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Evaluation of Composition and Architecture of the Platelet Rich Fibrin: An Observational Study

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Abstract:

Aim: To undertake a detailed examination of the cell composition and structure of Choukroun's platelet rich fibrin using hematologic counts, scanning electron microscope and light microscopy. Additionally the aim is to also find any differences in:

- Structure and morphology when PRF is produced using two varied kinds of collection tubes, namely, glass and plastic tubes.
- Structure and morphology when two different methods of compression of PRF clot are used to get a membrane (compressed/soft)

Materials and Methods: Sample set of 14 healthy subjects participated in this study. Blood collected from each of the subjects was subjected to centrifugation; the platelet rich clot/membrane obtained was processed and subjected to light microscopy and scanning electron microscopy examination. The residual plasma was subjected to blood analysis.

Results: The study revealed that the PRF clot contained most of the platelets and more than 50% of the leukocytes from the blood sample and their distribution adhered to a distinctive three-dimensional pattern. No significant difference in the PRF architecture and composition was found between groups using different tested collection tubes and compression techniques.

Conclusion: The PRF contained most of the platelets and the leukocytes from a blood harvest into a single autologous fibrin biomaterial.

Keywords: Platelet rich fibrin; platelets; leukocytes; biomaterial

Introduction:

The use of blood-derived products to seal wounds and stimulate healing started with the use of fibrin glues, which were first described 40 years ago and are constituted of concentrated fibrinogen (polymerization induced by thrombin and calcium[1]. Platelet-rich fibrin (PRF) is a second – generation platelet concentrate which allows one to obtain fibrin membrane enriched with platelets and growth factors, after an anticoagulant-free blood harvest. PRF was developed in France by Choukroun et al.in 2001 and is widely used to accelerate soft and hard tissue healing. The advantages of PRF over the platelet-rich plasma (PRP) include ease of preparation/application, minimal expense, and lack of biochemical modification (no bovine thrombin or anticoagulant is required). PRF is a strictly autologous fibrin matrix containing a

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large quantity of platelet and leukocyte cytokines[2] and the method is simple and inexpensive: blood is collected in glass or plastic tubes and immediately centrifuged. During the centrifugation process three layers are formed: a red blood cell (RBC) base at the bottom, acellular plasma (platelet-poor plasma [PPP]) as a supernatant, and a PRF clot in the middle. The biologic activity of the fibrin molecule is enough in itself to account for the significant cicatricial capacity of the PRF and the slow polymerization mode confers to the PRF membrane a particularly favourable physiologic architecture to support the healing process[3]. In addition, the clinical effects of PRF with or without membrane in extraction sockets was determined in mongrel dogs and it was found that PRF enhances healing and osseous fill in extraction socket taken place within 3 weeks[4]. PRF in various surgical procedures like, sinus floor augmentation during implant placement[5], with coronally displaced flap in multiple gingival recessions[6], and in facial plastic surgery procedures[7] have been shown to provide promising results. Though platelets and leukocyte cytokines play an important part in the biology of this biomaterial, the fibrin matrix supporting them certainly constitutes the determining element responsible for the real therapeutic potential of PRF[8]. Cytokines are immediatelyconsumed and destroyed in a healing wound. The synergy between cytokines and their supporting fibrin matrix has much more importance than any other parameter. A physiologic fibrin matrix (such as PRF) will have very different effects than a fibrin glue enriched with cytokines (such as PRP), which will have a massively uncontrollable and short-term effect[2].

The aim of this study was to perform an examination of the composition and architecture of the Choukroun's PRF clot using hematologic counts, photonic microscopy, and SEM. The secondary objective is to point out (if any) the structural and morphologic differences between PRFs commonly produced with two different kinds of collection tubes (glass and plastic tubes) and using two different methods for the compression of the PRF clot into the membrane (compressed or softly).

Materials and methods:

A total of 14 subjects (10 male and 4 female) were included in this study between March and May 2011. All subjects were medically and periodontally healthy volunteers recruited from department of periodontology, AECS Maaruti College of Dental Sciences and Research Centre, Bangalore. The study was approved by the Ethical Committee of AECS Maaruti College of Dental Sciences and Research Centre, Bangalore and conducted according to the guidelines for Good Clinical Practice. Careful consideration was adopted to ensure that the subjects were aged between 20 – 50 years with no previous history of aspirin intake or other medications for one month prior and presence of systemic disease. For each volunteer, the blood sample (11 tubes of 5 ml each) was obtained from the antecubital vein (in two stages: five tubes harvested on the right arm and six tubes harvested on the left arm). One tube contained an anticoagulant for whole blood analysis (control group). The 10 other tubes were taken without anticoagulant for PRF production (test groups): five were taken in glass tubes (series 1) and five in plastic tubes (series 2).

