomaterial with improved biocompatibility and mechanical properties by using PRF obtained through horizontal centrifugation under various mixing combinations (both Solid- and Liquid-PRF used either alone or together) with DBBM, b) to assess the solidification and degradation time of the novel PRF block, as well as of its microstructure and mechanical strength, and c) to evaluate the bioactivity of the novel bone block on osteogenic differentiation using the culture medium collected from each of the bone block groups.

## 2. Materials and methods

### 2.1. Preparation of PRF

Blood samples were collected after the informed consent of 6 volunteers (average age 26 years). All protocols used in this study were approved by the Ethics Committee of the School and Hospital of Stomatology, Wuhan University (B52/2020). All participants were in good general health as previously described [19].

In this study, PRF was prepared using plastic PET tubes to create Liquid-PRF, while glass tubes were used to obtain Solid-PRF as previously reported [20, 21]. Both Liquid-and Solid-PRF were obtained using a protocol of 700 RCF (g) for 8 min at room temperature utilizing a horizontal centrifugation (Bio-PRF, Venice, Florida) according to previous reports [13]. Six 10-mL tubes of blood were collected from each participant. Four 10-mL glass tubes were used to produce Solid-PRF (Bio-PRF, Venice, Florida) and two 10-mL plastic tubes (Bio-PRF, Venice, FL) were used to produce Liquid-PRF. After centrifugation, for Solid-PRF, the red blood clots attached to the PRF clots were gently removed by scraping. Then, a sterile Bio-PRF compression box was used to gently compress the clots into membranes. For Liquid-PRF, a sterile syringe was used to draw out the yellow plasma fluid.

### 2.2. Preparation of three kinds of bone blocks

The following 3 protocols were then utilized (Fig. 1):

BLOCK-1: Solid-PRF mixed with DBBM (Bio-Oss®, Geistlich, Wolhusen, Switzerland). Two Solid-PRF membranes were cut into small PRF fragments sized between 1-2 mm, and mixed thoroughly with 0.25 g of DBBM particles and stirred gently for 15 seconds before shaping into a bone block [22].

BLOCK-2: Liquid-PRF mixed with DBBM: 1mL Liquid-PRF obtained from the buffy coat layers was added drop by drop to the 0.25 g of DBBM bone grafting material and stirred gently for 15 seconds before shaping.

BLOCK-3: Solid-PRF + Liquid-PRF mixed DBBM: Two Solid-PRF membranes were cut into small PRF fragments sized between 1-2 mm and mixed thoroughly with 0.25 g of DBBM particles. Then 1 mL Liquid-PRF from the buffy coat layers was added drop by drop to the DBBM containing bone grafting complex and stirred gently for 15 seconds before shaping.

### 2.3. Scanning electron microscopy (SEM)

Use the 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer to fix the bone blocks from each group for 24 h, then rinse them with 0.2 M sodium cacodylate buffer and distilled water, and utilize ascending dilutions of ethanol (25%, 50%, 75%, 95%, and 100%) to dehydrate them [23]. And then freeze-drying them. Finally, coating the samples with gold automatically [22]. The surface of each group was then captured by SEM (VEGA 3 LMU, TESCAN, Brno, Czech Republic) and investigated for morphological differences.

### 2.4. Mechanical testing

The bone blocks from each group were also transferred for tensile testing. A tensile test involved loading a sample between two plates and then applying a force to the sample. Stretch the samples and record the relationship between the deformation and the applied load. A static material testing device (ZQ-21B-3, ZHIQU, Dongguan, China) was used to characterize the mechanical properties of the studied materials. Specifically, the tensile strength was carried out on each of the 3 groups with a constant crosshead speed of 1 mm/min [24]. Data was reported as means +/- standard deviation.

### 2.5. Solidification time

The bone blocks from each group were also put in a 6-cm dish immediately after preparation, then the dish was tilted at 45 degrees every 15 seconds to observe whether the DBBM mixture would dissolve/flow out of the bone block complex. If no particles were separated, the mixture was deemed to be completely solidified and a corresponding solidification time was recorded.

### 2.6. Degradation property of bone blocks

The bone blocks from each group were placed in separate wells of six-well plates, covered in 5 mL of Dulbecco's modified Eagle's medium (DMEM, HyClone, Thermo Fisher Scientific Inc, Waltham, MA), and incubated at 37 °C in a humidified atmosphere with 5% $CO_2$ for 4 days. Photos were taken at 0 h, 4 h, 12 h, 20 h, 28 h, 48 h and 96 h [25].

