



Effect of relative centrifugal force on the biological properties of liquid platelet-rich fibrin produced via horizontal centrifugation

Mengge Feng¹ · Yan Wei¹ · Hongjiang Wei¹ · Yunxiao Wang¹ · Yufeng Zhang^{1,2} · Richard J. Miron³ · Yulan Wang¹

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Abstract

Objectives Platelet-rich fibrin (PRF) in liquid form has shown advantages in tissue engineering including acting as injectable fillers and drug carriers. However, few studies have investigated the best relative centrifugal force (RCF) for preparing liquid PRF. The aim of the present study was to find out optimal centrifugation force for preparing liquid PRF.

Materials and methods Liquid PRF was prepared using horizontal centrifugation (liquid H-PRF) with RCF ranging from 100 g, 300 g, 500 g, to 700 g for 8 min. The volume, weight, solidification time, and tensile properties were subsequently investigated. Scanning electron microscopy (SEM) and rheologic tests were carried out to investigate the microstructure and rheologic properties of liquid H-PRF after natural polymerization. The total number, concentration, and distribution of cells within each liquid H-PRF was evaluated by complete blood count (CBC) analysis and hematoxylin–eosin staining.

Results As RCF values increased, the volume and weight of liquid H-PRF both increased accordingly. SEM images revealed that as the centrifugal force increased, the fibrin bundles became thinner with a denser fibrin network, and rheologic tests revealed improved mechanical properties. CBC analysis demonstrated that 500 g group had the highest number of leukocytes and neutrophils, whereas 100 g group yielded the highest concentration of leukocytes and platelets. Furthermore, histological analysis suggests that cells obtained by 500 g for 8 min were most evenly distributed in liquid H-PRF.

Conclusions In summary, the present study provided insights into the contents of liquid H-PRF prepared at different centrifugation forces, enabling clinicians to choose proper centrifugation forces based on their needs.

Clinical relevance The present findings provide theoretical basis for clinical choice of liquid H-PRF protocol from mechanical, cell contents, and histological aspects.

Keywords Blood · Liquid platelet-rich fibrin · Horizontal centrifugation · Regeneration · Centrifugal force

Introduction

In oral surgery and periodontology, many efforts have been made to promote the process of wound healing and bone formation [1]. Platelet concentrates in liquid form have been recently utilized widely in dental medicine such as

stimulating gingival papilla regeneration [2], accelerating orthodontic tooth movement [3] since they contain supra-physiological concentrations of autologous platelets as well as growth factors. The growth factors contained in platelet concentrates have been shown to increase cell migration, cell proliferation, collagen deposition, and angiogenesis during tissue remodeling and regeneration [4, 5].

Among injectable platelet concentrates, platelet-rich plasma (PRP) was utilized in regenerative medicine as first generation product with evidence supporting its use [6, 7]. Nevertheless, the preparation of PRP typically required two centrifugation steps and uses anticoagulants added to the blood collection tubes [8, 9]. However, the heterogeneity of PRP preparation and the concerns regarding the use of anticoagulants have since been shown to limit healing since clotting is an important step to healing [10]. As a result,

✉ Yulan Wang
wyl.dentistry@whu.edu.cn

¹ The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University, Wuhan, China

² Medical Research Institute, School of Medicine, Wuhan University, Wuhan 430071, China

³ Department of Periodontology, University of Bern, Bern, Switzerland

injectable platelet-rich fibrin (i-PRF), a second generation platelet concentrate, was developed in 2015 by modifying the relative centrifugal force (RCF), time of centrifugation and by using more hydrophobic plastic blood collection tubes [11]. This liquid-PRF formulation was originally prepared using a single centrifugation cycle (60 g, 3 min) without the need/addition of any anticoagulants [12]. By using lower centrifugation speeds, shorter centrifugation time, and hydrophobic plastic tubes, the fibrin clotting times could be drastically delayed, leaving a final liquid-PRF formulation following the centrifugation cycle. The resulting i-PRF was shown to contain thrombin and fibrinogen that remained as liquid for about 15 min after centrifugation prior to fibrin formation, thus making it suitable to be injected for periodontal injection [2], mixing with bone substitutes [13], or treating erosive oral lichen planus [14]. Liquid-PRF would then polymerize into its fibrin gel form after 15 min, which resulted in a three-dimensional fibrin scaffold containing plentiful autologous cells including platelets and leukocytes and showed promise as a volume enhancer for periodontal use and also utilized in various studies for periorbital/perioral rejuvenation and/or lip augmentation [15, 16].

Historically, i-PRF was prepared using fixed-angle centrifugation which has since been reported to cause cell damage and loss of platelets in the final PRF concentrate. In 2019, horizontal PRF (H-PRF) was shown to accumulate up to 4 times more cells when compared to fixed-angle devices which accumulates cells on the distal surfaces of blood collection tubes [17]. Nevertheless, study investigating suitable protocols for the production of liquid-PRF using horizontal centrifugation remains relatively unexplored.

For this reason, the aim of the present study was explore the optimum relative centrifugal force (RCF) for producing liquid H-PRF by the following methods: (a) assessing the final volume, weight, solidification time, and mechanical properties when centrifugation was carried out at 100 g, 300 g, 500 g, and 700 g for 8 min. (b) Thereafter, the investigation of the microstructure and rheologic behavior of liquid H-PRF at each protocol was investigated; (c) lastly, the evaluation of the cell distribution of liquid H-PRF at each protocol was quantified using complete blood count (CBC) analysis and hematoxylin–eosin staining.

Materials and methods

Preparation of liquid H-PRF

All protocols used were approved by the Ethics Committee of the School and Hospital of Stomatology, Wuhan University (B52/2020), in this study. Written informed consents were obtained from 8 healthy volunteers (average

age 26 years) with no blood-related disorders as previously described [18, 19].