After the blood collection, the tubes were centrifuged at 3,000 rpm for 10 minutes immediately with a specific table centrifuge (Remi R-8C laboratory centrifuge) at room temperature. After centrifugation, the PRF clot was removed from the tube using sterile tweezers, separated from the RBC base using scissors, and placed in a sterile metal cup. Each PRF clot was allowed to release its serum (PRF-clot exudate). In each series (glass or plastic tubes), two clots were emptied from their serum by compressing them with a metal spoon (forcible exudate extraction; method 1), and two clots were left aside to release their serum slowly during 20 minutes into a metal cup (soft exudate extraction; method 2) Fig A. Finally, in each series, the fifth clot was processed for SEM evaluation and fixed in 2.5% glutaraldehyde directly with its serum content without compression.

In each series, the four emptied membranes were sent for histologic examination; in each method group (forcible or soft exudate extraction), one membrane was analyzed using light microscopy, and one membrane was analyzed using SEM.

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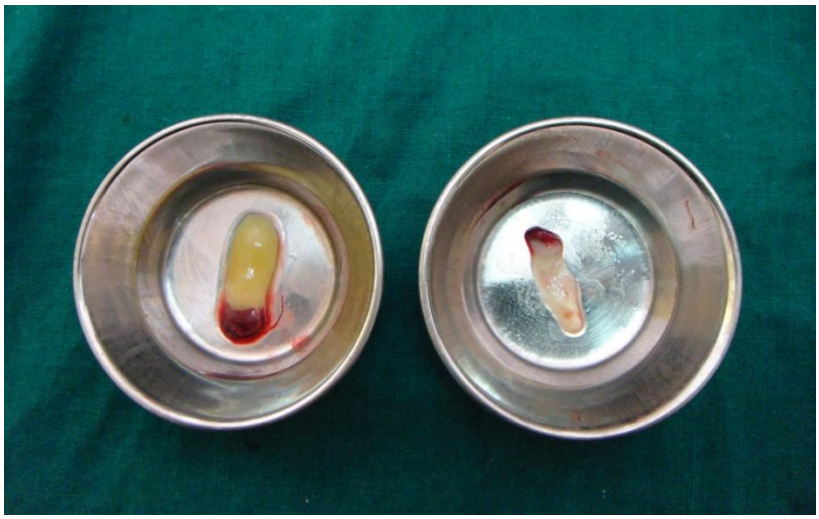
Fig A. Forcible exudate extraction; method 1 and Soft exudate extraction; method 2 – PRF clot



Leukocyte and Platelet Counts Analysis:

For each subject, the harvesting were analysed using an automatized counter (Beckman Coulter Automat, Beckman Coulter) at the hematologic laboratory after isotonic dilution:

- Whole blood with anticoagulant (control group): one sample per volunteer
- RBC base put back into solution using the PPP supernatant and the PRF clot exudate obtained by strong compression (method 1): four samples per volunteer (two in dry glass tubes and two in glass coated plastic tubes)
- RBC base put back into solution using the PPP supernatant and the PRF clot exudate obtained without compression (method 2): four samples per volunteer (two in dry glass tubes and two in glass coated plastic tubes).

The Haemogram and mean platelet volume was measured and the leukocyte formula was evaluated by flow cytometry.

Histologic Procedures for Light-Microscopy Evaluation:

PRF membranes were dehydrated in increasing gradients of alcohol (70%, 80%, 95%, and 100%) and placed in toluene before paraffin inclusion. After complete dehydration, the membrane was 0.5 mm thick. For each PRF membrane, a series of 10 successive 7-µm sections was performed according to the long axis of the membrane; i.e., 140 µm of the membrane thickness could be analyzed in a longitudinal and reliable manner. These 20 sections were stained using two different specific protocols: 10 sections with hematoxylin and eosin and 10 sections with Masson's trichrome.

Histologic Procedures for SEM Evaluation:

A morphologic evaluation of the PRF clot and membrane was conducted with a scanning electron microscope (Quanta 200 ESEM). The PRF clot and membrane were fixed in 2.5% glutaraldehyde for 1 hour and then treated with osmium tetraoxide and finally was kept in a dessicator for dessication. To observe the fibrin matrix, the PRF clot was cut longitudinally in its center, and the membrane was cut at the yellow end. Specimens were sputter coated with 20 nm gold and examined in a scanning electron microscope. Photographs were taken at 15 to 25 kV using 15 to 5000 magnifications. SEM was used to complete the observations of the photonic microscopy concerning the identification of the cell bodies trapped in the matrix (leukocytes, platelets, and RBCs) and to analyze the overall architecture of the fibrin network.

