Original Article

Evaluation of Histological and pH Changes in Platelet-Rich Fibrin and Platelet-Rich Fibrin Matrix: A *In vitro* Study

Abstract

Background: The autologous platelet concentrates (PCs), such as platelet-rich fibrin (PRF) and platelet-rich fibrin matrix **(**PRFM), are processed through different centrifugation protocols, which can affect their biological properties and in turn influence treatment outcome. The pH value can influence the process of wound healing directly or indirectly. Hence, a study was conducted to evaluate fibrin network pattern, initial pH of obtained matrix, and its changes during degradation matrix and to determine platelet and leukocyte count in PRF and PRFM. **Materials and Methods:** Blood from the volunteers was collected in blood vacutainers for processing PRF and PRFM. It was centrifuged as per the standard protocol. Serum from PRF and PRFM was subjected for analysis of platelet and leucocyte concentration using Hemo-Analyzer. The pH of PRF and PRFM were evaluated over 5 days using a pH meter. PRF and PRFM were subjected to histological and scanning electron microscopic analysis. **Results:** There was no significant variation in the platelet and leukocyte count between PRF and PRFM. A steady rise in the level of pH with respect to PRFM was observed, whereas there was a decrease in the pH levels in PRF. Fibrin network was denser in PRF compared to PRFM. **Conclusion:** The PCs undergo variation in pH upon degradation. Formation of fibrin matrix is influenced by the method of preparation. Fibrin pattern is crucial to facilitate adhesion of cells and transport of nutrients to enable proliferation and differentiation of mesenchymal cells and better wound healing.

Keywords: *pH, platelet concentrates, platelet count, platelet-rich fibrin matrix, platelet-rich fibrin, wound healing*

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Introduction

Platelet concentrates (PCs) have evolved as regenerative therapeutic materials of choice in most medical and dental procedures. It was developed to reinforce natural healing process, endorsed by inherent growth factors and cells. Currently, several techniques are available for the preparation of PCs. It is available as the first-generation platelet-rich plasma (PRP) and second-generation platelet-rich fibrin (PRF). Added to these, we have platelet-rich fibrin matrix (PRFM), advanced PRF, and recently injectable PRF.[1]

Wound healing involves complex process which involves degradation and reassembly of ectodermal, endodermal, and mesenchymal tissues to complete regeneration process. Literature indicates that the pH value can influence the process of wound healing directly or indirectly.[2] Traditional belief of low pH value for favorable healing is contrasted by alkaline environment favoring skin graft take up. Hence, pH of the wound has a potential influence over healing process and different pH ranges are required for certain distinct phases of wound healing.[2] Hence, it is important to understand the pH of biological dressing such as PCs and its variation as the matrix degrades.

Fibrin matrix in PRP and PRFM is obtained through activation process mediated by addition of bovine thrombin and calcium chloride, which leads to rapid formation of tetramolecular or bilateral junction, which constitutes thick rigid network, whereas slow physiological polymerization in PRF results in trimolecular or equilateral junction, constituting flexible fibrin network.[3] This process of external (PRFM) or internal (PRF) activation of clotting mechanism and speed at which the fibrin meshwork forms may influence the quantity of platelets or white cells getting

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encased within the matrix. The claims about the resultant fibrin matrix can also be verified through histological and scanning electron microscopic (SEM) evaluation. A large number of studies emphasize on platelet concentration, but the role of leukocytes and their interaction during the process of tissue regeneration is often neglected. The interaction of these native cells with the matrix is crucial for wound healing and depends primarily on the fibrin structure, such as the thickness of the fibers and branching pattern.[4] Hence, a study was undertaken with the objectives of evaluation of fibrin network pattern between PRF and PRFM, initial pH of obtained matrix, and its changes during degradation matrix and to determine platelet and leukocyte count in PRF and PRFM.

Materials and Methods

Source of data

This is a single-center, prospective observational study. Enrolled subjects were volunteers at Faculty of Dental Sciences (FDS), MS Ramaiah University of Applied Sciences (RUAS). Written informed consent was taken from all subjects for participation in the study after the clearance (FDS/EC/2014-2016_06) from ethical board of FDS, RUAS.