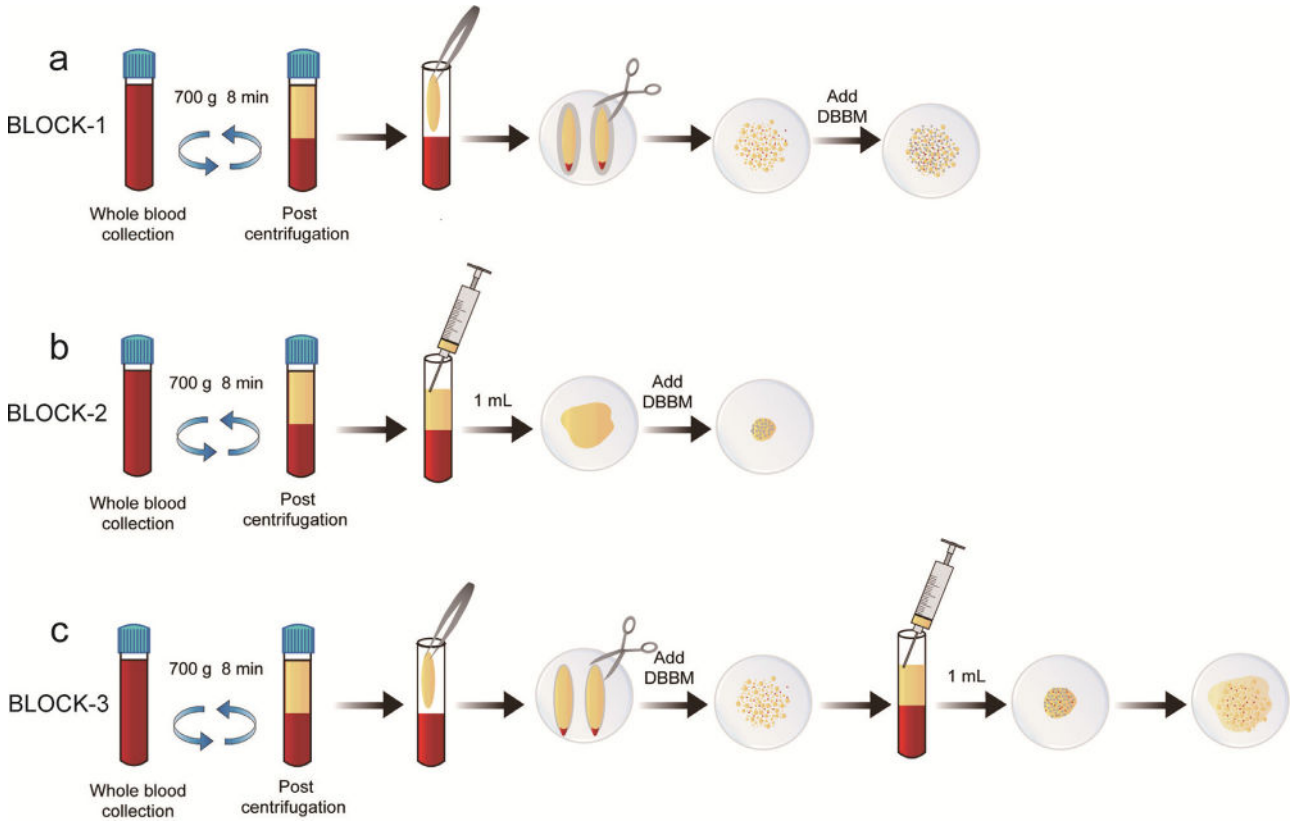


**Fig. 1. Schematic demonstrating the preparation of PRF blocks.** (a) BLOCK-1 was prepared by cutting the Solid-PRF membrane into fragments and mixing them with DBBM particles. (b) BLOCK-2 was prepared by mixing DBBM particles with Liquid-PRF.(c) BLOCK-3 was prepared by mixing DBBM particles with Solid-PRF fragments followed by addition of Liquid-PRF.

The photos revealed the ability for the bone graft complex to either stay intact or dissolve over time.

### 2.7. Preparation of conditioned medium

The supernatant of each of the bone block groups was collected after incubating the block in DMEM for 3 days in a humidified 5% $CO_2$ atmosphere at 37 ˚C. The conditioned medium was prepared and utilized for future experiments by adding the supernatant to culture medium at 20% of the total volume [26].

### 2.8. Cell culture

Human osteoblasts (hFOB 1.19) were purchased from Shanghai Yu Bo Biotech Co., Ltd. and cultured in a humidified atmosphere at 37 ˚C in DMEM supplemented with 10% fetal bovine serum (FBS, Beyotime Biotech) and 1% antibiotics (100 U/ml penicillin G, 100 $\mu$g/mL streptomycin, HyClone, Thermo Fisher Scientific Inc). For osteoblasts differentiation, osteogenic differentiation medium (ODM) was prepared by adding 5 nM dexamethasone, 10 mM $\beta$-glycerophosphate, and 50 $\mu$g/mL ascorbic acid in the conditioned medium as previously described [27].