Analyses of Platelet and Leukocyte Distribution:

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Each series of stained longitudinal sections observed by light microscopy was analyzed by counting the platelet aggregates and leukocytes (violet spots) in the different areas of the membrane. The distinction between platelet aggregates and leukocytes was only possible by morphologic examination in the microscope (operator - dependent).

Statistical Analyses:

The intent of this study was to be mainly descriptive in nature. However, results of blood counts were analyzed statistically to ensure deviation from the standard. Results obtained in dry glass tubes (series 1) and glass coated plastic tubes (series 2) were compared to each other globally and within each method group and finally compared to the control group. Moreover, in each series, the two methods were compared to one another. Statistical analyses were performed by one-way analysis of variance, and when there was a significant difference, the post hoc tukey test was used. P values <0.05 were considered statistically significant.

Results

Leukocyte and platelet counts:

Blood analyses are usually difficult to interpret because of large inter individual variations. In the test groups (series 1 and 2), the leukocyte levels dropped compared to the control group ($P < 0.01$); more than fifty percent of the leukocytes seemed to have disappeared (Table 1).

Table 1: Number of leukocytes, RBC and Platelets in whole blood (control group) and Residual plasma base in test groups

	Leukocytes/µl		**RBCs/µl**		**Platelets/µl**	
Sample	**Average**	**Range**	**Average**	**Range**	**Average**	**Range**
Control	**7382**	**6500 - 8400**	**5.05 (10^6)**	**5.5 – 5.52 (10^6)**	**2.02(10^5)**	**1.7– 2.35(10^5)**
Series 1	**3514.5**	**3100 - 4000**	**5.75 (10^6)**	**5.5 – 5.52 (10^6)**	**6308.5**	**4200 - 7800**
Series 2	**3603.25**	**3000 - 4200**	**5.81 (10^6) (10^6)**	**5.61 – 6.01**	**7488.25**	**6300 - 9500**

Most of the platelets (> 95%) were absent from the test group tubes after PRF membrane extraction. There was no difference between the two method groups ($P > 0.05$). The leukocyte counts was significantly different between the control group and test groups (Table 2).

Table 2: Leukocytes formula in whole blood (control group) and Residual plasma base in test groups

	Total Blood (%)		**Series 1 (%)**		**Series 2 (%)**	
Cell Type	**Average**	**Range**	**Average**	**Range**	**Average**	**Range**
Neutrophils	**53.2**	**51.4 – 55.6**	**70.18**	**65.4 – 78.3**	**67.74**	**61.5 – 72.8**
Eosinophils	**3.6**	**2.7 – 4.5**	**5.82**	**3 – 9.3**	**4.91**	**3.6 – 6.7**
Basophils	**0.5**	**0.3 – 0.7**	**0.1**	**0 – 0.4**	**0.5**	**0 – 0.7**
Lymphocytes	**38.35**	**36.3 – 40.1**	**19.85**	**17 – 22.6**	**23.18**	**20.7 – 28.3**
Monocytes	**7.85**	**7 – 8.6**	**4.06**	**1.2 – 7.8**	**4.02**	**2.5 – 5.4**

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In the test groups, lymphocyte proportions were significantly lower, and neutrophil-leukocyte proportions were significantly higher, than in the control group (P <0.01), indicating that lymphocytes were more likely to be trapped in the PRF matrix than the other leukocytes, which tended to be eliminated with the residual RBC base. The mean platelet volume decreased significantly between the control and test groups (P <0.01) (Table 1). It was not possible to demonstrate either a significant difference (P >0.05) in residual blood contents between method groups (forcible or soft exudate extraction) or any difference between the tube series (dry glass tubes and glass-coated plastic tubes), or within each method group (Tables 1 and 2).

Light-Microscopy Study:

The PRF clot can be described as composed of two main parts a fibrin yellow portion, constituting the main body, and a red RBC portion located at the end of the clot. Between these two areas, a whitish layer called the ‘‘buffy coat’’ is present. Histological sections were taken from these three layers.

Hematoxylin and eosin stain:

The fibrin matrix appeared homogeneous in light pink, and platelet aggregates were dark blue/violet (Fig. 1A). RBCs and leukocyte cytoplasm were not easily detectable: they were darker pink. The leukocyte nuclei were stained in dark blue with the hematoxylin, but showed similarity to platelet aggregates. Therefore, it was very difficult to distinguish the aggregates from the platelet aggregates (Fig. 1B).

