Original Article

Comparative Evaluation of Platelet Concentration and its Distribution in the Buffy Coat Region of Platelet Rich Fibrin in Non-diabetic and Controlled Diabetic Patients: A Light Microscopic Study

M Hemalata[1], D Jayanthi[2], Lalith Vivekanand[3], Swati[4], Zoya Mulla[4]

[1]Head and Professor, Department of Periodontics, M.R. Ambedkar Dental College and Hospital, Bengaluru, Karnataka, India, [2]Professor, Department of Periodontics, M.R. Ambedkar Dental College and Hospital, Bengaluru, Karnataka, India, [3]Reader, Department of Periodontics, M.R. Ambedkar Dental College and Hospital, Bengaluru, Karnataka, India, [4]Postgraduate Student, Department of Periodontics, M.R. Ambedkar Dental College and Hospital, Bengaluru, Karnataka, India

ABSTRACT

Introduction: Delayed wound healing might be a detrimental factor in the success of regeneration in a diabetic patient. The use of a biofuel like platelet rich fibrin (PRF) may compensate for the same and enhance the healing potential of the tissue. The variation in the concentration of platelet in diabetic patient will quantify the efficiency of the PRF obtained. Hence, this study has evaluated and compared the concentration of platelets and its distribution in the buffy coat region of PRF in non-diabetic and controlled diabetic patients.

Materials and Methods: A total of 20 patients were equally divided into two groups, depending on their blood glucose levels as Group A - non-diabetic and Group B - controlled diabetic. Blood sample was collected from each patient and PRF obtained according to the protocol. PRF membranes were then processed for light microscopic examination.

Results: The PRF membrane showed a similar macroscopic structure and microscopic distribution in both the groups. The concentration of platelets in the buffy coat region was significantly higher in Group A ($P < 0.05$). The distribution pattern also varied among both the groups, the platelet distribution in Group B was sparse when compared to Group A.

Conclusion: The Choukroun's PRF concept leads to specific clot architecture with platelets entrapped in the fibrin meshwork especially in the buffy coat region. The concentration of platelet was much dense in the non-diabetic patient as compared to diabetic patient. This study showed that with increased blood sugar levels there were changes in the concentration and distribution of platelets. But still, the PRF in controlled diabetics contained moderate platelets which might act as a potential adjunct in the regenerative procedure.

Keywords: Blood platelets, Diabetes mellitus, Light microscopy, Platelet rich fibrin

Corresponding Author: Dr. Swati, #13 D Burdwan Compound, Ranchi - 824 001, Jharkhand, India. E-mail: swatiprasad.jss@gmail.com

INTRODUCTION

Diabetes mellitus is a combination of heterogeneous disorders commonly presenting with episodes of hyperglycemia and glucose intolerance, as a result of lack of insulin, defective insulin action, or both. Such complications arise due to derangements in the regulatory systems for storage and mobilization of metabolic fuels including the catabolism and anabolism of carbohydrates, lipids, and proteins emanating from defective insulin secretion, insulin action, or both.[1] Diabetes mellitus alters the cellular microenvironment in multiple organ systems including the eyes, nerves, kidneys, and blood vessels. The oral cavity is not an

exception. Diabetes has profound effects on oral tissues; particularly in individuals with poor glycemic control.[2] Diabetes is a risk factor for periodontal diseases and oral infection. Diabetes protracts wound healing leading to secondary infection, hence regenerative surgical procedure should be cautionary undergone a change.[3]

Using platelet concentrates is a way to accelerate and enhance the body's natural wound healing mechanisms.[4] The use of platelet concentrate like platelet rich fibrin (PRF), during surgical procedures is a current treatment concept used to accelerate wound healing and tissue maturation. PRF is an immune and platelet concentrate collecting on a single fibrin membrane all the constituents of a blood sample favorable to healing and immunity.[5] PRF is a second-generation platelet concentrate widely used to accelerate soft and hard tissue healing and is a strictly autologous fibrin matrix containing a large quantity of platelet and leukocyte cytokines.[6] Many studies have documented the findings of increased platelet count and mean platelet volume in diabetics. The large platelets contain more dense granules are more potent than smaller platelets and hence more thrombogenic.[7] The altered platelet morphology and function may increase the risk of developing micro- and macro-vascular disease in diabetic patient.[8]

These variations of the platelets might be very detrimental when using PRF in a diabetic patient; hence in this study, we have evaluated and compared the concentration of platelets and its distribution in buffy coat region of the PRF in non-diabetic and controlled diabetic patient.