### 2.9. Cell proliferation assays

Human osteoblasts were inoculated with the conditioned medium from each of the 3 bone block groups at a density of 10,000 cells per well. At time points 1,3 and 5 days, use the Cell Counting Kit-8 assay (CCK-8; Beijing Solarbio Life Science) to determine the number of osteoblasts and a microplate reader scanning to measure them at 450 nm (PowerWave XS2, BioTek, Winooski, VT, USA) as previously described [27]. All samples were repeated three times independently.

### 2.10. Migration assay

Transwell chambers with a pore size of 8 $\mu$m (Corning Costar, USA) were used to quantify the migration ability of osteoblasts when cultured with conditioned medium from each of the 3 groups of bone blocks. 500 $\mu$L of the conditioned medium containing 10% FBS was added to the lower wells [27]. The osteoblasts were starved for 12 h in DMEM containing 0.5% FBS and then were resuspended and added to the upper compartment for migrating ($10^4$ cells / well). After 24 h, use 4% formaldehyde to fix cells and then stain the cells with 0.1% crystal violet solution (Good Bio Technology Co., Ltd, Wuhan, China) for 15 min, respectively. Then use the phosphate buffered saline (PBS, HyClone/Thermo Fisher Scientific) to rinse the samples for three times. Use a cotton swab to wipe out the unmigrated cells in the upper chamber. Use the Olympus DP71 microscope (Olympus Co., Tokyo, Japan) to capture the migrating cells in the lower chamber, and then use ImageJ software (ImageJ v2.1, National Institutes of Health, Bethesda, MD) to count. All samples were repeated three times independently.

### 2.11. ALP activity assay

Human osteoblasts were inoculated in osteogenic differentiation medium prepared with conditioned medium from each of the 3 bone block groups at a density of 50,000 cells per well for 7 days. Disslove the cells in 0.1% Triton X-100 (Merck, Darmstadt, Germany) at 4 ˚C. Then use an ALP activity assay (Beyotime, Shanghai, China) to quantitatively determine the ALP activity. And then quantify the total protein with BCA Protein Assay Kit (Thermo Fisher Scientific Inc.). Normalize the ALP activity to the total protein, and then measure it as OD405 nm/OD562 nm.

**Table 1**
**Primer sequences of various osteoblast differentiation markers.**

| Genes | Primer sequence |
|---|---|
| hGAPDH-F | GCACCGTCAAGGCTGAGAAC |
| hGAPDH-R | TGGTGAAGACGCCAGTGGA |
| hALP-F | CAACGAGGTCATCTCCGTGATG |
| hALP-R | TACCAGTTGCGGTTCACCGTGT |
| hRUNX2-F | CCCAGTATGAGAGTAGGTGTCC |
| hRUNX2-R | GGGTAAGACTGGTCATAGGACC |
| hOCN-F | CGCTACCTGTATCAATGGCTGG |
| hOCN-R | CTCCTGAAAGCCGATGTGGTCA |
| hCOL1A1-F | GATTCCCTGGACCTAAAGGTGC |
| hCOL1A1-R | AGCCTCTCCATCTTTGCCAGCA |

For ALP staining, human osteoblasts were fixed with 4% formaldehyde for 15 min, washed 3 times with PBS, and then stained with ALP dyes (Beyotime Biotech). Capture all images under the same microscope. All samples were repeated three times independently.

### 2.12. Alizarin red staining

Human osteoblasts were inoculated in osteogenic differentiation medium prepared with conditioned medium from each of the 3 bone block groups at a density of 50,000 cells per well for 14 days. Firstly, use the 4% formaldehyde to fix cells for 10 min and then stain them with 0.1% alizarin red solution (pH = 4.2; Sigma, MO, USA) for 1 h at 37 ˚C [27]. Capture all images under the same microscope. Dissolve the nodules with the 10% hexadecyl lpyridinium chloride for 1 h and measure the colorimetric assay at 562 nm for quantitative analysis. Samples were repeated three times independently.

### 2.13. Real-time PCR for osteoblast differentiation markers

In the real-time PCR experiments, seed 50,000 osteoblasts with the conditioned medium from each of the 3 bone block groups, respectively. After 7 and 14 days of culture, total RNA was isolated from osteoblasts using AxyPrep$^{TM}$ Multisource Total RNA Miniprep Kit (AXYGEN Inc, CA, USA) according to the manufacturer's protocol. Use a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA) to determine the RNA concentration as previously described [27]. A First Strand cDNA Synthesis Kit (GeneCopoeia, MD, USA) was immediately used to reversely transcribe a total of 1 $\mu g$ RNA to cDNA. The gene expression for collagen type I alpha1 (*COL1A1*), runt-related transcription factor 2 (*RUNX2*), alkaline phosphatase (*ALP*), osteocalcin (*OCN*) and GAPDH genes of human were performed with a CFX Connect$^{TM}$ Real-Time PCR Detection System (BIO-RAD, Hercules, CA). List the above-mentioned primer sequences of these markers in Table 1. Calculate gene expression levels relative to house-keeping gene GAPDH by using the delta-delta Ct method. All samples were performed in triplicate.