Fig 1A. Pink homogenous material with few clusters of cellular elements is seen (low magnification)

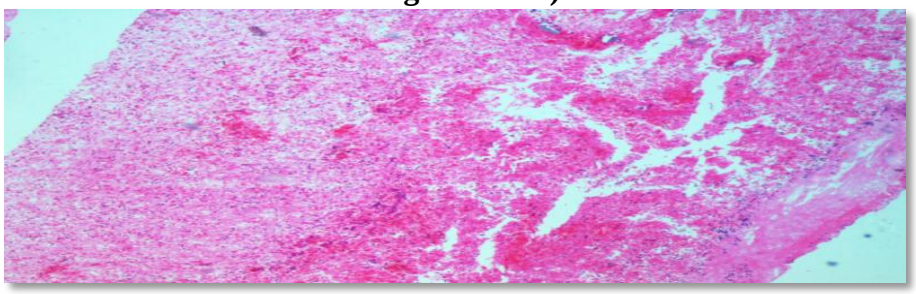
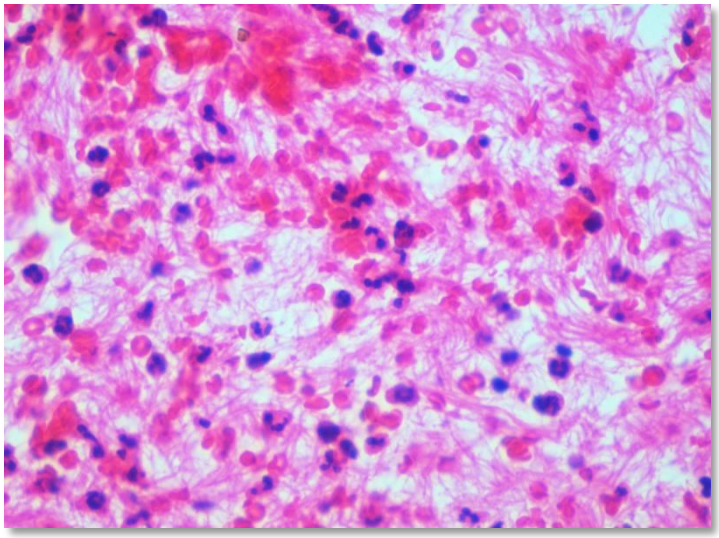


Fig 1B. Violet spots seen which indicate the leukocytes and platelets (high magnification)



Masson’s trichome stain

The platelet aggregates were still dark blue, but RBCs were stained in bright red and were easily identifiable (Fig 2A). Leukocytes were still difficult to

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separate within the stained platelet aggregates. At higher magnification (400x) the PMNs could be clearly distinguished from platelet aggregates (Fig 2B).

Fig 2A. Blue homogenous material with few clusters of cellular elements (pink and blue) are seen (low magnification)

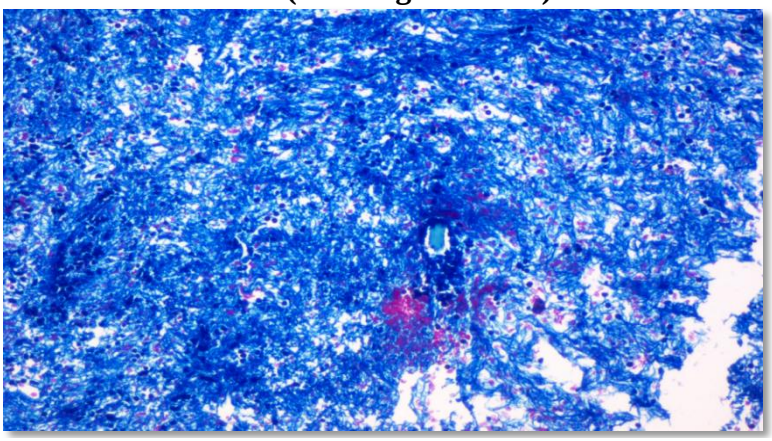
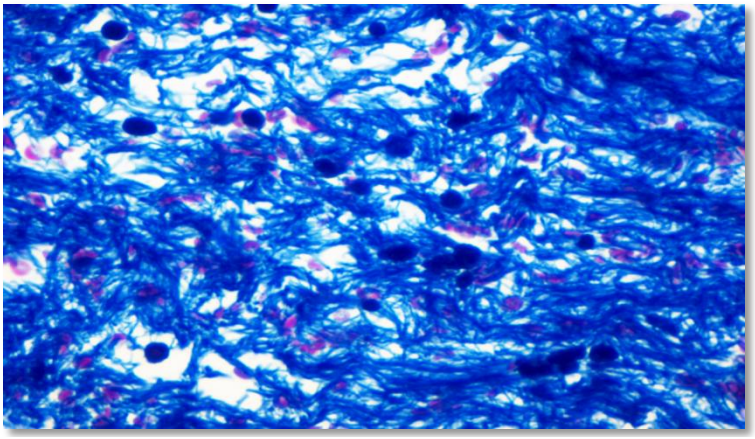