Venous blood was collected using plastic PET tubes 10-mL blood per tube (Plasmatrident, Weiyin Technology Co., Ltd., Wuhan, China) to prepare liquid H-PRF using a protocol of 100 g, 300 g, 500 g, and 700 g for 8 min by horizontal centrifugation (Plasmatrident, Weiyin Technology Co., Ltd., Wuhan, China) according to previous reports [18].

Determination of volume, weight, and solidification time of liquid H-PRF

Twelve blood tubes were centrifuged for 100 g, 300 g, 500 g, and 700 g for 8 min to prepare liquid H-PRF (sample size was 3 at each time point). After centrifugation, the upper yellow plasma liquid layer (including the buffy coat) was then transferred into 6-well cell culture plates for future tests. The volume of liquid H-PRF in each group was then recorded. Then, the amount of time required for solidification was recorded by observing the plates for fibrin clot formation and tilting the plates to 45° every 15 s. When the liquid H-PRF formed a whole gel, the mixture was defined as solidified, and the corresponding time was recorded as previously described [18, 19]. After the liquid H-PRF was solidified into a clot, it was weighed using a weighing meter (Shimadzu, UX420S, Japan), and the weight was recorded.

Mechanical testing

After weighing, the liquid H-PRF gels were then transferred for tensile test. The tensile test involves loading a sample between two plates and then applying mechanical force to the sample. The device (ZQ-21B, ZHIQU, Dongguan, China) was used to characterize the mechanical properties of all groups. Specifically, under the condition of constant crosshead speed of 1 mm/min, the tensile strength of 4 groups of specimens was tested respectively. Test success criteria: the specimen is stretched to the point where the fracture is located in the center of the specimen and not at both ends. Three specimens were successfully tested in each group, and the maximum load value at fracture, namely the maximum tensile force, was recorded [18].

Scanning electron microscopy (SEM)

Four tubes of blood were centrifuged for 100 g, 300 g, 500 g, and 700 g for 8 min to prepare liquid H-PRF. The liquid H-PRF obtained after centrifugation was aspirated and placed in a 6-well plate to stand for coagulation. Liquid H-PRF gels formed after natural polymerization from each group were fixed using the 2.5% glutaraldehyde at 4°C for 24 h and dehydrated utilizing ascending dilutions of ethanol (30, 50, 70, 80, 90, 95, and 100%) for 10 min. Then

the samples went through critical-point-drying. Finally, the samples were coated with gold coating. The microstructure of the gel was observed by scanning electron microscope at 5 kV and 10 kV, and the surfaces of each clot were then recorded by SEM (Zeiss SIGMA) and investigated for morphological differences [18].

Rheologic measurements

Four tubes of blood were centrifuged for 100 g, 300 g, 500 g, and 700 g for 8 min to prepare liquid H-PRF. The liquid H-PRF obtained after centrifugation was aspirated and placed in a 6-well plate to stand for coagulation. Stress-controlled rheometer (Kinexus ultra+, Malvern Instrument Ltd., UK) was used to measure the rheological properties of liquid H-PRF gels formed after natural polymerization from each group with a parallel plate (50 mm in diameter; the gap between the two parallel plates was set at 1 mm). Under the condition of a shear rate of 0.1 s, the viscosity of the liquid H-PRF gels was determined by a single shear rate test. The change of shear viscosity with the alteration of shear rate in each liquid H-PRF group was recorded. The test temperature was 25°C [20–23].

Quantification of cells found in liquid H-PRF

Four tubes of blood were centrifuged for 100 g, 300 g, 500 g, and 700 g for 8 min to prepare liquid H-PRF for cell quantifications using plastic centrifugation tubes (Chixin Biotech, Wuhan, China) containing anti-coagulants (2 mg/mL EDTA-K2). An additional whole blood sample was collected as a control group. A sequential pipetting protocol was utilized to precisely quantify the cells obtained in liquid H-PRF as previously described [17]. The protocol describes pipetting 1-mL layers sequentially from the top layer of H-PRF to the buffy coat layer, in addition, recording the total H-PRF volume (mL) of each protocol to quantify the total cell number and cell concentration of each protocol. Each 1-mL layer sample from liquid HPRF was transferred into 1.5-mL sterile EP tubes and sent for CBC analysis using a Sysmex XN-550 (Sysmex Corporation, Kobe, Japan) as previously described [24–26].

Histological sample preparation

Four tubes of blood were centrifuged for 100 g, 300 g, 500 g, and 700 g for 8 min to prepare liquid H-PRF. After the polymerization of liquid H-PRF in each group, we retrieved the PRF gels from each tube. Remove the red blood cell (RBC) fraction without damaging the bottom of the fibrin-rich gel. PRF gels were then fixed in 4% formaldehyde for 12 h followed by washing with phosphate-buffered saline (PBS) for 3 times. Then samples were embedded in deionized water overnight and thereafter placed into embedding

cassettes along the longitudinal axis. The samples were dehydrated in an alcohol series and xylene [27]. Then the samples were embedded in paraffin before sectioned at a thickness of 5 µm using a rotary microtome (Microm HM 325, Thermo scientific, USA). Prior to staining, the sample was sequentially immersed in xylene, and then the ethanol concentration for dewaxing and rehydration. Hematoxylin eosin staining (Servicebio, Wuhan, China) was performed on the sections before taking images using microscope (Aperio VERSA 8, Leica, Germany) [28–30].

Statistical analysis

One-way analysis of variance (ANOVA) was utilized to analyze the data via GraphPad Prism software 8.0 (La Jolla, CA). Shapiro-Wilk test confirmed that the data were in line with normal distribution; Brown-Forsythe test confirmed that the data were homogeneity of variance, one-way analysis of variance Tukey test for each group of data. Data are graphed as mean ± SD. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ are considered statistically significant.