## 3. Statistical analysis

Use GraphPad Prism software 7.0 (La Jolla, CA) to analyze the data by t test and ANOVA. Data are graphed as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ are considered statistically significant.

## 4. Results

### 4.1. Bone block solidification and degradation time

As shown in Table 2, statistically significant and substantial differences were observed in the solidification time among the 3 methods to prepare bone blocks. BLOCK-3, which comprised mixing Solid-PRF fragments with DBBM followed by the addition of Liquid-PRF, demonstrated by far the fastest solidification time into a PRF block graft when compared to the other groups, while the BLOCK-1 never truly solidated. The solidification time of BLOCK-3 and BLOCK-2 was 2.1 and 25.6 min, respectively (over 10-fold faster when Solid-PRF fragments were combined with Liquid-PRF). As for degradation time, BLOCK-3 retained the initial shape for up to 96 hours, while BLOCK-1 hardly remained compact for 4 hours (Fig. 2).

**Table 2**
**Solidification time for BLOCK-1, BLOCK-2 and BLOCK-3 in min (Samples were performed in triplicate).**

| Sample | Solidification time (min) | | |
|---|---|---|---|
| | BLOCK-1 | BLOCK-2 | BLOCK-3 |
| **1** | / | 26.8 | 2.5 |
| **2** | / | 24 | 1.6 |
| **3** | / | 25.9 | 2.2 |
| **Mean** | / | 25.6 | 2.1 |

### 4.2. SEM analysis

Interestingly, SEM images revealed very different patterns between the 3 groups of PRF blocks. BLOCK-3 showed a dense fibrin mesh covering the entire surface of the bone block. The fibrin mesh entrapped the components of the block including the DBBM particles and PRF fragments as well as contained numerous visible cells as depicted in Fig. 3. The SEM images of BLOCK-2 showed a less dense fibrin mesh when compared to BLOCK-3 but also showed cells and a fibrin structure. BLOCK-1 showed the least amount of cells contained and by far the most loosely connected fibrin structure among all 3 groups.

### 4.3. Mechanical analysis

The results demonstrated that BLOCK-3 led to a composite scaffold with an increased tensile resistance 10-fold greater when compared with the value of the BLOCK-2 group (Fig. 3c, d). The tensile resistance of BLOCK-1 showed no ability to resist mechanical strength tests and could never shape into a complete PRF block.

### 4.4. Osteoblast proliferation and migration

No statistically significant differences in terms of cell proliferation were observed among the groups at day 1. Compared with the control group with the normal culture medium, the conditioned medium from BLOCK-3 increased the proliferation rates of human osteoblasts at day 3 and day 5 (Fig. 3e). The conditioned medium from all 3 groups promoted cell migration at 24 h, with BLOCK-3 demonstrating the highest ability of promoting osteoblast migration as shown in Fig. 4a, b.

### 4.5. Osteoblast differentiation

Lastly, use the osteoblast differentiation media to culture human osteoblasts for 7 and 14 days and then stain the cells with ALP and ARS to evaluate the osteogenic potential of each of the three groups of PRF blocks. At day 7, compared with the control group and the other two bone block groups, BLOCK-3 showed significantly higher ALP activity (Fig. 4c, d). ARS also had the same trend. While conditioned medium from BLOCK-1 and BLOCK-2 showed additional mineralized nodules when compared to the control group, the conditioned medium from BLOCK-3 induced the highest number of mineralized nodules when compared to all other groups (Fig. 4e). ARS quantitative analysis confirmed the above results (Fig. 4f).