MATERIALS AND METHODS

Participants

An approval from the Institutional Ethics Committee was achieved at the beginning of the study in accordance with the Helsinki Declaration of 1975, as revised in 2000. Subjects were recruited from the Department of Periodontics at M.R. Ambedkar Dental College and Hospital, Bengaluru, Karnataka, India. It was mandatory for all the patient to have read and signed an informed consent form before their inclusion in the study. A total of 20 subjects in the age range of 18-55 years belonging to either gender were equally divided into two groups (10 in each group) depending on their blood sugar level.

1. Group A - Non-diabetic patient
2. Group B - Controlled diabetic patient.

Hematological Investigation and Eligibility Criteria

All the subjects underwent random blood sugar (RBS) analysis, subjects with RBS level below 150 mg/dl, were considered non-diabetic and included in Group A, whereas subjects with a history of Type 2 diabetes for more than 6 months with hemoglobin A1c of ≤6.5, and on medication were included in Group B. Subjects with (1) any systemic disorders for Group A and other than diabetes for Group B, (2) any diagnosed malignancy, and (3) history of aspirin intake or other medications that might interfere with coagulation over the previous 2 weeks and pregnant and lactating female were excluded.

Preparation of PRF

PRF was prepared according to the protocol developed by Choukroun *et al*.[9] Intravenous blood was collected by venipuncture of the antecubital vein in 10 ml sterile tubes without anticoagulant and immediately centrifuged at 3000 rpm (400 g) for 10 min. After centrifugation, the PRF clot was removed from the tube using sterile tweezers, separated from the red blood cell (RBC) base using scissors, and placed in a sterile metal cup. It is then fixed in 10% formalin for 24 h.

Histological Procedures for Light-microscopy Evaluation

PRF membranes were dehydrated in increasing gradients of alcohol (60%, 80%, 100% and 100%) and then placed in two grades of xylene before paraffin inclusion according to tissue processing protocol.[10] After complete dehydration, the membrane was 0.5 mm thick. For each PRF membrane, a series of 2 successive 7-μm sections was performed according to the long axis of the membrane, i.e., 14 μm of the membrane thickness which could be analyzed in a longitudinal and reliable manner.[11] These 40 sections were stained using the following two different stains following their specific protocols. 20 sections (10 from each group):

1. Hemalaun and eosin
2. Masson's trichrome (modified by Goldner).

Evaluation by Light Microscopy

The PRF clot can be described macroscopically as composed of two main parts observable with the naked eye, 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.

Each series of stained longitudinal sections were observed by light microscopy and platelets concentration, and distribution was analyzed in the buffy coat region. After complete analysis, each section was graded according to Table 1.

With the hemalaun and eosin staining, platelet aggregates were dark blue/violet whereas RBCs and leukocyte cytoplasm were not easily detectable; they were dark pink. The leukocyte nuclei were stained in

dark blue with the hemalaun, closely resembling platelet aggregates. Based on the morphology the platelets and leukocytes were differentiated.

With Masson's trichrome (modified by Goldner) staining, platelet aggregates were dark blue, but RBCs were stained in bright red and became easily identifiable. Leukocytes were still difficult to separate within the stained platelet aggregates. In the transition layer, platelet aggregates, leukocytes, and RBCs were mixed together (Table 2).

Statistical Analyses

Statistical analyses were performed by Student's *t*-test and Pearson correlation. $P < 0.05$ was considered statistically significant.

RESULTS

Platelet Distribution

In this study, each series under light microscopy was analyzed by counting the violet spots in the different areas of the membrane. These spots represented platelet aggregates. In Group A, dense aggregate of platelet was seen which appeared as violet spots with the hemalaun and eosin staining whereas in Group B the density was less (Figure 1). With Masson's trichrome (modified by Goldner) staining (Figure 2), these platelet aggregates appeared dark blue hence differentiation between platelets and leukocyte was easier in this stain. We noted significant differences between Groups A and B ($P < 0.01$) with respect to platelet concentration (Table 3 and Graph 1).

Both the groups shared common distribution pattern in the buffy coat region, i.e., the platelet distribution was homogeneous throughout the clot width in the buffy coat. When compared between the two groups, the platelet distribution was significantly higher in Group A ($P < 0.05$) than compared to Group B (Table 4).

Table 1: The distribution of platelets was graded as follows in both the groups

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

Table 2: Appearance of platelet and WBCs in the both stains

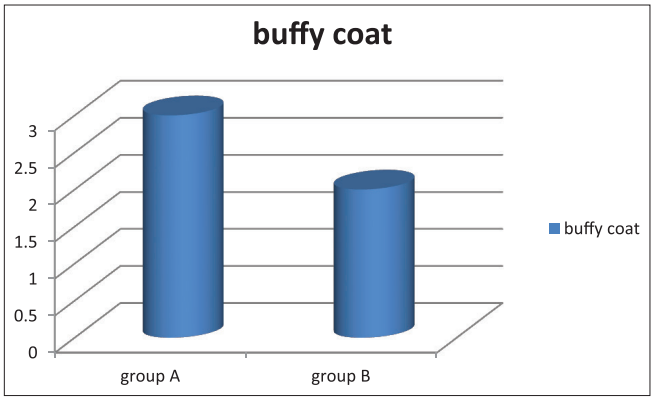
Cells	Hemalaun and eosin	Mansson trichrome
Platelets	Dark blue/violet	Dark blue
WBCs	Dark blue with pink cytoplasm	Dark blue/purple

WBC: White blood cell

DISCUSSION

The purpose of the study was to evaluate the concentration of platelets and its distribution in the buffy coat region of the PRF. The same was compared in healthy and controlled diabetic patients.

