Original Article

# Qualitative Analysis of Modified Advanced-Platelet-Rich Fibrin Buffy Coat among Diabetic Patients and Tobacco Smokers with Chronic Periodontitis: A Cell Block Cytology Study

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## Abstract

**Aim:** The aim of this study was to evaluate and compare histologically the pattern of distribution of platelet and leukocyte concentration, quality of fibrin network, and the aggregation of platelets in the three zones of modified advanced platelet-rich fibrin (A-PRF) buffy coat among uncontrolled type 2 diabetic patients, tobacco smokers, and healthy individuals with chronic periodontitis. **Materials and Methods:** In this cross-sectional cytology study, 180 generalized chronic periodontitis patients (46–55 years) were enrolled – Group 1 (control group): 60 systemically healthy participants, Group 2 (test group): 60 heavy tobacco smokers, and Group 3 (test group): 60 uncontrolled type 2 diabetic patients. Fifteen milliliters of blood was drawn from all study participants. Modified A-PRF membrane was prepared and then processed histologically. **Results:** The distribution pattern of platelet and leukocyte concentration in modified (A-PRF) gradually declines from the serum to the red blood cell (RBC) end of a clot in all groups. We have assessed that the serum and middle end of modified (A-PRF) membrane had an increasingly moderate distribution of platelets and leukocytes in both type 2 diabetics and tobacco smokers. RBC end had more of sparse distribution in all the three groups. Healthy individuals exhibited 95% of reversible pattern, whereas tobacco smokers had 78.33% and uncontrolled type 2 diabetic patients had 93.33% of irreversible aggregation pattern of platelets. Loose fibrin network pattern was seen in all the three groups. These observations conclude that tobacco smokers had a high percentage of loose fibrin network with sparse distribution of cells. Males showed more loose fibrin network pattern of modified (A-PRF) membrane than compared to females. **Conclusion:** In the present study, it can be concluded that the application of modified (A-PRF) may provide enhanced periodontal healing in uncontrolled type 2 diabetic patients and tobacco smokers; furthermore, females may have better regenerative capacity compared to males.

**Keywords:** *Fibrin network patterns, leukocyte concentration, light microscopy, modified (advanced platelet-rich fibrin), platelet aggregation*

## Introduction

Platelet concentrates are defined as autologous platelet derivatives with a platelet concentration higher than baseline, and they are widely used in different areas of regenerative medicine in order to enhance wound healing processes.[1] After tissue injury, platelet concentrates hold on to the growth factors enmeshed in the fibrin network resulting in their sustained release over a period of time, thus accelerating the wound healing process.

Choukroun *et al*. introduced a recent type of platelet concentrate termed as leukocyte platelet-rich fibrin (L-PRF), is a second-generation platelet concentrate and is fundamentally a fibrin matrix containing platelets, white blood cells (WBCs), serum and growth factors that can be considered as a healing biomaterials.[2-4] Histologically, the three-dimensional fibrin network of L-PRF first reported by Dohan *et al*. was the catalyst for the incorporation of platelets, leukocytes, and cytokines into the mesh architecture.[5] The concentration of platelets, leukocytes, and cytokines in a PRF is not uniform. The red blood cell (RBC) end of a PRF contains the highest concentration of platelets, leukocytes, and cytokines, which was thus defined as the essence of PRF. In addition, a modification of the preparation setting based on the low-speed centrifugation concept (LSCC) is the first step in the reduction of the relative centrifugation force (RCF). This



**How to cite this article:** Das N, Amaranath BJ. Qualitative analysis of modified advanced-platelet-rich fibrin buffy coat among diabetic patients and tobacco smokers with chronic periodontitis: A cell block cytology study. Contemp Clin Dent 2022;13:173-82.

**Submitted:** 23-Dec-2020
**Revised:** 19-Feb-2021
**Accepted:** 24-Feb-2021
**Published:** 21-Jun-2022

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**Access this article online**

**Website:** www.contempclindent.org

**DOI:** 10.4103/ccd.ccd_1018_20

**Quick Response Code:**

step was accompanied by a mild increase of centrifugation time, resulting in a so-called advanced-PRF (A-PRF). The influence of the two specific parameters, i.e., reduced centrifugation speed (G-force)/ RCF and increased centrifugation time in order to enhances the growth factor, platelet, and leukocyte contents which were released from the A-PRF clot.[6-8] Decreasing the rpm while increasing the centrifugation time in the A-PRF gave an enhanced presence of platelets and neutrophilic granulocytes in the distal/serum end of the clot away from the buffy coat. T- and B-lymphocytes, stem cells, and monocytes were detected in the surroundings of the buffy coat.[9] To the best of our knowledge, the latest addition to the third-generation of advanced PRF family is the modified A-PRF (Modified A-PRF PROCESS©, France) which represents the latest evolution. In modified (A-PRF) per unit area entrapment of cells is more, these findings could be related to the LSCC as well as binding affinity of the various growth factors, i.e., platelets, leukocytes, and cytokines to fibrin and fibrinogen and to the impact of the centrifugation time on the activated cells within the modified (A-PRF) clot. Thus, it might influence bone and soft-tissue regeneration.

It is now well established from an epidemiologic evidence that type 2 diabetes mellitus (DM) and tobacco smoking are considered as risk factors for periodontal disease.[10,11] Delayed wound healing might be a detrimental factor in the success of regeneration in tobacco smokers and diabetic patients. The use of biofuel like PRF may enhance the healing potential of the tissues in these patients.

Hence, this study was undertaken to evaluate and compare histologically the pattern of distribution of platelet and leukocyte concentration, the aggregation pattern of platelets, and the quality of fibrin network pattern in the three zones of modified A-PRF buffy coat among uncontrolled type 2 diabetic patients and tobacco smokers with chronic periodontitis.