Subsequently, the mRNA expressions of osteoblast differentiation markers were evaluated by real-time PCR. BLOCK-3 showed the highest increase in *COL1A1* mRNA expression while BLOCK-2 and BLOCK-1 showed no statistically significant changes at the two time points. At

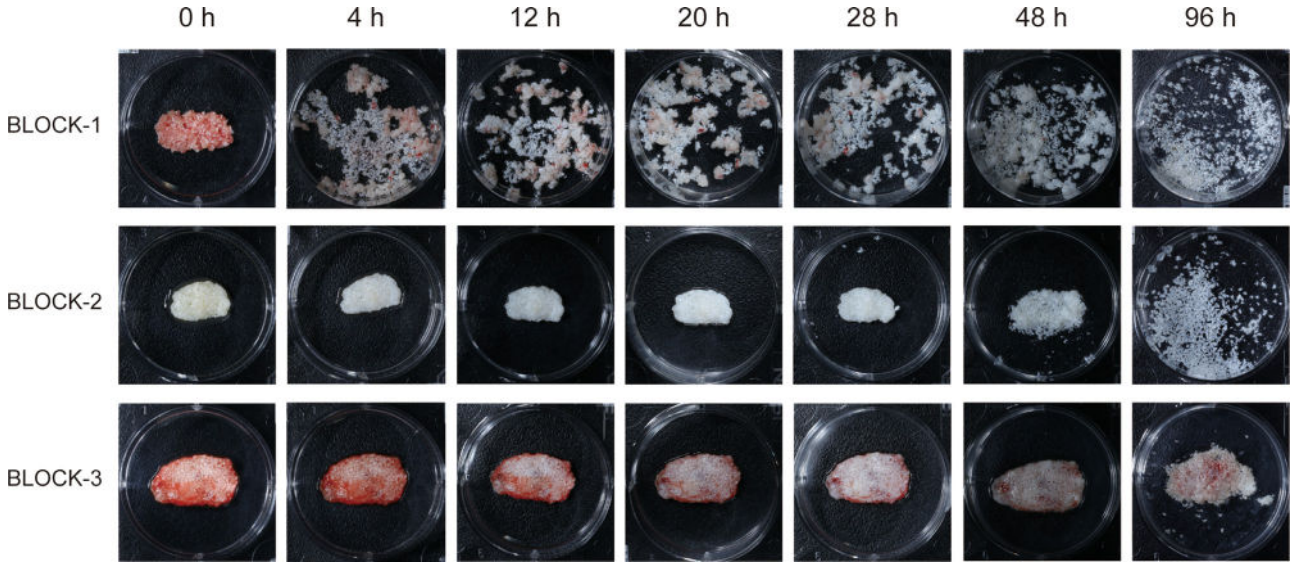


**Fig. 2. Photographic time profile of PRF blocks demonstrating their ability to maintain their shape over time (Samples were performed in triplicate).**

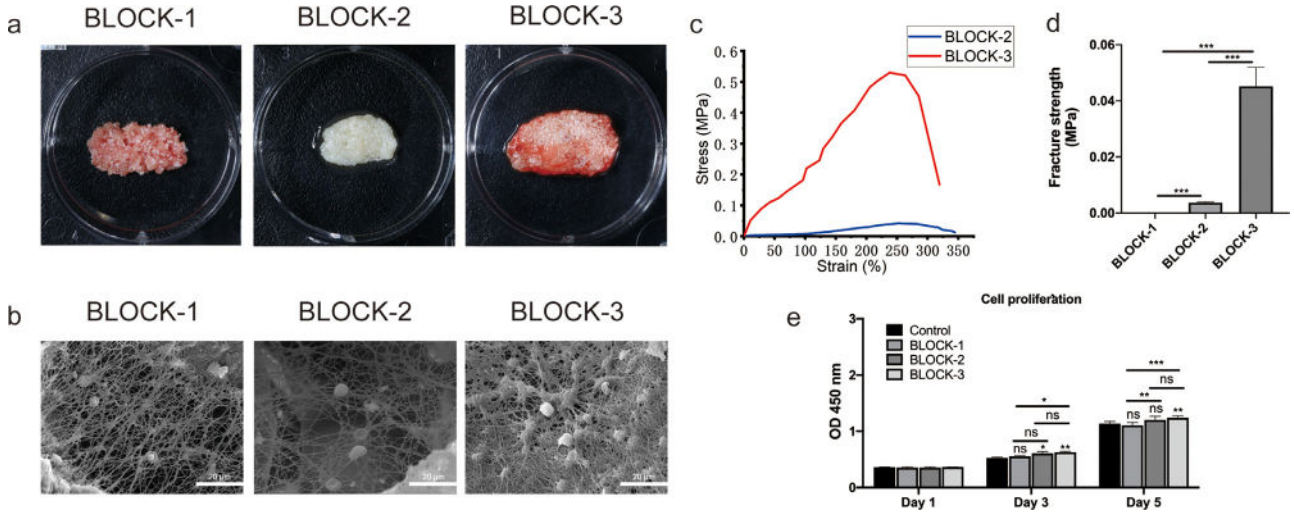


**Fig. 3. Characteristics of PRF blocks**. (a) Photos of BLOCK-1, BLOCK-2, BLOCK-3. (b) SEM image of BLOCK-1, BLOCK-2, BLOCK-3, scale bar = 20 $\mu$m. (c, d) The tensile resistance and fracture strength of BLOCK-1, BLOCK-2, and BLOCK-3 (Samples were performed in triplicate). (e) Proliferation assay of osteoblasts at day 1, 3 and 5 using control culture medium and conditioned culture medium from all three PRF blocks (* denotes P<0.05, ** denotes P<0.01, *** denotes P<0.001; Samples were performed in triplicate with 3 independent experiments).