When compared to a healthy patient, diabetic is characterized by chronic hyperglycemia which may result in various complications. Delayed healing is one such potential complication which might be very detrimental in the treatment of such patients. The outcome of the surgical procedures is largely dependent on the tissue response and the healing mechanism of an individual. Delayed wound healing might act as a



Graph 1: Comparison of platelet concentration in buffy coat region of platelet rich fibrin in both the groups

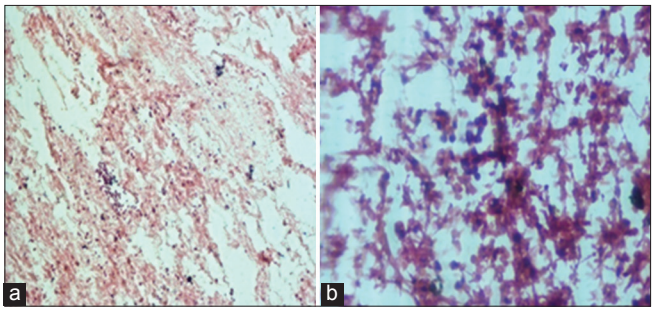


Figure 1: Hemalaun and eosin stained in (a) non-diabetic (b) diabetic

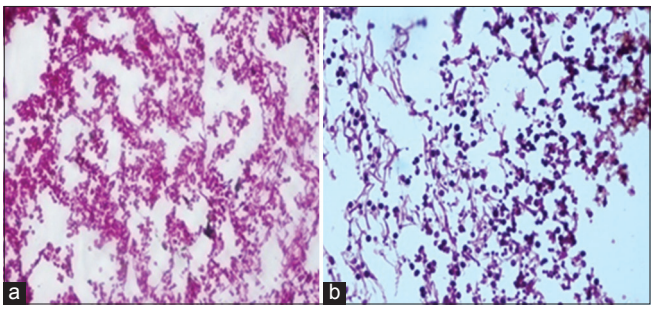


Figure 2: Masson's trichrome (modified by Goldner) stain in (a) non-diabetic (b) diabetic

Table 3: Intergroup comparison of platelet concentration in buffy coat region of PRF

Groups	Mean	SD	Mean±SD
Group A	2.6	0.51	2.6±0.51
Group B	2.2	0.51	2.2±0.51
P value			0.001

SD: Standard deviation, PRF: Platelet rich fibrin

Table 4: Intergroup comparison of platelet distribution in the buffy coat region of the PRF

Groups	Mean	SD	Mean±SD
Group A	2.8	0.42	2.8±0.42
Group B	2.2	0.42	2.2±0.42
P value			0.001

SD: Standard deviation, PRF: Platelet rich fibrin

drawback when treating a diabetic patient.

The use of platelet concentrate like PRF is a way to accelerate and enhance the body's natural wound healing mechanism. PRF was first developed in France by Choukroun *et al*. and dwells among a new generation of platelet concentrate that jump-starts the healing process to maximize predictability.[9] It consists of the platelets, cytokines, and the fibrin matrix.[12] The crux of PRF efficiency lies in the attempt to accumulate platelets and release cytokines in a fibrin clot. Degranulation of platelets entails the release of cytokines able to stimulate cell migration and proliferation within the fibrin matrix, launching the first stages of healing.[4,6]

In this light microscopic study, the platelet distribution in the PRF was not uniform and it was more concentrated in an intermediate layer located between RBCs and the fibrin clot which represented the macroscopic buffy coat on the PRF clot surface. This finding was in accordance to the study done by Dohan Ehrenfest *et al*.[11]

Hence, when harvesting clot for the surgical use, practitioners should collect this intermediate whitish layer. It is necessary to preserve a small RBC layer and the PRF clot end to collect as many platelets and leukocytes as possible. An accurate knowledge of the clot architecture is required for adequate PRF preparation because the exact same biologic and clinical effects from the two extremities of a PRF membrane cannot be expected. Thus, the surgical techniques should be carefully adapted to the membrane composition for some delicate applications such as periodontal surgery.

Many studies[5,9,11] have documented the concentration of platelets to be maximum in the buffy coat, hence our study was aimed at evaluating the concentration and distribution of platelets in the buffy coat region of the PRF in healthy and controlled diabetic patient.