## Materials and Methods

### Study population and design

The cross-sectional study comprised 3 groups with 180 chronic periodontitis patients aged between 46 and 55 years, out of these 2 were test groups and 1 was control group with equal distribution of genders. All the participants were informed about the study, and written consent was obtained. Ethical approval was taken by the institutional ethics committee.

- Group 1 (control group) – 60 systemically healthy individuals with generalized chronic periodontitis
- Group 2 (test group) – 60 heavy tobacco smokers with generalized chronic periodontitis
- Group 3 (test group) – 60 uncontrolled type 2 diabetic patients with generalized chronic periodontitis.

### Hematological investigation and eligibility criteria

The following inclusion criteria were included in the study: (1) patients with generalized chronic periodontitis with minimum 20 teeth having probing pocket depth (PPD) of ≥6 mm, clinical attachment (CAL) loss of ≥5 mm, and radiographic bone loss extending to the mid-third of the root and beyond; (2) patients with a history of uncontrolled type 2 diabetes for more than 6 months with (random blood sugar level ≥200 mg/dl or ≥11.1 mmol/l or glycated hemoglobin ≥7%), and on medication; (3) individuals who are smoking more than 20 cigarettes per day for the past 5 years or more; and (4) patients who have not undergone systemic or periodontal surgery. Exclusion criteria were as follows: (1) patients with bleeding disorders or any systemic disorders; (2) pregnant and lactating mothers; (3) individuals with a decrease or increase in platelet count; (4) patients with any diagnosed malignancy; (5) individuals with adverse habits such as pan chewing and alcohol; and (6) patients on antibiotics, anticoagulants, immunosuppressive medications, or cytotoxic medications from the past 6 months.

### Periodontal examination

Periodontal examination was done using mouth mirror and HU-Friedy HU-Friedy University of North Carolina-15 (UNC-15) Periodontal Probe to assess the following indices and clinical parameters, they were Plaque Index-Silness and Loe, 1964;[12] Gingival Bleeding Index-Ainamo and Bay, 1975;[13] and PPD by Grant *et al*., 1965.[14] The probe was passed within the gingival sulcus along the circumference of the tooth. CAL was measured from the cementoenamel junction to the base of the pocket using UNC-15 periodontal probe.[12] A total of six sites per tooth (mesiobuccal, midbuccal, distobuccal, mesiolingual, midlingual, and distolingual) were recorded for PPD and CAL.

### Collection and preparation of samples

The blood sample was collected to prepare modified (A-PRF) for histological analysis. A 15-ml disposable syringe and tourniquet were used to draw 15-ml intravenous blood from the antecubital vein from all the participants [Figure 1a and b].

### Preparation of modified (advanced platelet-rich fibrin) clot

The 15-ml blood was immediately (within 1 min) transferred into the sterile glass tubes (nonsilica coated) (Borosilicate Glass, New Delhi, India) for preparing modified (A-PRF) by centrifugation (Remi R-8C, New Delhi, India) in a standard benchtop, based on Ghanaati *et al*.,[9] 2014 protocol centrifuge at (208 g, 1300 rpm for 14 min) [Figure 1c and d] due to the difference in densities, it resulted in the separation of three basic fractions: a base of RBCs at the bottom,

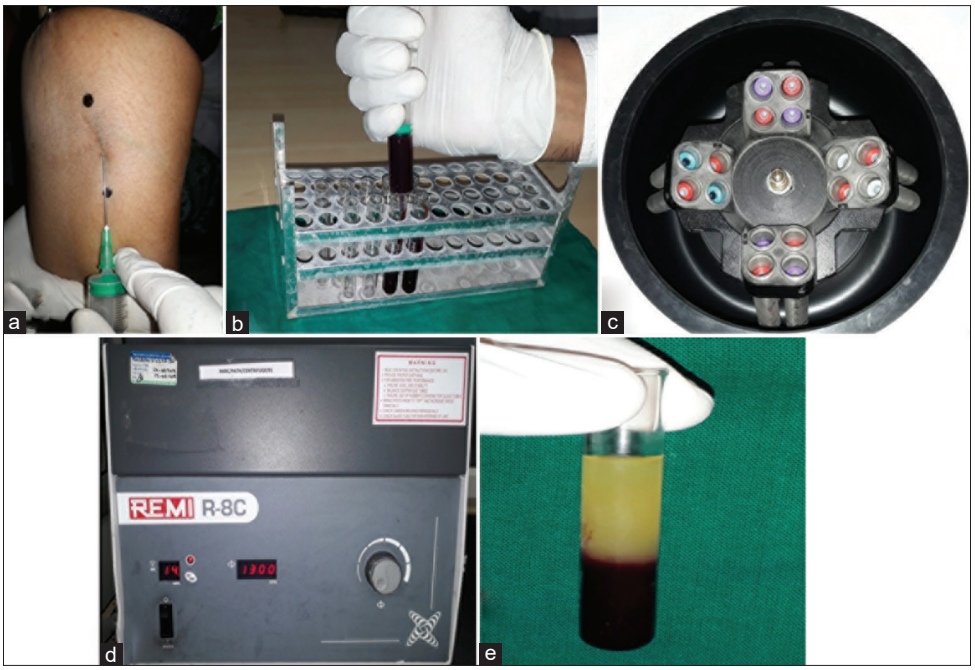



**Figure 1: Preparation of modified (advanced platelet-rich fibrin) clot: (a) Collection of blood sample. (b) Transfer of blood into test tube. (c) Nonsilica-coated test tubes in centrifugation machine. (d) Centrifuged at 1300 rpm for 14 min. (e) Modified (advanced platelet-rich fibrin)**

acellular plasma on the surface, and finally, a platelet concentrate clot between these two [Figure 1e]. The clot thus formed is obtained with the help of sterile tweezer and was segmented by scissor in such a manner as to preserve a small RBC layer which is the most potential regenerative area, i.e., platelets and WBCs concentrated in an intermediate layer located between RBCs and modified (A-PRF) clot [Figure 2a and b].