Results

Liquid H-PRF volume, weight, and solidification time

As shown in Fig. 1, with the increase of RCF, the length of liquid H-PRF in the tube rose accordingly. When the liquid H-PRF was transferred to 6-well plates, the 500-g group seems more homogeneous when compared with other groups. The quantitative analysis showed that the volume and weight of liquid H-PRF also increased along with the increase of RCF (Fig. 2a,b), and these increase in volume and weight was statistically significant. Regarding the time required for liquid H-PRF solidification, the solidification time was slowest when the lowest centrifugal force was used (100 g), and was highest at 300 g (Fig. 2c). The liquid H-PRF in 500 g and 700 g group solidates slightly slower than 300 g group but still faster than that in 100 g group.

Mechanical analysis

The mechanical strength of liquid H-PRF gels was represented by their fracture force. The tensile resistance has shown that liquid H-PRF gels became significantly stronger shown when the RCF was at or above 500 g. No statistical significance was observed in the tensile resistance of liquid H-PRF gels prepared for 500 g and 700 g centrifugal force (Fig. 2d).

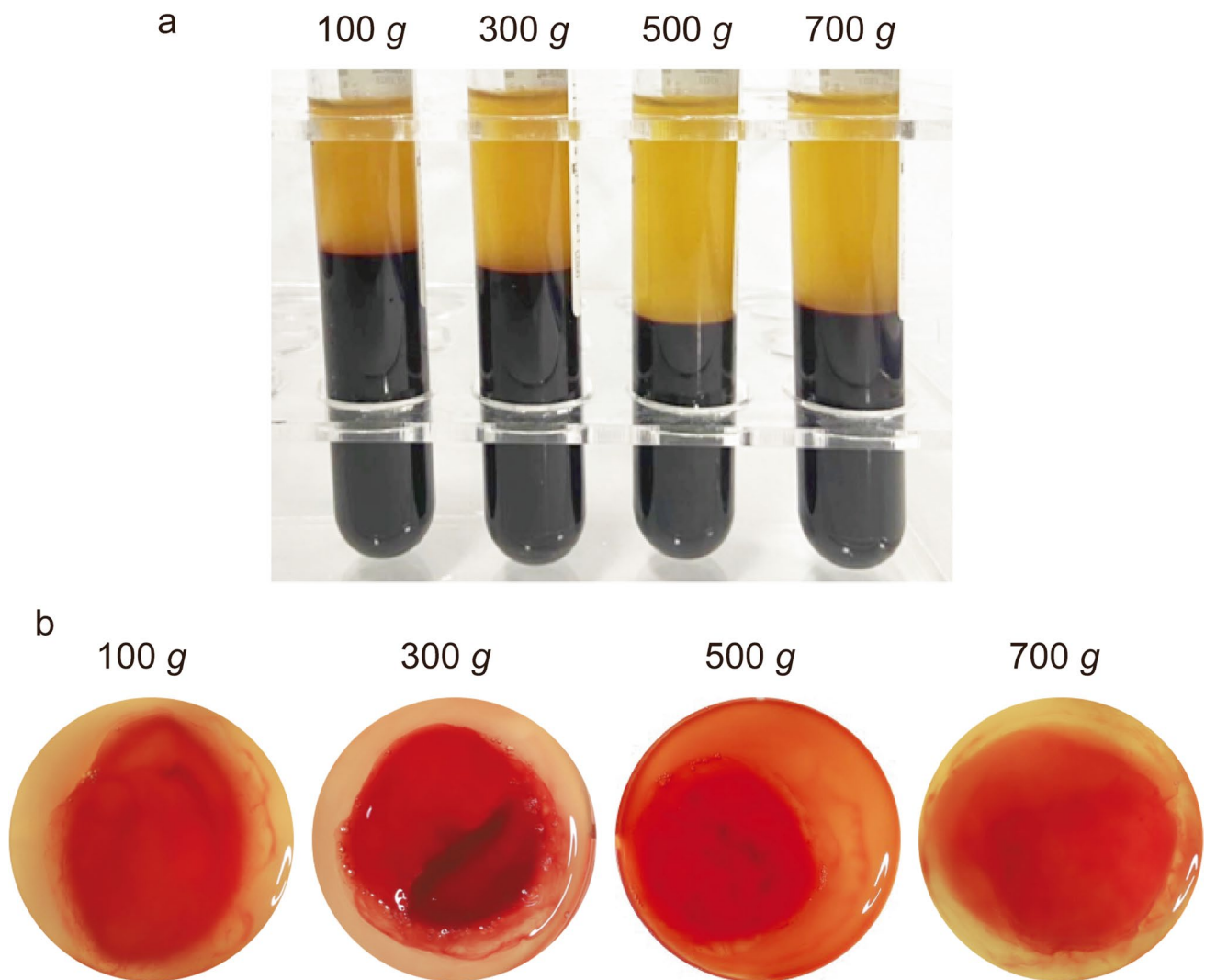


Fig. 1 Photos demonstrating the preparation of liquid H-PRF after various centrifuge force treatments. **a** The state of liquid H-PRF after centrifugation at different centrifuge force. **b** Liquid H-PRF gels in final ready form

SEM analysis

Ultrastructural evaluation of liquid H-PRF gels was performed through SEM as depicted in Fig. 3. Interestingly, SEM images revealed that as the centrifugal force increased from 100 to 700 g, the fibrin bundles became thicker with a denser fibrin network. That is, the fibrin network that forms inside the clot becomes denser as centrifugal force rose.