Patient recruitment

Ten healthy volunteers who were not on any medication were chosen. Subjects having history of blood dyscrasias, any anticoagulant or immunosuppressive therapy, and alcoholics were excluded. Blood samples were obtained from the antecubital vein using sterile standard phlebotomy procedures.

Preparation of platelet-rich fibrin

The PRF preparation was adopted from the protocol by Choukroun *et al*.[5] The sample blood was drawn in 4 ml tubes (BD Vacutainer, USA) and centrifuged at 400 g for 10 min in a centrifuge machine (Remi Elektrotechnik Limited Vasai, Bharat). The resultant fibrin is separated from red corpuscles using a scissor. Noncellular plasma at the top is aspirated through a syringe and kept for the determination of platelet and leucocyte count [Figure 1]. Of 10 samples, five samples were stored in Dulbecco modified culture medium (DMEM) for pH analysis, while four samples for histological analysis and one sample for SEM were fixed in 10% formaldehyde for 24 h for histological analysis, and one sample was washed in phosphate buffered saline ([PBS], Thermo Scientific, USA) and fixed with 2.5% glutaraldehyde (Sigma-Aldrich, USA) for 20 min at room temperature for SEM analysis. Exudate was used for the determination of platelet and leukocyte count.

Preparation of platelet-rich fibrin matrix

Blood was collected in sterile collection tubes (BD Vacutainer, buffered sodium citrate 0.109M, 3.2%, USA). On mixing gently, the tubes were centrifuged at 1100 *g* for 6 min to get PRP. Later, the resultant PRP was transferred into a glass containing calcium chloride (0.25 mL $CaCl_2$ 1 M) in sterile conditions. The tubes were gently swirled and centrifuged at 4500 *g* for 25 min. A semitransparent, yellow-white platelet-fibrin matrix PRFM formed at the bottom of the tube [Figure 1]. Of 10 samples, five samples were stored in DMEM for pH analysis, while four samples for histological analysis and one sample for SEM analysis were fixed in 10% formaldehyde for 24 h

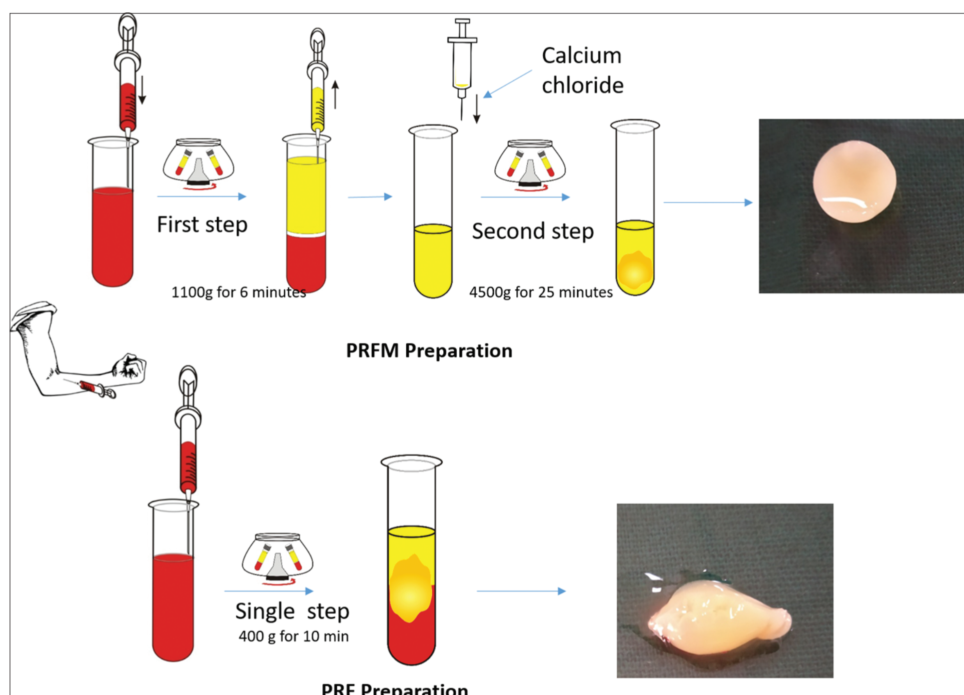


Figure 1: Schematic diagram representing preparation of platelet-rich fibrin and platelet-rich fibrin matrix with resultant matrix

for histological analysis, and one sample was washed in PBS (Thermo Scientific, USA) and fixed with 2.5% glutaraldehyde (Sigma-Aldrich, USA) for 20 min at room temperature for SEM analysis. The remaining serum was used for the determination of platelet and leukocyte count.