### Standardized and efficient preparation of modified (advanced platelet-rich fibrin) membrane

The modified (A-PRF) clots were made into membranes using the Modified (A-PRF) Box (Box Grid, Sialkot, Punjab) which allows the adequate preparation of homogeneous membranes with higher growth factor content and prevents shrinkage of the fibrin matrix architecture. It was designed to collect and transform up to 16 modified (A-PRF) clots into membranes [Figure 2c and d].

### Histological procedures for light microscopy evaluation of modified (advanced platelet-rich fibrin) membrane

The prepared modified (A-PRF) membrane was placed in a bottle containing 10% formalin for 24 h for fixing [Figure 2e]. Later, these membranes were processed as per the tissue processing protocol, dehydration done by increasing gradients of alcohol (60%, 70%, 80%, 90%, and 100%) and placed in two grades of xylene to achieve complete dehydration to yield 0.5 mm thick membrane followed by paraffin inclusion. For each modified (A-PRF) membrane, a series of 2 successive 7-µm sections were performed along the long axis of the membrane, i.e., 14 µm of the membrane thickness which could be analyzed in a longitudinal and reliable manner to get 360 sections which were stained with Hemalaun and Eosin (Organo Biotech, New Delhi, India) and Masson's trichrome (BioMarq Life Sciences Pvt. Ltd., Hyderabad, Telangana).

### Steps for obtaining the modified (advanced platelet-rich fibrin) slides by cell Block cytology method

The isolated modified (A-PRF) membranes were prepared into slides as per the following [Figures 3 and 4]:

(1) Fixing – The modified (A-PRF) membranes were transferred into a perforated stainless steel cassette alongside identification chit marked with lead pencil which is transferred to 10% formalin-containing container and fixed for 24 h to prevent autolysis and to preserve the biological tissues [Figure 3a]. (2) Tissue processing – After 24 h of fixation, the cassette-containing membranes were transferred into a perforated stainless steel cylindrical container where it was subjected to dehydration, clearing, and infiltration of wax into the membranes as they passed through various processing solutions such as 10% formalin, 60%, 70%, 80%, 90%, and 100% isopropanol alcohol, xylene (two changes), and paraffin wax in an orderly manner. This procedure was proceeded for 16 h in an automated tissue processor (Leica) which enables to remove the water from tissue and replaces in a medium that after solidification will allow thin sections to be cut [Figure 3b-d]. (3) Embedding – Leuchars blocks were prepared by embedding tissue using paraffin wax. It enabled the tissue section for sectioning and in preparation of the slide [Figure 3e-g]. (4)

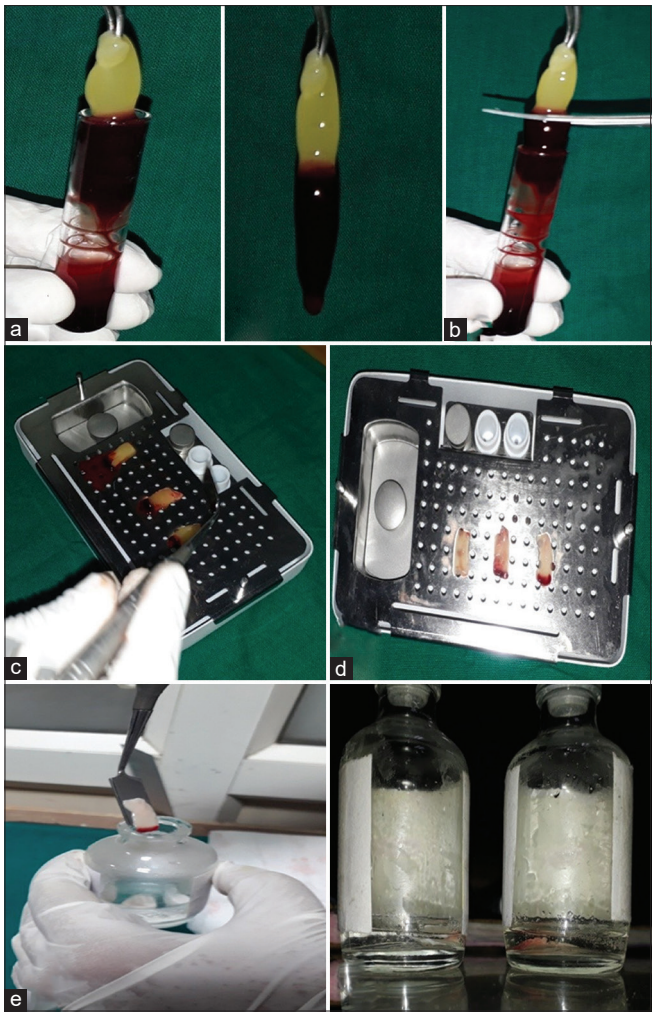



**Figure 2: Preparation of modified (advanced platelet-rich fibrin) membrane: (a) Preparation of modified (advanced platelet-rich fibrin) clot. (b) Separation of modified (advanced platelet-rich fibrin) clot. (c) Modified (advanced platelet-rich fibrin) clot. (d) Modified (advanced platelet-rich fibrin) membrane. (e) Modified (advanced platelet-rich fibrin) Membrane in a 10% formalin for 24 h**

Tissue sectioning – Using Leica microtome, 4 μm thickness of tissue slice was obtained [Figure 3h and i]. (5) Dewaxing – The deparaffinization of the slides was done by heating it for about 55°C, followed by dropping into xylene to eliminate wax enabling the tissues to be stained [Figure 3j and k]. (6) Tissue staining – Hemalaun and Eosin and Masson's trichrome stains and sections were used for staining [Figure 4a]. (7) Slide numbering – The slides were numbered based on the order for record [Figure 4a]. (8) Histological slide analysis – Prepared slides were assessed with compound microscope at ×20, ×40, and ×100 magnifications [Figure 4b].