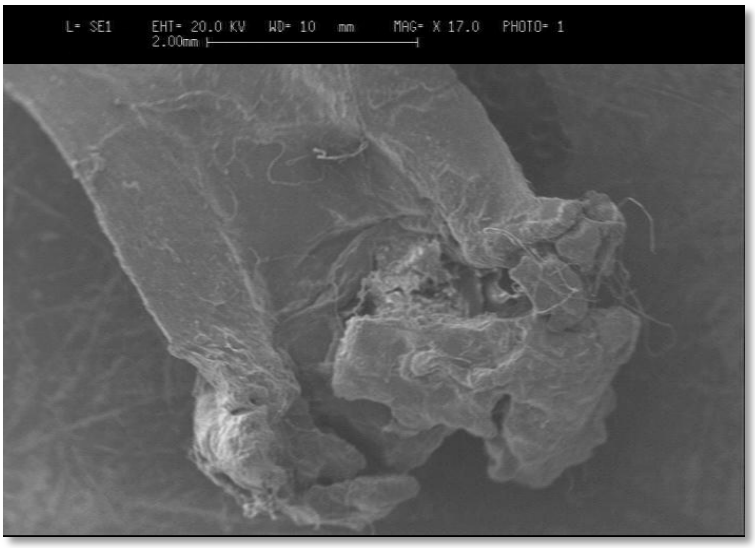
Fig 2B. RBCs are stained red and the leukocytes and platelets are blue (high magnification)



SEM Evaluation

Again the PRF clot/membrane (Fig 3) was analysed in three parts: Red end of the PRF, at borderline between RBC area and yellow clot and yellow part of the clot.

Fig 3. PRF clot analysed using SEM at low magnification (17x)



Red area of the PRF:

The red area of the clot contained clusters of RBCs which was easily identified due to its

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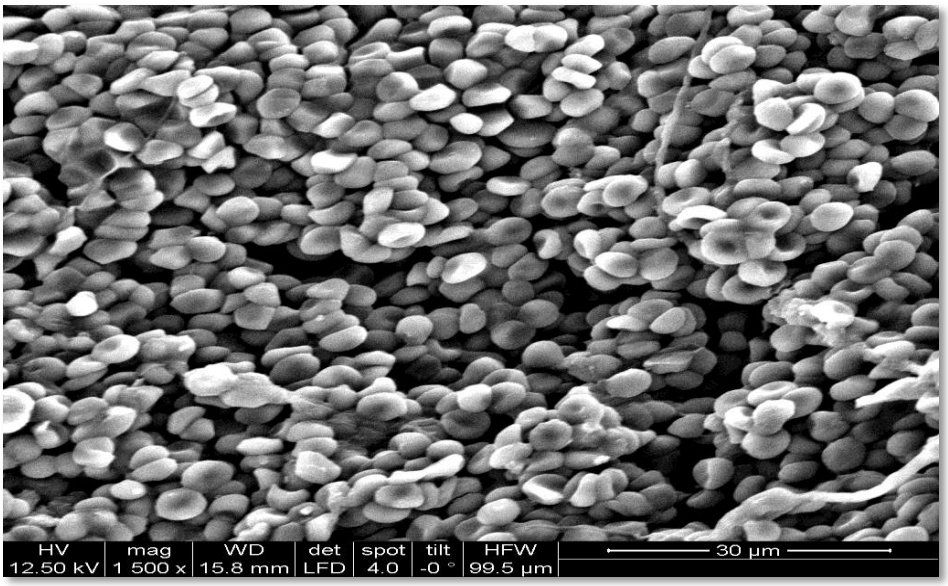
morphology i.e. the biconcave shape of the cells (Fig 4)