Tests performed

Determination of platelet and leukocyte count in platelet-rich fibrin and platelet-rich fibrin matrix

Four milliliters of blood was drawn from five volunteers as control values. Platelet and leukocyte counts were performed on patient's whole blood, exudate of PRF, and the residual serum remaining following PRFM preparation after centrifugation using automized counter at the laboratory.

Determination of pH of platelet-rich fibrin and platelet-rich fibrin matrix over a 5-day period

PRF and PRFM samples collected from different donors and stored in DMEM in a CO_2 incubator at 37°C. The baseline pH value of DMEM was noted using a pH meter (Eutech instruments pH 700) before storing the specimens. The surface pH of PRF and PRFM was evaluated over 24 h and day 2, 3, 4, and 5 using the same instrument.

Histological characteristics of platelet-rich fibrin and platelet-rich fibrin matrix

The samples were fixed in 10% neutral-buffered formalin for 24 h and processed through graded concentration of ethyl alcohol and then embedded in paraffin wax. Sections of 4-µm thickness were obtained and stained with Leishman stain to observe blood cells. Leishman stain is based on a methanolic mixture of polychromed methylene blue and eosin. This has both acidic and basic dyes. Acidic dye eosin stains basic component of the cells, i.e., cytoplasm and granules, whereas basic dye methylene blue stains acidic component, i.e., nucleus. Platelets appear as purple bodies. Slides were viewed under an Olympus optical microscope (BX53F2, Tokyo, Japan). The photomicrographs were captured with a Jenoptik Progress Gryphax Arktur USB 3.0 microscope camera, Jena, Germany.

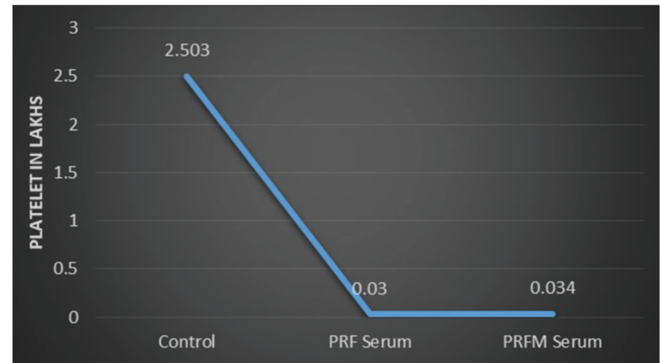
Scanning electron microscopic analysis

One sample each of PRF and PRFM were subjected to drying protocol using hexamethyldisilazane (Thermo Scientific, USA), mounted on stubs, sputter coated with gold, and observed under SEM (ESEM Quanta 200, FEI) at an accelerating voltage of 15 kV. SEM images were analyzed using Image J software, Version 1.48 (National Institute of Health, Bethesda, MD) for pattern fibrin matrix.

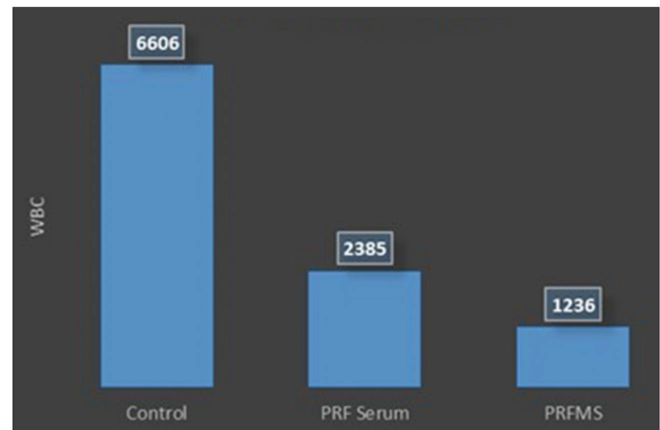
Results

The average platelet count in control group was 2.503, whereas the average platelet count in PRF serum was 0.03 and in PRFM was 0.34 [Graphs 1 and 2]. There was no significant variation in the residual serum platelet count between PRF and PRFM. There was a drop in leukocyte count from that of whole blood.