### Scoring criteria for assessing microscopic parameters in modified (advanced platelet-rich fibrin) membrane by light microscopy

The modified (A-PRF) membrane can be described macroscopically as composed of two main parts: a fibrin yellow portion constituting the main body and a red portion located at the end of the clot (full of RBCs). Between these two areas, a whitish layer called the "buffy coat" can be observed with the naked eye [Figure 5].

Each series of stained longitudinal sections were observed for distribution of platelet, leukocyte concentration, and type of fibrin network pattern by light microscopy in the buffy coat region. Each section was graded according to [Tables 1 and 2]. To study the distribution of platelet and leukocyte concentration, we used two different stains, namely Hemalaun and Eosin and Masson's trichrome; these two stains easily differentiate platelets and leukocytes and shared the same microscopic appearance. Hence, based on the morphology, the platelets and leukocytes are differentiated according to Table 3.

The grades of platelet aggregates were assessed semi-quantitatively, and differentiated according to grading system, as shown in Table 4. The reversible platelet aggregation was taken as normal and the irreversible platelet aggregation as pathological aggregability. Reversible platelet aggregation means the sticking of platelets to each other without fusion, with their membranes intact. In irreversible platelet aggregation, platelets are fused and membranes are partly destroyed.

### Statistical analysis

The IBM Statistical Package for the Social Sciences (SPSS) Statistics 21.0, United states, was used to analyze the data collected. Comparisons and difference between the test and control groups were carried out using percentage distribution method. The percentage of mean was estimated for different variables in two study groups and control groups with gender. To achieve 85% power (instituted by G*power, version 3.0.1; Franz Faul Universitat, Kiel, Germany) and detect significant differences with effect size of 0.47 ($P \leq 0.05$), a total of 60 participants were required in each group.

## Results

In this study, the pattern of distribution of platelet and leukocyte concentration, quality of fibrin network, and the aggregation of platelets were observed in the three zones, i.e., serum (distal or tail)/middle/RBC (proximal or face) of modified A-PRF buffy coat region among all the three study groups (Group 1 [healthy individuals], Group 2 [tobacco smokers], and Group 3 [uncontrolled type 2 diabetic patients]) with chronic periodontitis.

### Distribution of platelet concentrations in serum/middle/red blood cell zones of modified (advanced platelet-rich fibrin) buffy coat

The values of mean percentage distribution for serum, middle, and RBC end showed statistically significant differences among all the study groups and were shown in Graph 1a-c. It was found that serum and middle end has highly moderate, whereas RBC end has a more sparse distribution of platelets.

**Figure 3: Steps of cell block cytology method: (a) Modified (advanced platelet-rich fibrin) membrane in 10% formalin bottle for 24 h. (b) Perforated stainless steel cassette. (c) Modified (advanced platelet-rich fibrin) membranes are transferred into a cassette with patient details. (d) Tissue Processor® e) Embedding of tissue using paraffin wax. (f) Embedding (g) Paraffin blocks (h) Tissue sectioning by Leica microtome (i) 4-µm thickness of tissue slice obtained (j) Dewaxing (k) Deparaffinization of the slides is done by heating it for about 55°C**

**Figure 4: Preparation of slides: (a) Prepared slide. (b) Slide box**

## Distribution of platelet aggregation in the buffy coat region of modified (advanced platelet-rich fibrin) membrane

Percentage distribution method was applied, and statistically significant differences were seen among the three groups. Healthy individuals exhibited 95% of reversible pattern, whereas tobacco smokers had 78.33% and uncontrolled type 2 diabetic patients had 93.33% of irreversible aggregation pattern of platelets, as shown in Graph 2.

## Distribution of leukocyte concentrations in serum end/middle end/red blood cell end zone of buffy coat region of modified (advanced platelet-rich fibrin)

The values of mean percentage distribution for serum, middle, and RBC end showed statistically significant differences among all the study groups and were shown in Graph 3a-c. It was found that serum end has highly moderate and middle and RBC end has a more sparse distribution of leukocytes.

**Table 1: Criteria of the distribution of platelet and leukocyte concentrations were graded as follows (Hemalata *et al*., 2016)[15]**

| Grade | Criteria | Distribution |
|---|---|---|
| 0 | Absence of platelets and leukocytes | Absence |
| 1 | Platelets and leukocytes covering≤25% of the respective zone | Sparse |
| 2 | Platelets and leukocytes covering 25%50% of the respective zones | Moderate |
| 3 | Platelets and leukocytes covering 50%100% of the respective zones | Dense |

## Assessment of quality of fibrin network pattern of modified (advanced platelet-rich fibrin)

Percentage distribution method was applied, and statistically significant differences were seen among all the study groups. It was found that loose/porous fibrin network pattern of modified (A-PRF) membrane was seen among all the study groups, but tobacco smoker group showed the maximum, as shown in Graph 4.

## Gender-based assessment of quality of fibrin network pattern of modified (advanced platelet-rich fibrin)

It was observed that loose/porous fibrin network pattern of modified A-PRF membrane was obtained in all the study groups. Statistically significant differences were seen. Males showed more loose/porous fibrin network pattern of modified (A-PRF) membrane than compared to females in all the groups, as shown in Table 5.