Rheologic measurements analysis

Rheologic measurement analysis was used to find out the suitability of liquid H-PRF gels for injection use. Figure 4a reveals that when the time reached 200 s, the shear viscosity of liquid H-PRF gels obtained by 100 g,

300 g, 500 g, and 700 g is 35.4 Pa s, 84.7 Pa s, 154 Pa s, and 83.2 Pa s, respectively. The results indicated that the internal cross-linking of PRF formed a more stable three-dimensional network structure as the centrifugal force increased. And the shear viscosity of liquid H-PRF gels obtained by 500 g was the largest, requiring more external forces to move it. Figure 4b demonstrates the shear-thinning rate of liquid H-PRF gels. The shear thinning of the liquid H-PRF gels prepared by 100 g, 300 g, 500 g, and 700 g was 0.87, 2.04, 3.71, and 2.32, respectively.

Cell numbers following centrifugation

Figure 5 and 6 demonstrate the total number and concentrations of leukocytes, platelets, neutrophils, monocytes, and lymphocytes in liquid H-PRF prepared by different

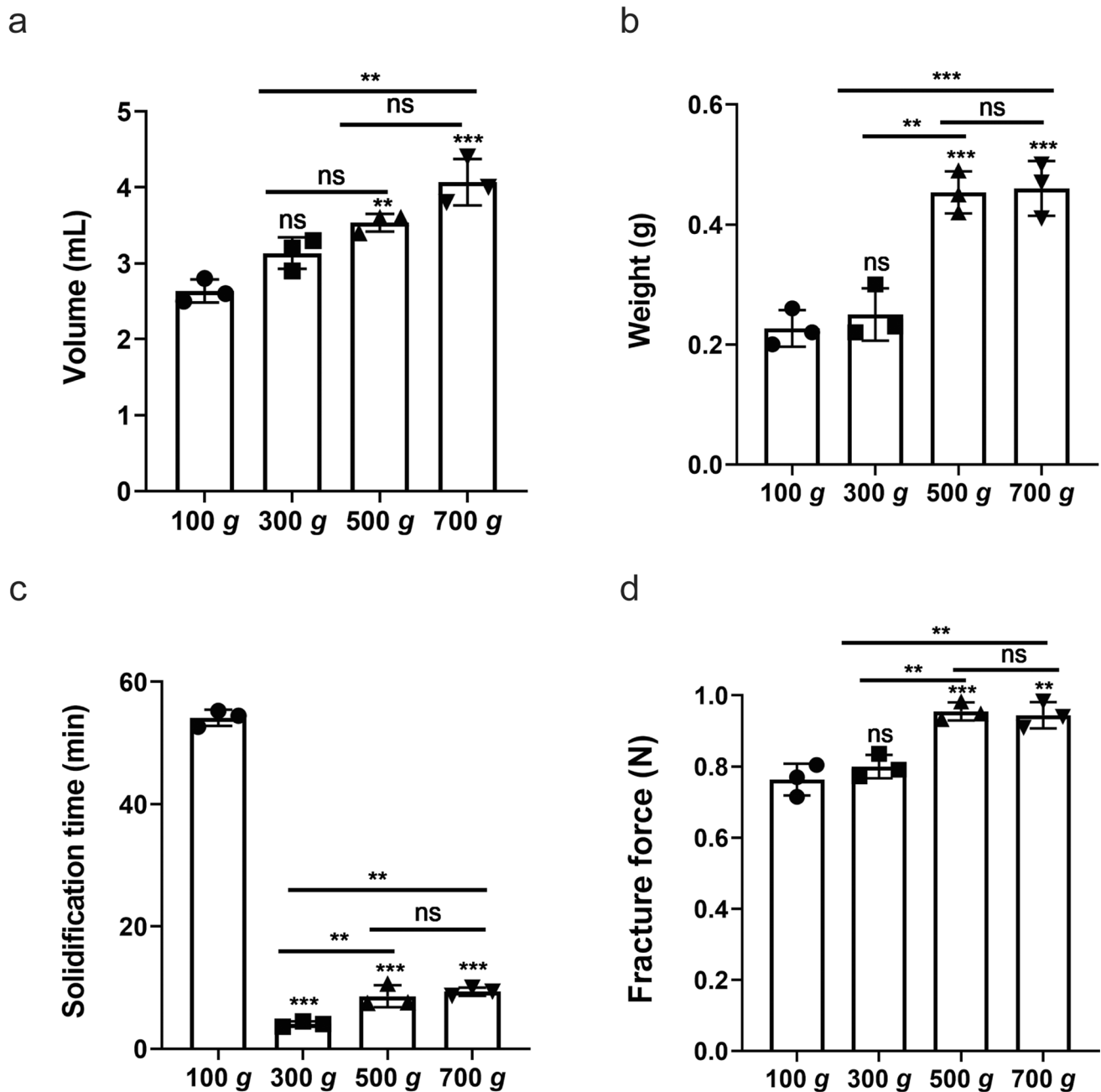


Fig. 2 Quantitative analysis **a** volume, **b** weight, **c** solidification time and **d** fracture strength of liquid H-PRF after various centrifuge force treatments (samples were performed in triplicate)

centrifugation protocols. Note that of all the protocols, a 100 g for 8 min protocol led to the highest concentration of leukocytes, monocytes, and lymphocytes. This is due to the fact that when the centrifugal force is 100 g, less volume is obtained by liquid H-PRF and therefore, a higher cell concentration of cells is obtained. Moreover, centrifugation for 8 min utilizing the 500 g protocol yielded the highest yield/number of leukocytes and neutrophils collected in total liquid H-PRF.