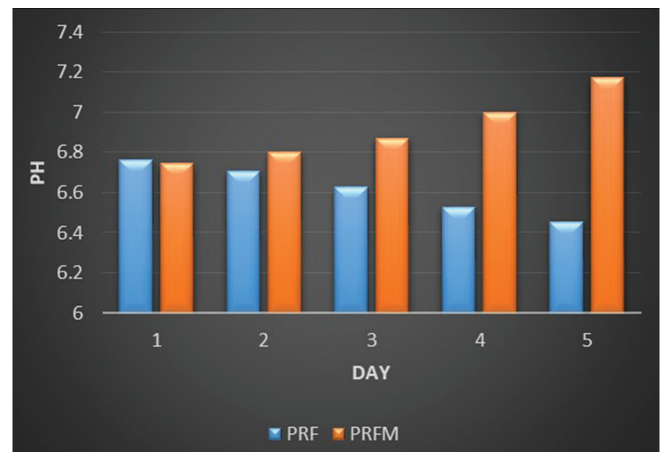
There was a steady rise in the level of pH with respect to PRFM in contrast to PRF, whereas there was a decrease in the pH levels [Graph 3]. This indicates the increasing alkalinity of PRFM and increasing acidity of PRF over 5 days.



Graph 1: Platelet count in controls and supernatant serum of platelet-rich fibrin and platelet-rich fibrin matrix



Graph 2: Leukocyte count in controls and supernatant serum of platelet-rich fibrin and platelet-rich fibrin matrix



Graph 3: Basic pH value and subsequent changes during degradation of platelet-rich fibrin and platelet-rich fibrin matrix

On microscopic examination, PRFM showed loosely arranged fibrinous matrix in association with abundant leukocytes, predominantly lymphocytes, and platelets [Figure 2a and b]. PRFM revealed dense fibrin matrix, abundant platelets diffusely spread in a fibrinous matrix with sparse leukocytes [Figure 3].

SEM analysis of PRF showed less dense fibrin network with thin fibrinous strands [Figure 4] in contrast to PRFM which showed dense fibrin network with thick fibrinous strands [Figure 5].

Discussion

Wound healing is a metabolically active process of biochemical reactions. This process is influenced directly or indirectly by pH. Among many factors which affect wound healing, extracellular and intracellular pH is significant which impacts enzymatic activity, macromolecular synthesis, transportation of nutrients, and cell cycle progression. The pH also influences different stages of wound healing such as angiogenesis, collagen formation, and macrophage activity. Matrix metalloproteinases which are involved in remodeling of extracellular matrix are also shown to be sensitive to variations in pH.[6] Under normal conditions, the pH of the skin is essentially acidic, i.e. average 4.7,[7] and may vary according to the anatomical location. It is important for the barrier function as many pathogenic bacteria are inhibited by lower pH value. However, too much lower pH may reduce antibiotic efficiency.[8]

Healing wounds exhibits temporary physiological acidosis. This may lead to Bohr effect wherein decreased binding capacity of hemoglobin leads to release of normally still bound oxygen to the tissues. This results in higher concentration of oxygen at wound edge promoting healing phase. Contrasting acute wounds such as chronic venous leg ulcers and pressure sores exhibit alkaline pH (7.3) but shift to acidic medium during reepithelialization. The nature of dressing applied to the wound can also affect the pH underneath it.

For the first time, a study of pH of PC was evaluated. In our study, we found PRF turned acidic at the day 5 as compared to day 1. Evidence has shown that alkaline pH has lower healing rate compared to acidic pH.[9] Hence, wound healing can be partially controlled by altering the pH. Depending on the type of wound, the biomaterial to promote wound healing is selected; hence, it can be inferred that PRF helps in better wound healing because of its acidic pH which promotes epithelialization and angiogenesis.[9] PRFM was observed to be alkaline on day 1 and alkalinity increased on day 5. Hence, it may not be