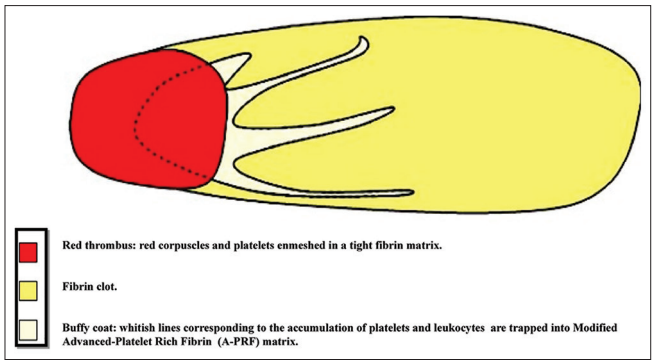



**Figure 5: Schematic description of modified (advanced platelet-rich fibrin) clot. The process protocol is divided into three parts: A red thrombus in contact with the red blood corpuscle base, an acellular fibrin gel, and a network of buffy columns corresponding to platelets and leukocytes accumulation**

**Table 2: The variation in fibrin meshwork pattern of modified (advanced platelet-rich fibrin) membrane evaluated through histological analysis by light microscopy in the buffy coat region was graded as follows**

| Types of fibrin network pattern |
|---|
| 1) Dense fibrin network pattern |
| 2) Loose/porous fibrin network pattern |

**Table 3: Appearance of platelets and leukocytes in the stains**

| Cells | Hemalaun and Eosin stain | Manson's trichrome stain |
|---|---|---|
| Platelets | Dark blue/violet | Dark blue |
| Leukocytes | Dark blue with pink cytoplasm | Dark blue/purple |

**Table 4: Criteria of the platelet aggregation were differentiated according to grading system as follows (Breddin and Bauke, 1965)[16]**

| Grade | Criteria |
|---|---|
| Normal | Reversible platelet aggregation |
| Pathological | Irreversible platelet aggregation |

### Histologic assessment of microscopic features of modified (advanced platelet-rich fibrin) membrane among three groups

Distribution of platelets and leukocytes in three zones of modified (A-PRF) membrane buffy coat region showed that the sections from the serum end (A1, B1, and C1), middle end (A2, B2, and C2), and RBC end (A3, B3, and C3) exhibit decreasing compactness and increasing porosity in healthy individuals, tobacco smokers, and uncontrolled type 2 DM patients, as shown in Figure 6a, b, d and e.

The stained sections of PRF clot in all the three groups were assessed for:

i. Dense fibrin network pattern
ii. Loose/porous fibrin network pattern
iii. The entrapment pattern of platelets and WBCs within the dense and loose type of fibrin networks.

The histologic sections of all the three groups showed an outermost layer of RBCs, followed by the dense fibrin network layer consisting of maximum number of platelets and WBCs entrapped and the inner layers as moving away from RBC were seen to be dominated by loose fibrin network pattern with moderate platelets and WBCs entrapment.

#### *Modified (advanced platelet-rich fibrin) membrane in healthy individuals*

In the modified (A-PRF) membrane, the presence of both dense and loose fibrin networks was present [Figure 6f - a]. The percentage of loose fibrin network was more than compared to dense fibrin network, and but we found that entrapment of platelets and WBC cells was more layered within the dense fibrin network whereas fewer cells were diffusely arranged in loose fibrin network, as shown in Figure 6a, b, d, and e. A prominent fibrin border of considerable thickness is appreciated in Figure 6f - b.

#### *Modified (advanced platelet-rich fibrin) membrane in tobacco smokers*

The modified (A-PRF) membrane was occupied with higher percentage of loose fibrin network [Figure 6f - a]. The leukocyte and platelet aggregates were seen moderate in serum and middle end and more sparse in RBC end of the loose fibrin network and the rest part of the clot; the enmeshment of cells was diffusely scattered, as shown in Figure 6a, b, d, and e. The fibrin border could not be appreciated in modified (A-PRF) membrane of tobacco smokers, as depicted in Figure 6f - b.

#### *Modified (advanced platelet-rich fibrin) membrane in uncontrolled type 2 diabetes mellitus patients*

The distribution of loose fibrin networks was present, and platelet and leukocyte were seen at the periphery near to the dense fibrin network which was present in a reduced quantity, as illustrated in Figure 6f - a. The leukocyte and platelet aggregates were seen more moderate in serum and middle end and less sparse in RBC end of the loose fibrin network, as shown in Figure 6a, b, d, and e. The presence of a fragile fibrin border is shown in Figure 6f - b.

## Discussion

In the present research, we used two distinctive stains, namely Hemalaun and Eosin and Masson's trichrome (modified by Goldner), to study the distribution of platelet and leukocyte concentration' these two stains easily differentiate platelets and leukocytes and shared the same microscopic appearance. Because of certain limitations of Hemalaun and Eosin stain further, we use its modification Masson's trichrome – a three-color staining protocol used in histology method remains one of the best,