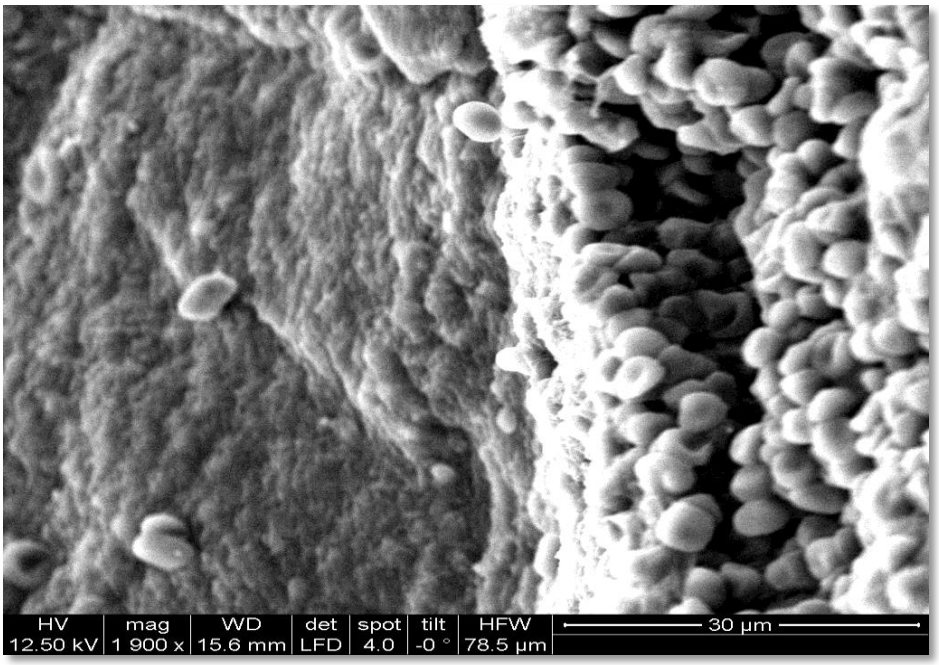
Fig 4. RBC area of the clot



Borderline between RBC area and yellow clot:

This area represents the buffy coat region. The area was composed of thick fibrin strands and a few scattered RBCs (probably from contamination during clot handling). The fibrin network appeared to be mature. It also contained platelets and fibrin that formed large and dense clusters due to extensive aggregation and clotting (Fig.5).

Fig 5. PRF clot – The RBC area and yellow part of the clot



This aggregate formed a solid and thick mesh. The maximum density of platelet/leukocyte ratio was analysed in the buffy region and throughout the clot width the distribution of platelet/leukocyte was homogeneous (Fig 6A). The cells were grouped according to central or centrifugal platelet-concentration veins and these veins in their cellular free matrix offered a high platelet/leukocyte densities (Fig 6B).

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Fig 6A. Borderline area of red and yellow part (buffy coat region)

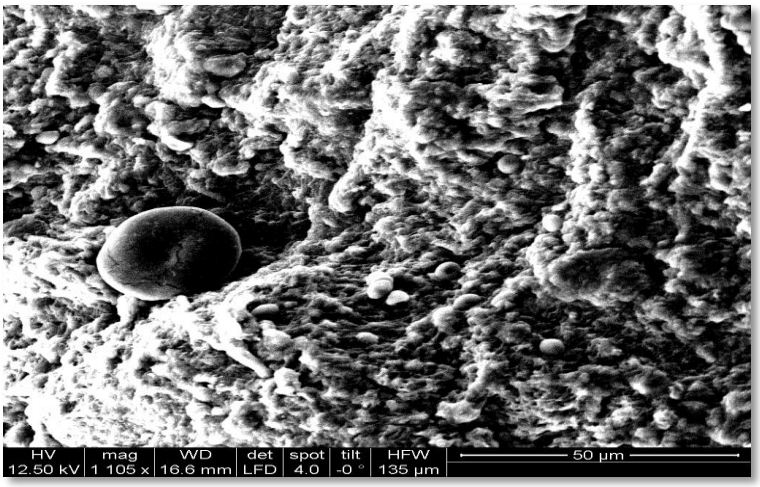
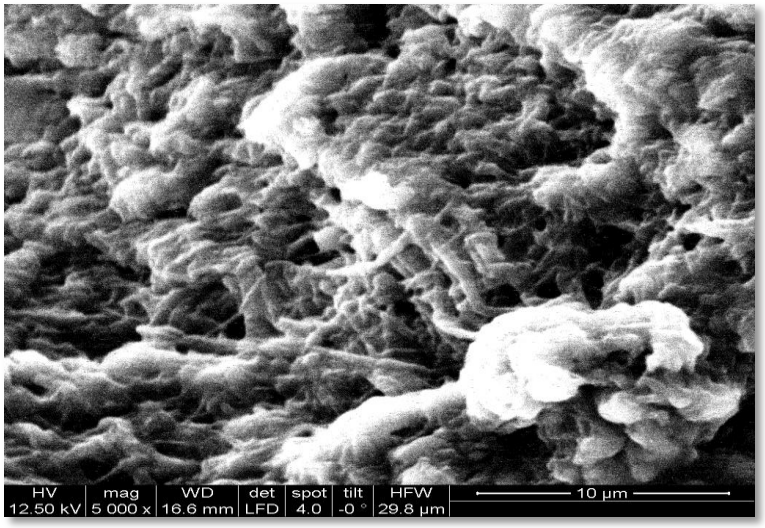