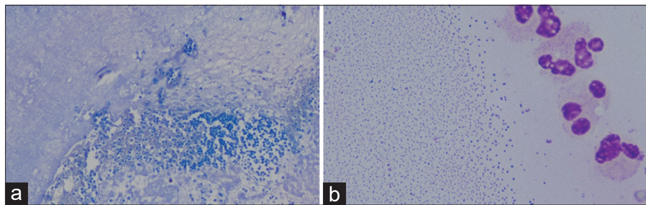


Figure 2: (a) Photomicrograph of platelet-rich fibrin (×10) showing fibrinous material with leukocytes and platelets (Leishman stain; ×100) (b) Photomicrograph of platelet-rich fibrin (×100) showing lymphocytes and platelets in a fibrinous background (Leishman stain; ×1000)

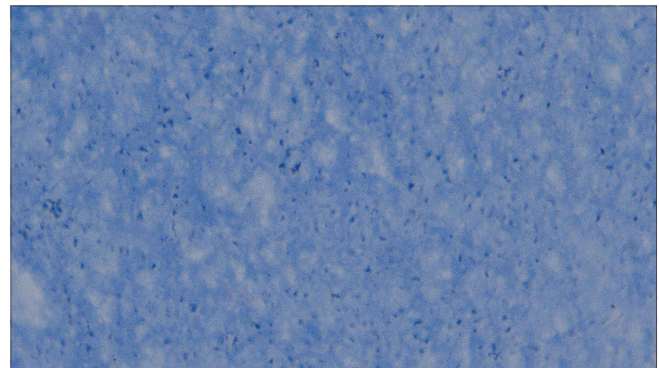


Figure 3: Photomicrograph of platelet-rich fibrin matrix (×100) showing numerous platelets in a fibrinous background (Leishman stain; ×1000)

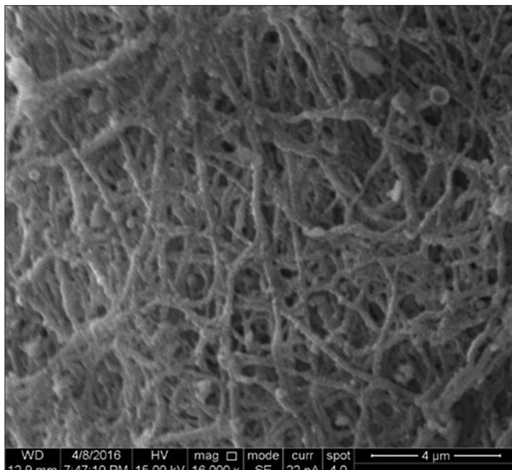


Figure 4: Scanning electron microscopy of platelet-rich fibrin showing thin fibers

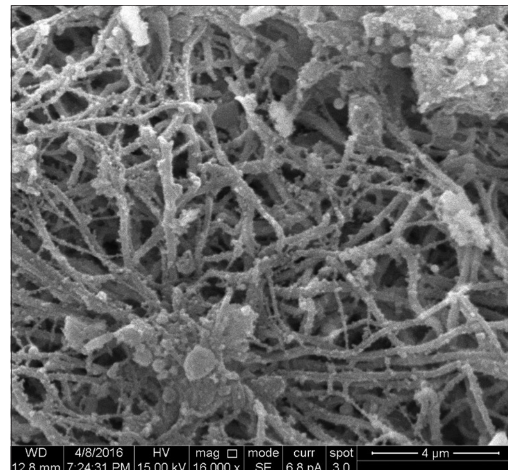


Figure 5: Scanning electron microscopy of platelet-rich fibrin matrix showing dense fibers

beneficial in wound healing except for the cases where the pus predominates and wound pH lowers significantly. The PRFM may increase pH and may restore normalcy. However, other related factors should be considered before making a choice of a biomaterial.