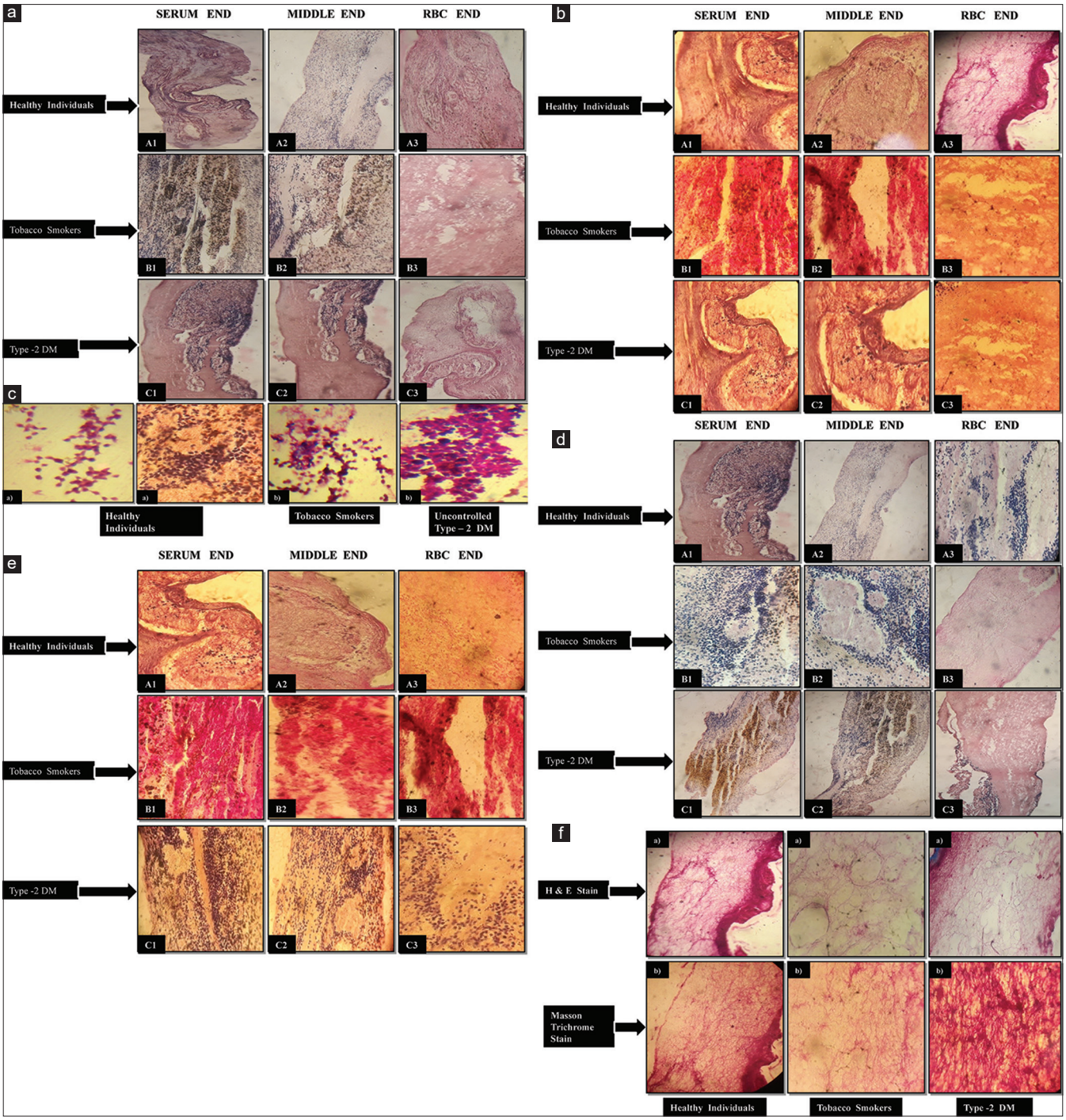
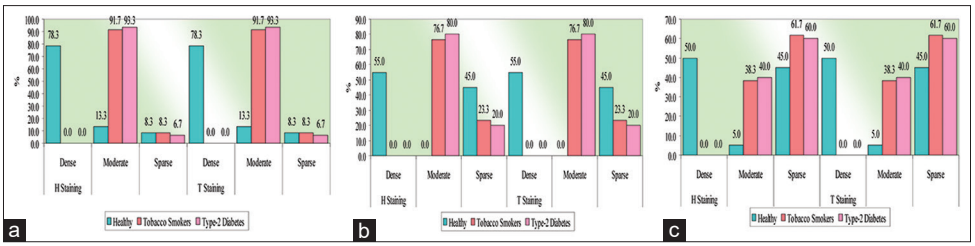
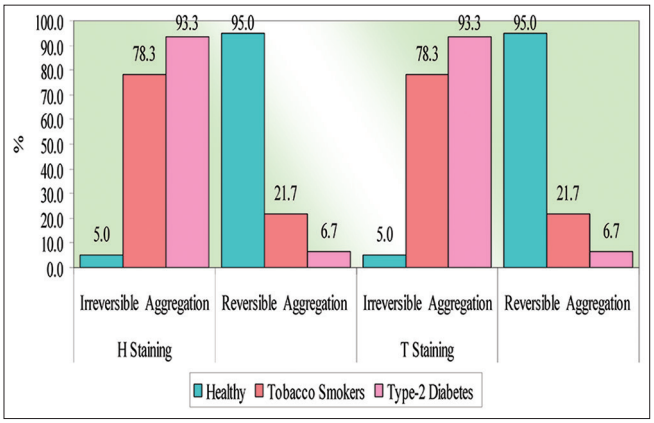



**Figure 6: Histologic presentation of modified (advanced platelet-rich fibrin) membrane: (a) Distribution of platelets in three zones of buffy coat by H and E stain. The sections from the serum end (A1, B1, and C1), middle end (A2, B2, and C2), and red blood cell end (A3, B3, and C3) exhibit decreasing compactness and increasing porosity in healthy individuals, tobacco smokers, and uncontrolled type 2 diabetes mellitus patients. (b) Distribution of platelets in three zones of buffy coat by Masson's trichrome stain. The sections from the serum end (A1, B1, and C1), middle end (A2, B2, and C2), and red blood cell end (A3, B3, and C3) exhibit decreasing compactness and increasing porosity in healthy individuals, tobacco smokers, and uncontrolled type 2 diabetes mellitus patients. (c) Platelet aggregation pattern. (a) Reversible platelet aggregation (physiological aggregation) in healthy individuals (control group). (b) Irreversible platelet aggregation with ruptured membrane in tobacco smokers and uncontrolled type 2 diabetes mellitus patients (test groups). (d) Distribution of leukocytes in three zones of buffy coat by H and E stain. The sections from the serum end (A1, B1, and C1), middle end (A2, B2, and C2), and red blood cell end (A3, B3, and C3) exhibit decreasing compactness and increasing porosity in healthy individuals, tobacco smokers, and uncontrolled type 2 diabetes mellitus patients. (e) Distribution of leukocytes in three zones of buffy coat by Masson's trichrome stain. The sections from the serum end (A1, B1, and C1), middle end (A2, B2, and C2), and red blood cell end (A3, B3, and C3) exhibit decreasing compactness and increasing porosity in healthy individuals, tobacco smokers, and uncontrolled type 2 diabetes mellitus patients. (f) Assessment of quality of fibrin network pattern between healthy individuals, tobacco smokers, and uncontrolled type 2 diabetes mellitus patients by both (a) H and E stain and (b) Masson's trichrome stain**