Histological analysis

Qualitative histological observation of liquid H-PRF gels from each group was performed, with a particular emphasis placed on the distribution of leucocytes and platelets within the fibrin clots. The loose matrix structure presented polymerized fibrin with many platelets and leukocytes entrapped within the fibrin fibers. H&E staining was conducted to determine the cell

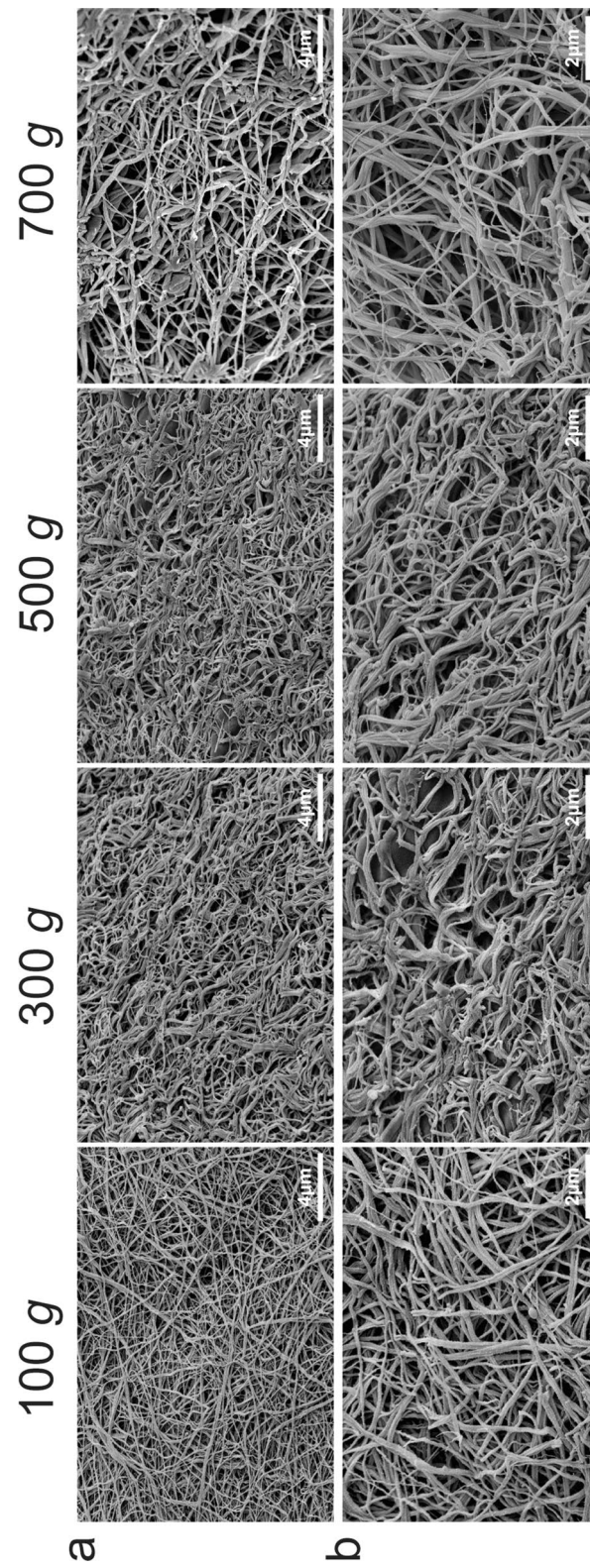


Fig. 3 SEM image of liquid H-PRF gels after various centrifuge force treatments at low/ (scale bar = 4 μm) and high magnification (scale bar = 2 μm) (samples were performed in one)

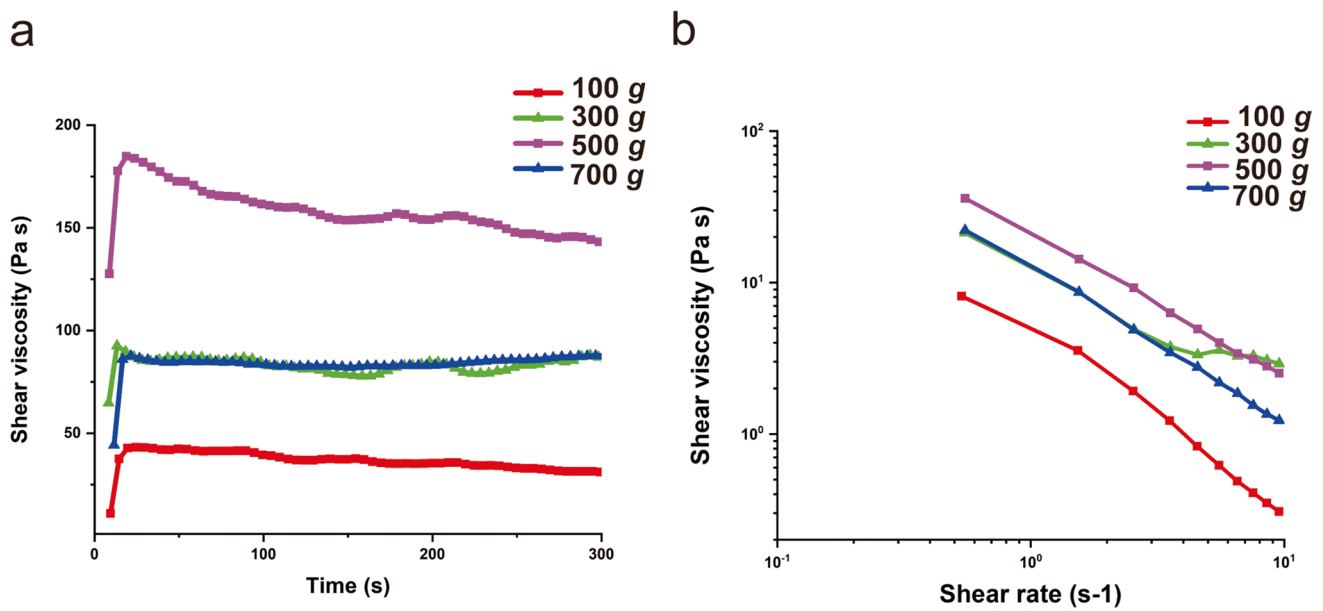


Fig. 4 Rheologic properties of liquid H-PRF gels. **a** The variations in shear viscosity with time are shown. **b** The variations in shear viscosity with shear rate are shown. (samples were performed in one)