Quality of platelet is of significance in clinical use. Platelet counts cannot be directly determined in solid fibrin clots unlike the liquid type of PRP and blood.[10] They are instead calculated by subtraction method wherein platelet counts in different liquids such as clot exudate; l the supernatant liquid is subtracted from those in whole blood samples.[11] In our study, there was a decrease in the platelet and leukocyte count in PRF serum and PRFM serum in comparison to the platelet count of the whole blood group. Hence, by the subtraction method, we assume that platelet is incorporated in both PRF and PRFM. A similar method was adopted in other studies.[12,13] A study by Alefiya S Mamajiwala *et al*. also used Leishman stain to estimate the platelets and showed more than 90% inclusion of platelets in PRF matrix.[14] They proposed that increase in centrifugation speed and time leads to reduction of platelet inclusion in matrix, contrary to the study by Ghanaati *et al*. who proposed reduction in speed but increase in time of centrifugation.[15] However, histological sections of PRFM did not show any presence of platelets. Subtraction method does not envision the possibility of platelet contamination within the red blood cells (RBC) fraction or the potential loss of platelets during the process of centrifugation. To ensure the quality of PCs, a simple and accurate chair-side platelet-counting method should be developed.

Most of the studies have concentrated on the release of growth factors from the PC.[16-18] Three-dimensional fibrin architecture plays a key role in tissue repair and regeneration. Fibrin matrix acts as a scaffold for the cells to adhere and agglomerate at the site of injury. Fibrin network density, blood platelet, and white blood cells are interdependent. Platelets adhere, aggregate, and form a procoagulant surface favoring thrombin generation and fibrin formation in the healing of wounds.[19] In addition, platelets express and release growth factors such as Transforming growth factor – beta 1 and Platelet derived growth factor. Sustained release of these factors maintains bioactivity over healing period. These growth factors interact with tumour necrosis factor-α and Interleukin-1, attract neutrophils to the site of injury and upregulate expression of adhesion molecules and promotes tissue repair. These events influence processes such as angiogenesis, inflammation and the immune response[18,19,20] The interaction of cell surface receptors of fibrin matrix such as syndecans and integrins maintains cellular morphology and promotes cell adhesion, migration, and differentiation.[21,22] In our study, we used the hematoxylin and eosin staining technique to identify the fibrin pattern, density, and distribution of cell components in fibrin. The results were satisfactory in identifying the density of fibrin; however, we noticed inability in distinguishing platelets and leukocytes entrapped in fibrin. Further, the individual fibrin thickness and morphology could not be analyzed.

We used the cell block histological method to study the morphological aspects of the fibrin clot and cells trapped in a fibrin clot.[23] To our knowledge, this is the first comparative study of fibrin thread architecture between PRF and PRFM. Leucocyte and platelet were unevenly distributed in PRF, Majority of the cells were found concentrated near the junction of fibrin clot with RBC's [Picture 2a and b]. In PRFM, distribution of platelet and leucocyte was scanty [Figure 3]. PRF clot and membrane contain leukocytes from the initial blood harvest, and these cells are enmeshed within the fibrin matrix and lymphocytes are more often observed. The leukocytes have an excellent impact on the intrinsic biology and on properties of the PCs, due to their immune reactions and antibacterial potential. Leukocytes influence proliferation and differentiation patterns of the cells in culture. The influence of the leukocytes on the mechanism of PC action has not gained much-needed attention; its supporters claim that their influence on the inflammatory state is beneficial, while others, on the other hand, warn against their negative effect in the form of solid enzymes release.[24] Few studies have reported that inflammatory reactions and an increase in mast cells and macrophages promotes scar formation.[25]

The limitation of the study is that we used *in vitro* model to study the static cells entrapped, and we need better study model and also clinical investigations to assess PCs *in vivo* and influence of structure, cellular communication, viable cells, and bioactive elements in a dynamic environment. However, knowledge of histology, cellular concentration, and surface pH of PC can provide a guideline in the choice material for repair and regeneration.

Conclusion

Based on the study conducted, we conclude that the decrease in platelet and leukocyte count within the serum indicates that it has been transferred to the scaffold. However, histological analysis proves otherwise in case of PRFM. Hence, the subtraction method may not be the ideal method to assess the platelet concentration. The result has a strong clinical impact because the quantity of platelets and leukocytes implanted within each membrane is particularly efficient in the regulation of inflammatory reaction. Looking at the various aspects in which the pH value can influence wound healing, it clearly proves to be a potentially influential factor. The PCs have varied pH, and further clinical investigations on the impact of their pH in wound healing are required. Further, fibrin pattern is crucial to facilitate adhesion of cells and transport of nutrients to enable proliferation and differentiation of mesenchymal cells and better wound healing.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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