**Graph 1: Comparison of platelet concentrations in (a) serum end/distal end/tail zone, (b) middle end zone, (c) red blood cell end/proximal end/face zone of buffy coat region of modified (advanced platelet-rich fibrin) membrane among healthy individuals, tobacco smokers, and uncontrolled type 2 diabetic patients with chronic periodontitis**




**Graph 2: Comparison of platelet aggregation grading in the buffy coat region of modified (advanced platelet-rich fibrin) membrane among healthy individuals, tobacco smokers, and uncontrolled type 2 diabetic patients with chronic periodontitis**

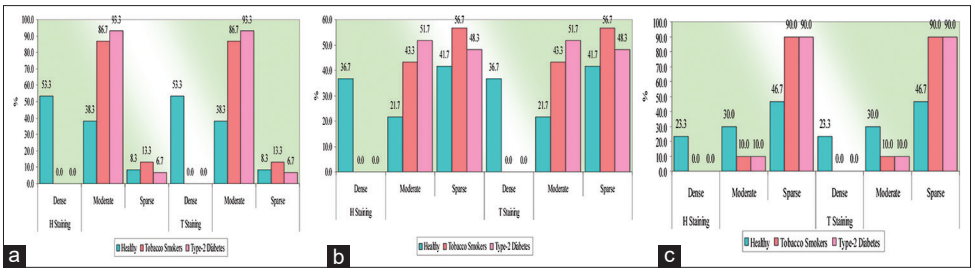
**Table 5: Gender-based comparison of quality of fibrin network pattern among healthy individuals, tobacco smokers, and uncontrolled type 2 diabetic patients with chronic periodontitis**

| Staining | Quality of fibrin network pattern | Male | | | Female | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Healthy individuals (Group 1) | Tobacco Smokers (Group 2) | Type-2 Diabetes (Group 3) | Healthy individuals (Group 1) | Tobacco Smokers (Group 2) | Type-2 Diabetes (Group 3) | Healthy individuals (Group 1) | Tobacco Smokers (Group 2) | Type-2 Diabetes (Group 3) |
| H & E stain | Dense | 9 | 3 | 5 | 10 | 5 | 6 | 19 | 8 | 11 |
| | Loose | 21 | 27 | 25 | 20 | 25 | 24 | 41 | 52 | 49 |
| | Total | 30 | 30 | 30 | 30 | 30 | 30 | 60 | 60 | 60 |
| Trichrome stain | Dense | 8 | 4 | 4 | 10 | 5 | 6 | 18 | 9 | 10 |
| | Loose | 22 | 26 | 26 | 20 | 25 | 24 | 42 | 51 | 50 |
| | Total | 30 | 30 | 30 | 30 | 30 | 30 | 60 | 60 | 60 |
| Total | Dense | 17 | 7 | 9 | 20 | 10 | 12 | 37 | 17 | 21 |
| | Loose | 43 | 53 | 51 | 40 | 50 | 48 | 83 | 103 | 99 |
| | Total | 60 | 60 | 60 | 60 | 60 | 60 | 120 | 120 | 120 |

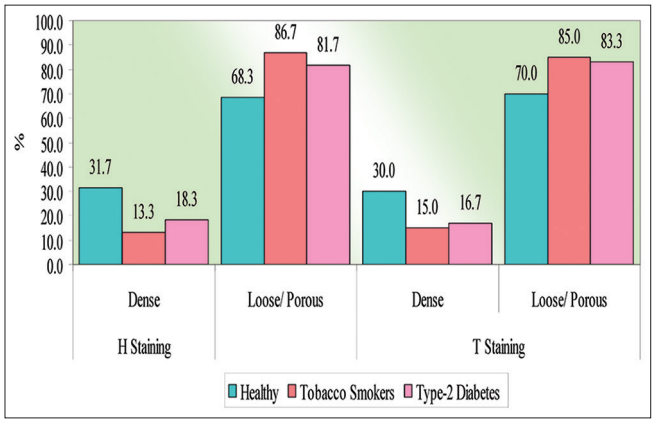
as it does the most precise of hematoxylins (Heidenhain's iron hematoxylin) with a reliable cytoplasmic stain. The trichrome is applied by immersion of the fixated sample into Weigert's iron hematoxylin as it stains the nuclei. In the present study, platelet aggregates appeared dark purple and RBCs were stained in bright red and became easily identifiable. Leukocytes were able to separate from stained platelet aggregates as its cytoplasm was stained dark pink with the dark blue nucleus. Masson's trichrome (modified by Goldner) stain proved to be a better stain as it was able to differentiate between platelets, RBCs, and leukocytes which had been in accordance with the Yajamanya *et al*. where they used similar histological processing and staining procedures.[17]