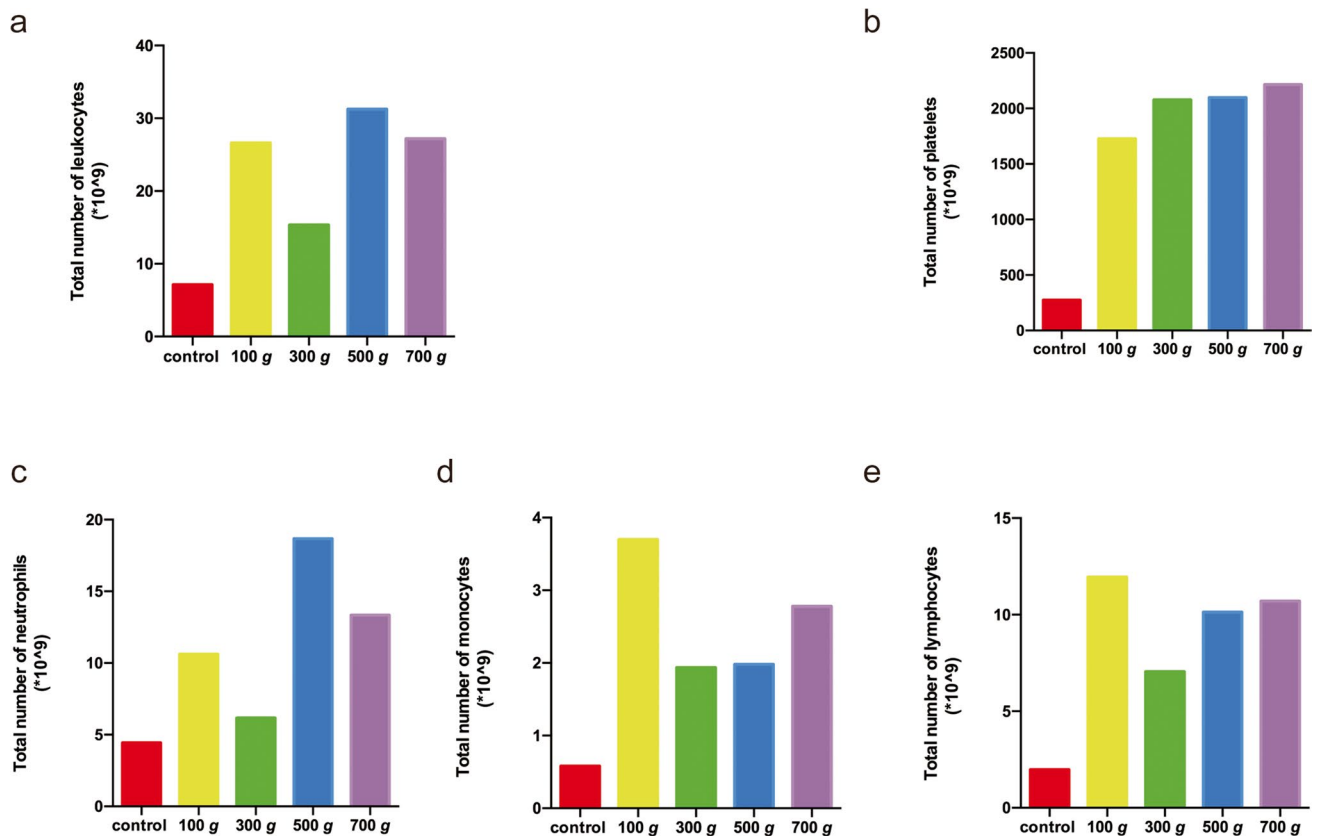


Fig. 5 The total number of cell types in each layer of liquid H-PRF from 1 mL down to the buffy coat layer utilizing different centrifuge force (samples were performed in one)