day 7, only BLOCK-3 increased the mRNA expression of *ALP*. At day 14, all block groups showed increase in *ALP* expression compared to the control group, with BLOCK-3 once again demonstrating the significantly highest levels. The mRNA expression of *RUNX2* followed a similar trend with *COL1A1* (Fig. 5). For *OCN*, all groups showed similar mRNA expression at day 7, with BLOCK-1 and BLOCK-3 demonstrating a statistically significant increase when compared to the control group.

## 5. Discussion

PRF has been utilized in various disciplines of medicine as a potential strategy to enhance tissue regeneration [10, 28] with a number of studies confirming its regenerative potential [29-31]. PRF is a mixture of cells (platelets and various white blood cells), embedded in a three-dimensional fibrin scaffold containing a variety of active growth factors and proteins, which have beneficial effects on tissue regeneration [18, 32, 33]. However, the current literature suggests that PRF alone is much more suitable for soft tissue regeneration when compared to bone augmentation [34-36]. Therefore, the aim of the present study was to investigate methods to assemble a bone graft complex (PRF Block) composed of bone grafting particles and PRF either formulated from Liquid-PRF and/or Solid-PRF.

This study demonstrated that mixing Solid-PRF fragments or Liquid-PRF alone with DBBM particles could not form an ideal bone block. Instead, it was found that the bone block made by mixing DBBM particles with Solid-PRF fragments followed by additional use of Liquid-PRF formed by far the fastest solidification results as well as the PRF bone block most resistant to degradation. By following this order, the clinician was able to shape the bone graft in customized shapes prior to solidification which occurred in roughly 2 minutes. The novel bone block made using Solid-PRF fragments, Liquid-PRF and DBBM further demonstrated an ability to resist degradation by showing a much longer degradation time when compared to the blocks formed with either Liquid- / or Solid-PRF alone. Furthermore, the longer degradation time, denser