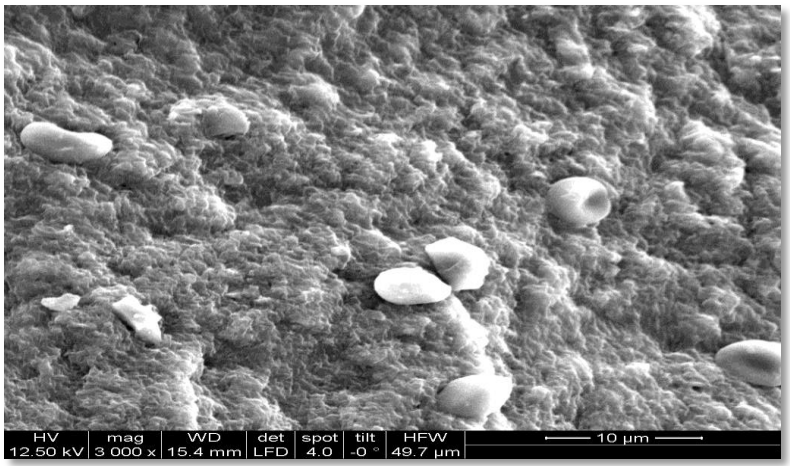
Fig 6B. The centrifugal platelet concentration veins seen in the buffy coat region



Yellow part of the clot:

This region constitutes the majority of the clot. A dense fibrin matrix can be seen along with a few cells (RBCs) which would probably be artifacts due to clot handling. (Fig. 7).

Fig 7. Yellow region of the clot



The PRF membrane showed fibres to be arranged compactly and the bundles were arranged in a parallel fashion. The cells were embedded in this compact fibre matrix. This is due to the compression technique that was employed to make the membrane (Fig 8A & 8B).

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Fig 8A. PRF membrane – arrangement of fibres

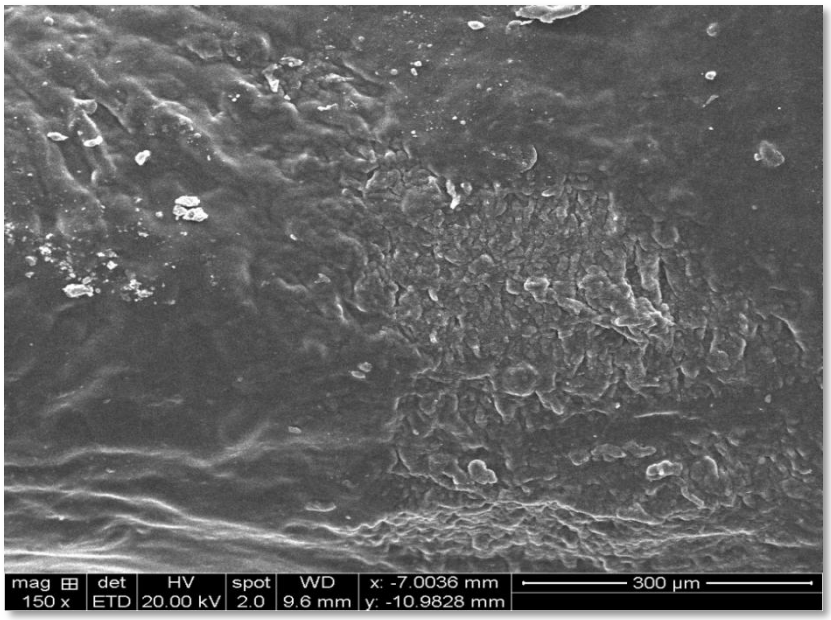
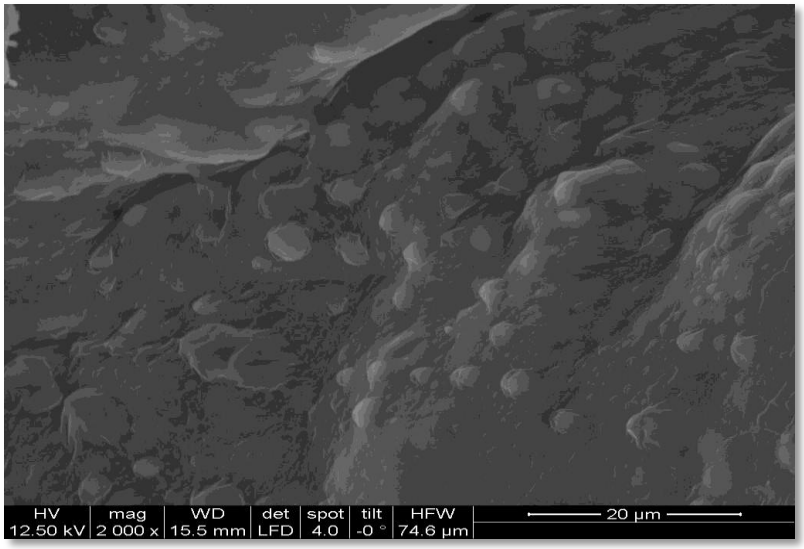