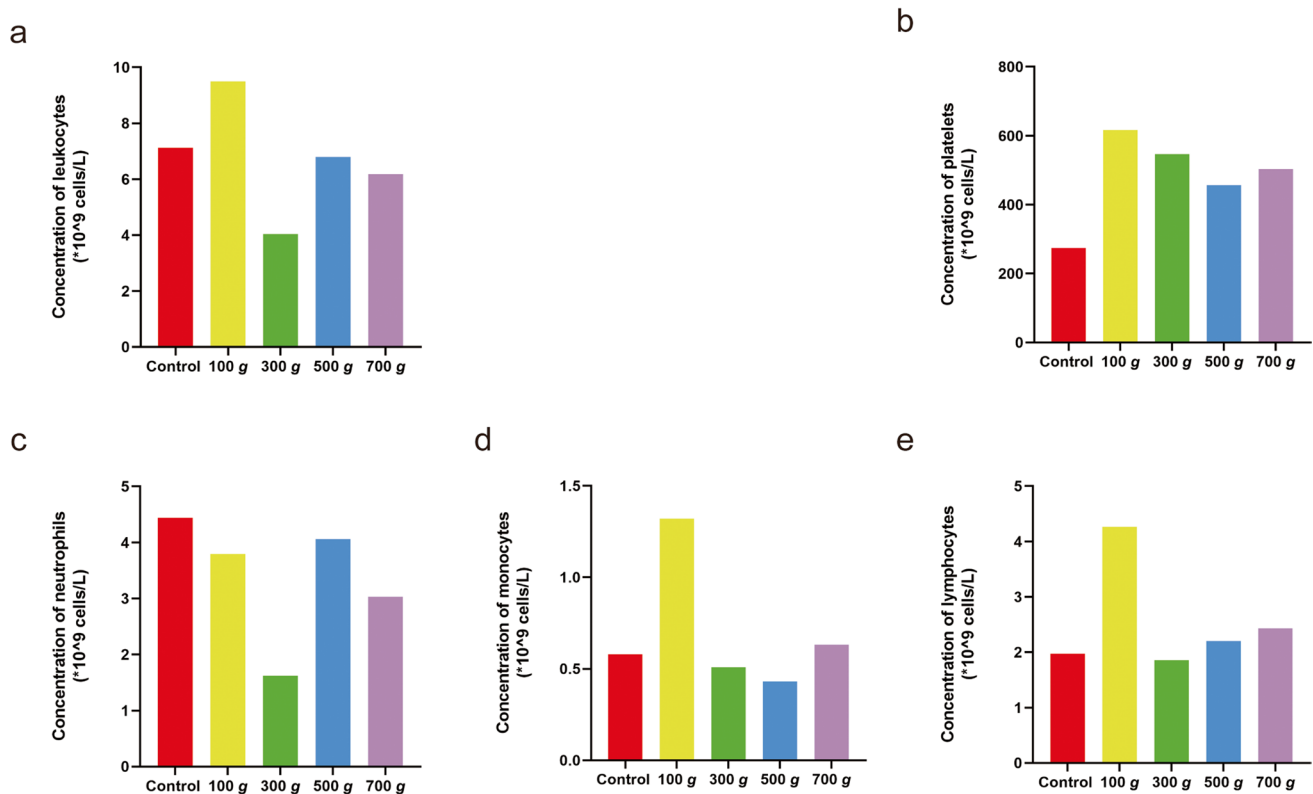


Fig. 6 The concentration of cell types in each layer of liquid H-PRF from 1 mL down to the buffy coat layer utilizing different centrifuge force (samples were performed in one)

distribution in cross sections of the four PRF-based matrices. In general, the highest concentration of cells aggregated was found in the lower half of the four PRF gels (Fig. 7). In the present study, protocols at 100 g and 300 g were inadequately effective at separating cell layers, and even the 8 min protocol did not result in a sufficient yield of platelets/leukocytes. Although the centrifugal force was short, most of the cells remained in the lower red blood cell portion due to the low RCF values applied (Fig. 7a, b). Liquid H-PRF, prepared using a higher RCF at both 500 g and 700 g, showed more even distribution pattern of cells with the most optimum results found at the 500 g centrifuge protocol. While the 700 g obtained the highest yield of cells, the volume of liquid H-PRF obtained at 700 g was also the greatest among all groups thereby lowering the concentration of cells (Fig. 7d). In contrast, a 500 g for 8 min protocol led to a higher concentration of leukocytes and platelets more noticeably spread within the upper layers of the liquid-PRF (Fig. 7c).

Discussion

Liquid PRF is an entirely 100% natural, autologous, and unaltered platelet concentrate that requires no additives or anticoagulants [31]. A large number of studies (in vitro,

in vivo, and clinical trials) across many fields of medicine and dentistry have now shown its superior wound healing ability. Thus, in the present study, it was also anticipated that local injection of liquid PRF could potentiate gingival fibroblast improvements or alleviate inflammation leading to satisfying clinical outcome [2, 14]. This is mainly because compared with exogenous growth factors and biodegradable materials, using autologous PRF is considered a natural way of regenerating tissues and restoring skin degradation by the natural polymerizing of PRF into its gel form. This has previously been demonstrated to significantly improve the release of growth factors found within PRF over time in much higher numbers when compared to PRP [10]. In addition, platelet concentrates release many cytokines that enhance extracellular matrix proteins that are either involved in or contribute to cell migration and proliferation, as well as the production of additional extracellular matrix proteins, which is difficult to achieve by other artificial fillers [12, 32, 33].

In our study, the morphology of a flowable and injectable liquid H-PRF was evaluated by mechanical testing, rheologic measurements, and scanning electron microscopy. We carefully inspected the fibrin network embedding cells to evaluate the biological and mechanical behavior of liquid H-PRF gel for soft and hard tissue repair. Li et al.