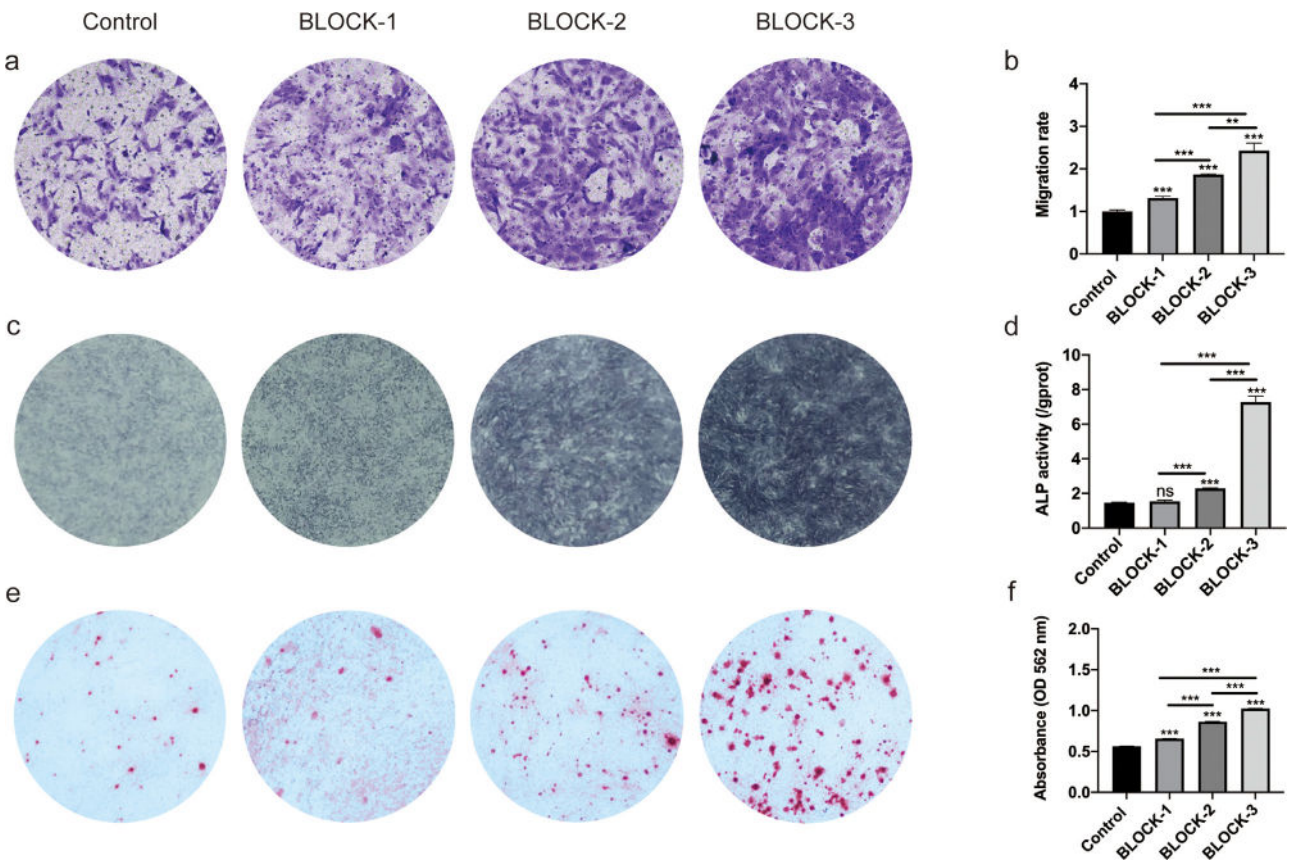


**Fig. 4. Osteoblast migration and differentiation assays**. (a) Light microscopic images of osteoblast migration using a Transwell assay at 24 hours cultured with the normal culture medium or conditioned medium from all three PRF block groups. (b) Quantitative analysis of osteoblasts migrated in (a) (** denotes $p<0.01$, *** denotes $p<0.001$; Samples were performed in triplicate with 3 independent experiments). (c, d) Light microscopic image and quantitative analysis of ALP activity representative of osteoblast differentiation at day 7 (** denotes $p<0.01$, *** denotes $p<0.001$; Samples were performed in triplicate with 3 independent experiments). (e, f) Light microscopic image and quantitative analysis of mineralization nodules formed at 14 days by osteoblasts using Alizarin Red Staining (*** denotes $p<0.001$; Samples were repeated 3 times independently).

microstructure and better mechanical strength of the bone blocks prepared via the combination approach appears to indicate a greater ability in maintaining the defect volume and stability of graft during bone augmentation procedures to ensure satisfactory clinical outcomes. A number of clinical studies have demonstrated the pivotal importance of bone graft stability on the outcomes of bone regeneration procedures [37]. In the present study, we compared each of the 3 bone blocks from blood collected from the same person for each group. Nevertheless, it is important to note that variability exists between a normal population, the weight and size of Solid-PRF were different among participants, and therefore the mechanical strength of each bone block had slight variability. This could also be affected simply by each of the patients standard levels of fibrinogen prior to centrifugation. Further study to address these differences and potentially even normalize the ratio of Solid-PRF, Liquid-PRF in combination with DBBM may be warranted.

In this study, the biocompatibility and osteogenic potential of each group of bone blocks was compared in vitro using human osteoblasts. Compared to Liquid-PRF alone + DBBM block and Solid-PRF alone + DBBM block, the combination approach incorporating Solid-PRF fragments with Liquid-PRF significantly improved the activity of human osteoblasts, including their enhanced cell migration, proliferation, and differentiation. Nevertheless, it should also be noted that each group contributed to superior outcomes when compared to the control group, highlighting that PRF enhances the bioactivity of DBBM (which typically is devoid of growth factors and extracellular matrix) when combined into a final bone block.

Biomaterials are manufactured in different forms with replacement bone grafts being developed and introduced to market as blocks or granules/particles in order to adapt to the various bone defects faced in routine clinical practice. Bone grafting particles have less-than-optimal mechanical properties, but are still able to fill bone defects with various shapes and sizes. Bone blocks have better mechanical properties, but they must be modified according to the defect shape in order to be placed into bone defects and may further be deemed less vascular (greater difficulty for cells and vessels to grow within the bone block), which may inhibit bone formation. For these reasons, bone particles are far more frequently utilized in routine clinical practice.

Nevertheless, the main disadvantages encountered in clinical practice while using particulate bone grafts are: (1) they may migrate to other or nearby tissues if not stabilized; (2) they have low structural integrity to deformation – especially to compression of the graft (i.e. vertical augmentations for instance); and (3) they are also less resistant to complete collapse of the bone graft when compared to bone blocks [38]. Therefore, novel strategies are still needed in order to overcome these drawbacks.