In this study, the concentration of platelets and leukocytes was found to be maximum in serum and middle end as compared to RBC end of modified (A-PRF) membrane buffy coat in tobacco smokers, uncontrolled type 2 diabetic patients, and healthy individuals. This finding was in accordance with Ghanaati *et al*. where it was concluded that, due to LSCC, less centrifugation force and more time would reduce cells pull down by G-forces, which increases a total number of cells contained within the top layer of modified A-PRF membrane enabling a higher number of platelets and leukocytes "trapped" within the fibrin matrix. Therefore, it is found that per unit area entrapment of cells more in modified A-PRF membrane.[18] Another possible reason for the variation in platelet and leukocyte concentration seen in this study may be that the recommended centrifuge time is too short to allow for complete clot formation to occur. Therefore, allowing the modified (A-PRF) clot to remain in the tube for a period longer than the recommended time may result in a more complete physiological reaction taking place.[19]

The platelet aggregation pattern was found to be irreversible in tobacco smokers and uncontrolled type 2 diabetic patients, whereas healthy individuals showed a reversible pattern which indicates activation of platelets and leukocytes. This finding is in accordance with the observation of Forner *et al*. They found that irreversible platelet aggregation pattern in diabetic and reversible in healthy individuals with chronic periodontitis.[20] The possible scientific reason for irreversible platelet aggregation pattern present in uncontrolled type 2 diabetic patients could be because of elevated levels of platelet and leukocyte. Second, the higher incidence of inflammatory cytokines in periodontitis patients has the potential to activate platelets and leukocytes. This could be possibly due to elevated serum sP-selectin level induced by systemic inflammation.[21]




**Graph 3: Comparison of leukocyte concentrations in (a) serum end/distal end/tail zone, (b) middle rend zone, (c) red blood cell end/proximal end/face zone of buffy coat region of modified (advanced platelet-rich fibrin) membrane among healthy individuals, tobacco smokers, and uncontrolled type 2 diabetic patients with chronic periodontitis**




**Graph 4: Comparison of quality of fibrin network pattern among healthy individuals, tobacco smokers, and uncontrolled Type 2 diabetic patients with chronic periodontitis**

The importance of understanding the quality of fibrin meshwork in modified (A-PRF) membrane in the platelet concentrates could be because it contains the highest concentration of growth factors in the first 1 mm of the yellow clot, just above the red clot which plays an immense role in the regeneration of the lost tissues.[22] Dohan *et al*. observed that the potential benefits of using platelet and leukocyte concentrate in regeneration process was due to the cytokines present in it along with a dense fibrin network pattern which further help in the wound stabilization.[23] Laurens *et al*. stated that the importance of wound healing greatly depends on the fibrin structure, such as the thickness of the fibers, the number of branch points, the porosity, and the permeability.[24] Hence, the present study focused on the histologic evaluation of fibrin mesh pattern and its interaction with platelets and leukocytes. The result obtained from the healthy participants in the present study was in accordance with Tunali *et al*. in 2014.[25]

The tobacco smoker participants in general were known to cause the downregulation of immune response. Rival *et al*. in 1987 observed a decrease in platelet aggregation which further affected the membrane formation.[26] *In vitro* study done by Eichel and Shahrik[27] in 1969 has shown an alteration in chemotaxis, phagocytosis, and killing activity of neutrophil when exposed to nicotine. In accordance with our study, the observations in the modified (A-PRF) membrane in tobacco smoker participants could be explained due to changes in the cellular component on chronic exposure of nicotine. In smoker participants, the clot histologically has shown a dominancy of loose fibrin network. The presence of declined cellular components and increase loose fibrin mesh in tobacco smoker group which was in accordance with a study of Yajamanya *et al*. in 2016.[17]

As per our understanding, this research work is the first of its kind which has assessed the gender-based variation in fibrin meshwork pattern of modified (A-PRF) membrane in uncontrolled type 2 DM patients, tobacco smokers, and healthy individuals through histological analysis. It was observed that the presence of loose/porous fibrin network pattern of modified A-PRF membrane in all the study groups. Males showed more loose/porous fibrin network pattern of modified (A-PRF) membrane than compared to females in all the groups. The possible scientific reason for finding a loose fibrin meshwork among the groups may be because of advanced age and a Low Speed Centrifugation Concept used in modified A-PRF preparation, as we know that, the age of the individual progress, the fibrin network pattern becomes more loose and irregular. Each fibrin pattern become thinner in nature and also the entrapment of the platelets and leukocytes within this fibrin network is progressively reduced to moderate and this finding was in accordance with Miron et al. study.[28] This leads to less entrapment of cells in the fibrin network was because of increasing age of the individual, reduced G-force and increased centrifugation time.

From this study, it can be stated that the two major constituents of modified (A-PRF), i.e., the platelets and fibrin network, are interdependent. To our understanding, this is a single observer study with certain limitations included evaluation of fibrin meshwork of individuals having uncontrolled diabetes and smoking could have yielded a better validation in terms of regeneration of the periodontium. The study shall be more insightful if electron microscopic analysis is done.

## Conclusion and Future Perspectives

The regeneration of the periodontium in diabetic patients and tobacco smokers is a challenging task to the periodontist.

The observations of the present study were concluded that the application of modified (A-PRF) membrane may provide enhanced periodontal healing in uncontrolled type 2 diabetic patients and tobacco smokers; furthermore, females may have better regenerative capacity compared to males. Thus, in the future, a long-term, multicenter, randomized controlled clinical trial is needed to analyze the real-time qualitative changes in modified (A-PRF) of periodontitis patients and also evaluate the clinical efficacy of modified (A-PRF) membrane in the field of periodontal regeneration.

### Acknowledgments

The authors were thankful to all the study participants for their cooperation in completing this study.

### Financial support and sponsorship

Nil.

### Conflicts of interest

There are no conflicts of interest.

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