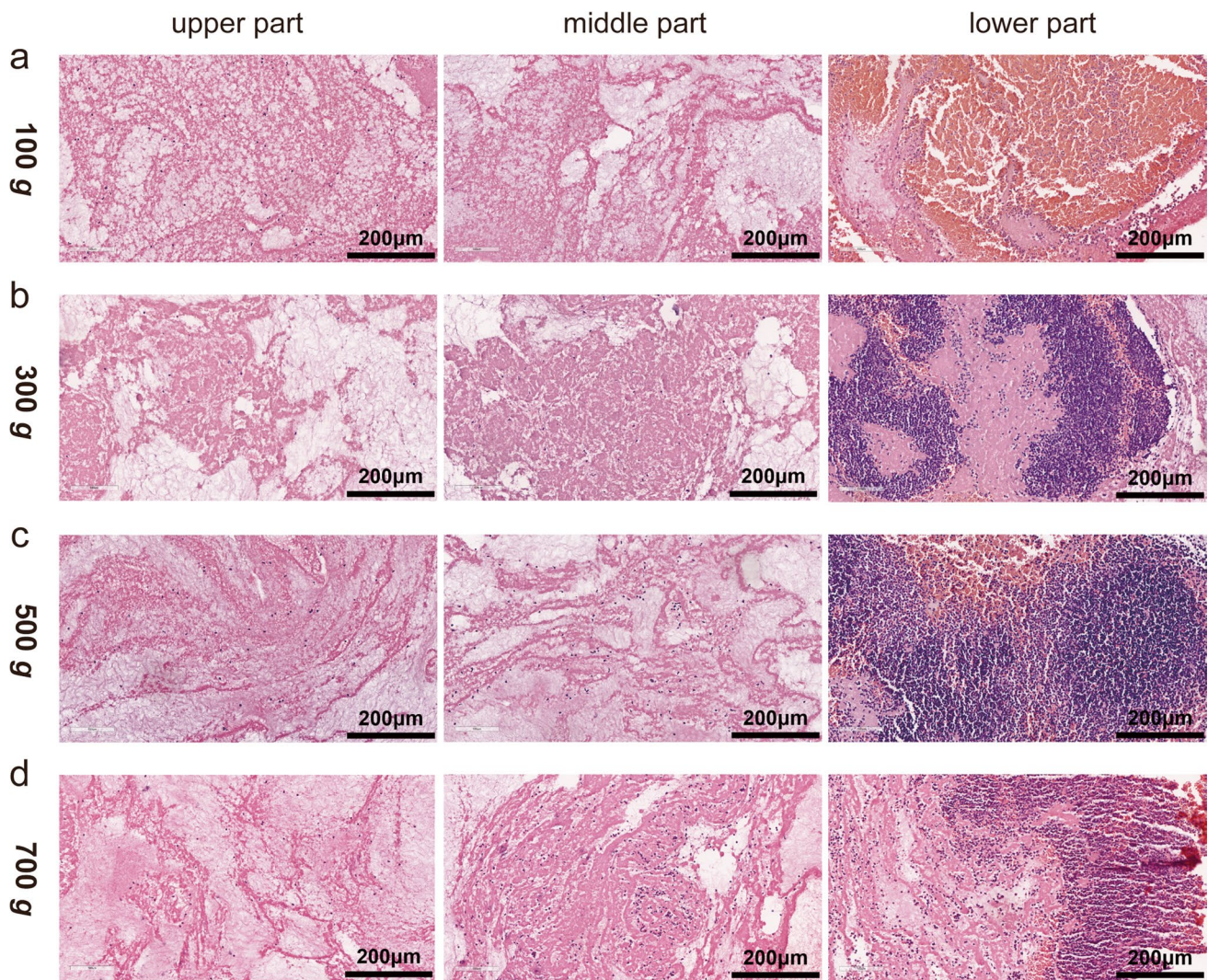


Fig. 7 H&E staining observation the three parts of liquid H-PRF gels sectioned. **a, b, d** Most of the cells remained in the lower red blood cell portion at the centrifuge force of 100 g and 300 g. **c** Cells

obtained by the centrifugation protocol of 500 g were most evenly distributed in liquid H-PRF (samples were performed in one)

found that fibrin fiber stiffness is strongly affected by its fibrin diameter [34]. From the rheologic measurements and mechanical analysis, we observed that as the centrifugal force increased, the mechanical properties of PRF also became stronger. Thus, data from the rheological properties of liquid H-PRF gels appears to point to the fact that higher RCF values may be more beneficial and more suitable for applications such as injectable material. When injecting into the targeted area for treatment purpose, the physical properties of the filler and the layer to be injected are factors that need to be considered. The biomaterial that has been injected should remain where it is and not migrate to other unwanted area after injection. In order to understand the physical properties of fillers, clinicians should consider the number, concentration, injection force, particle size, manufacturing process differences, and rheological properties of

biomaterials. Rheological properties are known as objective parameters for soft tissue fillers. Cohesiveness can be defined as a resistance to compressive/tensile forces in the vertical plane after implantation of the product. When the physical properties of the filler are too soft, the filler is relatively easy to diffuse, making it difficult to shape the target area [35–37]. In the present rheological study, we observed that the rheological properties of the fibrin clots made to be injected can be substantially altered and modified. Thus, it may be that future clinical research could better define which protocols may be better for injections into various areas of the face depending on the rheological need of the area. The results also found that the 500 g protocol seemed to provide the best biological advantage when compared to others with more cells and high modulus strength when compared to protocols at lower RCF values.

This study also demonstrated histological results indicating that more platelets and leukocytes in PRF were detected in the lower portions of liquid H-PRF gels irrespective of RCF values investigated. Therefore, this layer where the buffy coat is located is especially useful for the adequate concentration of liquid H-PRF. It was previously shown that even some cells were found in the red buffy coat zone which is this area is needed for optimal concentration of liquid-PRF. Lastly, compared with the centrifugal force of 100 g, 300 g, and 700 g, the centrifugal force of 500 g demonstrated histologically the most even cell distribution among the upper PRF layers. This is particularly relevant when the liquid H-PRF gels are compressed and utilized as bioactive membrane for regenerative purposes. The present study also has limitations for the lack of cell studies and animal studies. In the future, it is necessary to further explore the effect of liquid H-PRF gels obtained by this scheme on promoting the regeneration of hard and soft tissues in vivo, so as to provide reference for the clinical application of liquid H-PRF gels.

Conclusion

In summary, our results demonstrated that the liquid H-PRF gels obtained using a 500 g for 8 min could produce a uniform, moldable clot with great rheologic, high cell volume, and concentration evenly distributed throughout the upper plasma layers. This study demonstrates that liquid H-PRF obtained using a 500 g for 8 min protocol might be most suitable for using as injectable fillers with the most proper mechanical properties as well as good cell contents. However, further animal studies as well as clinical studies with larger sample size in the future would be needed to comprehensively study the short- and long-term effects of liquid H-PRF on periodontal injection.

Author contribution All authors contributed to the study conception and design. The study was conducted under the supervision of Yulan Wang and Yufeng Zhang. Material preparation, data collection, and analysis were performed by Mengge Feng, Yan Wei, Hongjiang Wei, and Yunxiao Wang. The first draft of the manuscript was written by Mengge Feng, revised by Yulan Wang and Richard J Miron. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Ethical approval The research involves collecting blood from human participants. All protocols used were approved by the Ethics Committee of the School and Hospital of Stomatology, Wuhan University (B52/2020) in this study.

Consent to participate Written informed consents were obtained from volunteers.

Conflict of interest The authors declare no competing interests.

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