The novel bone block fabricated and tested in the present study has attempted to address some of the common problems encountered during routine bone grafting procedures such as shaping of traditional bone blocks along with the inconvenience and low bioactivity of commercial products purchased 'off the shelf'. When the bone block presented in this

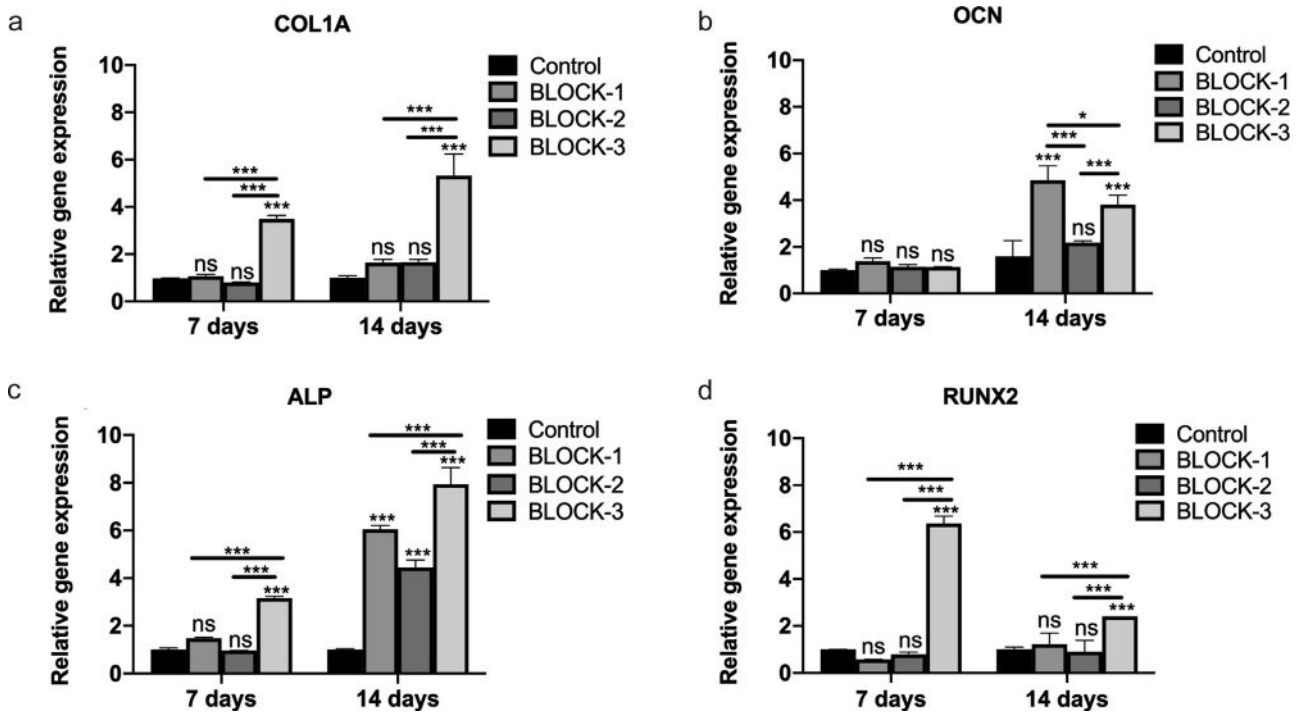


**Fig. 5. The expression of osteoblast differentiation markers induced by PRF blocks**. The mRNA expression of *COL1A*(a), *OCN*(b), *ALP*(c), *RUNX*2(d) of osteoblasts at day 7 and day 14 using the normal culture medium or conditioned medium from all three PRF block groups (*** denotes $p<0.001$; Samples were repeated 3 times independently).

study was fabricated, Solid-PRF fragments were mixed within the bone graft allowing for cells and growth factors to be found within the entirety of the bone graft complex. Thereafter, the additional use of Liquid-PRF allowed for the role of liquid fibrinogen to further enhance the properties of BLOCK-3. Two main advantages have been proposed: Liquid-PRF (liquid fibrinogen and thrombin) mechanically 'glues' all the components of the Solid-PRF + Liquid-PRF bone block together. Furthermore, it provided far greater bioactive properties on osteoblasts. This strategy is far superior for grafting purposes when compared to the use of a commercially ready-made bone block completely devoid of many regenerative cells and growth factors compared to the strategy proposed in the current study.

## 6. Conclusion

Our results demonstrated that the combined use of Solid-PRF fragments and Liquid-PRF mixed with DBBM particles produced a 3D flexible, moldable sticky bone block with a short time for solidification, great mechanical strength, and ability to induce osteogenic differentiation of osteoblasts. By following the recommended protocol presented in this study, a promising use of the commonly utilized bovine bone particles mixed with concentrated blood cells and growth factors was achieved facilitating bone grafting material handling and preventing degradation. Future clinical studies are warranted to further support the clinical application of PRF bone blocks in bone regeneration procedures.

## Declaration of Competing Interest

The authors declare that they have no conflicts of interest in this work.

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