Fig 8B. PRF membrane cells embedded in the fibrin matrix



The PRF harvested showed that it's morphology was similar to each other and was not altered by the different parameters i.e individual patients, sample collecting tubes and method of PRF clot collection or usage

Discussion

A global classification of platelet concentrates was published, and these products are now classified in 4 families related to their leucocytes and fibrin contents[10]. Choukroun's PRF is currently the biomaterial in L-PRF class (Leukocyte and Platelet-Rich Fibrin), with both high leukocyte and strong fibrin architecture. Considering that research has proved that PRF protocol is a way to transform a natural blood clot into a clinically usable bioactive membrane, it was aimed at evaluating this protocol to understand if it offers reproducible results if the main production principles are followed. The purpose of this study was to determine the cell composition and organization of PRF and to evaluate the influence of different sample collection tubes (glass or plastic tubes) and compression procedures (forcible or soft) on the final PRF-membrane architecture. Platelet counts showed that there was minimal platelet present in the RBC layer, the PPP or the exudate provided by compressing the PRF clot. Thus, proving that most of the platelets originating from the whole-blood sample were collected in the PRF membranes. This is because

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of close relationship between fibrin and platelets after clotting on concentrations of platelet growth factor in PRF membranes[8,10].

Leukocyte counts confirmed that more than 50% of the leukocytes were trapped in PRF membranes, and small lymphocytes seemed mainly collected, as confirmed by the SEM examination. The photonic microscopy study showed that the platelet and leukocyte distribution within the clot was not uniform and that they were concentrated in an intermediate layer located between RBCs and the fibrin clot and represents the buffy coat on the PRF-clot surface. The dense aggregate of activated platelets was situated on a mature fibrin background in the buffy coat area. Platelet morphology was totally modified by aggregation and clotting processes hence only a large aggregate of platelet-fibrin polymers were analysed. Therefore, when harvesting clots for surgical use, clinicians should collect this intermediate whitish layer as it has been demonstrated in vitro that PRF enhances proliferation of many different cell types such as fibroblasts, osteoblasts, adipocytes, keratinocytes and angiogenesis[11,12,13]. Thus, it is necessary to preserve a small RBC layer at the PRF clot end to collect as many platelets and leukocytes as possible.

The SEM evaluation showed that RBCs were widely predominant in the red part of the PRF clot, and the leukocytes were distributed at the junction between the red and yellow parts of the clot. Only a few RBCs were identified in the rest of the clot, which were probably artifacts due to clot handling. Platelet morphology is totally modified by aggregation and clotting processes. Therefore, it was not possible to identify non-activated platelets (discoid bodies) but only a large aggregate of platelet-fibrin polymers. Kawasaki et al.2004 obtained the same results with thrombin-activated PRP and showed the contribution of platelets to the structural rigidity of the fibrin network[14]. The PRF-membrane examination showed the fibrin strands were condensed and stuck to each other.

The Choukroun's PRF concept is founded on a mechanical concentration process during clot formation and leads to a specific clot architecture that is very different from a simple fibrin bulk.

The PRF clot is yielded by a natural polymerization process during centrifugation, and its natural fibrin architecture seems responsible for a slow release of growth factors and matrix glycoproteins during ≥7 days[11]. The original protocol if not followed correctly might lead to PRF-like clots with inadequate fibrin and platelet and leukocyte concentrations which in turn can hamper the incorporation of growth factors within the fibrin network, and yield variations in the sample harvest

Summary of key findings:

The primary outcome of the study was to to perform an examination of the composition and architecture of the Choukroun's PRF clot using hematologic counts, photonic microscopy, and SEM. The secondary objective of this work was to point out the structural and morphologic differences between PRFs commonly produced with two different kinds of collection tubes (glass and plastic tubes) and using two different methods for the compression of the PRF clot into the membrane (forcibly or softly).

The results of this study correlate with a prior study conducted by Ehrenfest D. et al in 2010 which showed that the type of test tube (dry glass or glass – coated plastic tubes) and the compression process of the clot (forcible or soft) did not seem to influence the architecture of the PRF[9].

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