Macroscopically both the groups showed similar PRF clot membrane structure. On microscopic evaluation concentrated platelets were trapped in a dense fibrin nexus in both the groups. This result was expected because of the close relationship between fibrin and platelets after clotting, and seemed to confirm the first studies[11] on concentrations of platelets in PRF membranes.

While comparing the platelet concentration and distribution, the density of platelets was significantly higher in non-diabetic group with homogeneous distribution all over the buffy coat as compared to controlled diabetic group where platelets density was less and distributed as aggregated clusters. This may be due to faster aggregation and disintegration of platelets as they are hyperactive in diabetic patient.

To study the platelet concentration and distribution, we used two different stains namely hemalaun and eosin and Masson's trichrome (modified by Goldner). Both the groups shared the same micrcoscopic appearance with these two stains.

In hemalaun and eosin staining, hematoxylin is a dark blue or violet stain that is basic/positive and binds to basophilic substances (such nucleus DNA/RNA-which are acidic and negatively charged) and eosin is a red or pink stain that is acidic/negative which binds to acidophilic substances such as positively charged amino acid side chains (e.g. lysine, arginine). In our study, the fibrin matrix appeared homogeneous in light pink as it takes up eosin stain and platelets aggregates were dark blue/violet because the nucleus takes up hemaluan stain. RBCs and leukocyte cytoplasm were not easily detectable: They took up eosin stains and appeared darker pink. The leukocyte nuclei were stained in dark blue with the hemalaun, but they looked like platelet aggregates. Therefore, it was very difficult to distinguish them from the platelet aggregates.

Masson's trichrome-a three-colour staining protocol used in histology method remains one of the best, as it does the most precise of hematoxylins (Heidenhain's iron hematoxylin) with a reliable cytoplasmic stain that gives a wealth of detail (acid fuchsin with ponceau de xylidine). The trichrome is applied by immersion of the fixated sample into Weigert's iron hematoxylin as it stain the nuclei., and then three different solutions, labeled A, B, and C which gives light red or pink cytoplasm, dark blue to black cell nuclei, red keratin and muscle fibers, blue or green collagen and bone. In our 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 are in accordance to the previous study[11] where they used similar histological processing and staining procedures.

The concentration of platelets showed statistically significant differences between the two groups tested. Further the study can be extended including a bigger sample size and using scanning electron microscope analysis for more descriptive results. But nevertheless, this study gives a glimpse of the possible variation in the concentration and distribution of the platelets among healthy and diabetic individuals. Fewer studies are evident evaluating the details of PRF in a diabetic. Again this study provides an insight to elaborate the findings in patients with diabetes for whom the wound healing is altered, and the success of the treatment largely depends on the response of the tissues.

The cell composition of PRF implies that this biomaterial is a blood-derived living tissue and must be handled carefully to keep its cellular content alive and stable.[13] The effectiveness of PRF in promoting the healing depends on the platelet growth factors that can improve the vascularization of the surgical site, promoting neoangiogenesis, as these platelets are well trapped in fibrin network of diabetic patient PRF can be used to accelerate soft and hard tissue healing as well as a potential adjunct for regenerative procedures in these patients.

CONCLUSION

PRF known to work on the principle of tissue engineering as it combines three key elements to enhance regeneration, i.e., platelet concentrate/cells, fibrin membrane/scaffold and growth factors/signaling molecule. The regeneration of the periodontium in diabetic patients is a challenging task to the clinicians. Thus, the development of new therapies which will enhance the regenerative potential in the tissues, such as the use of PRF as a tissue engineered scaffolds opened a new era of the periodontal regeneration. The application of PRF could present more possibilities for enhanced healing and functional recovery in diabetic patient.

REFERENCES

1. Piero MN, Nzaro GM, Njagi JM. Diabetes mellitus - a devastating metabolic disorder. Asian J Biomed Pharm Sci 2014;4:1-7.
2. Radhika T, Kannan R. Diabetes mellitus and oral health. J Orofac Sci 2012;4:7-11.
3. Abiko Y, Selimovic D. The mechanism of protracted wound healing on oral mucosa in diabetes. Review. Bosn J Basic Med Sci 2010;10:186-91.
4. Gupta V, Bains VK, Singh GP, Mathur A, Bains R. Regenerative potential of platelet rich fibrin in dentistry: Literature review. Asian J Oral Health Allied Sci 2011;1:22-8.
5. Choukroun J, Diss A, Simonpieri A, Girard MO, Schoeffler C, Dohan SL, *et al.* Platelet-rich fibrin (PRF): A second-generation platelet concentrate. Part IV: Clinical effects on tissue healing. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;101:e56-60.
6. Toffler M, Toscano N, Holtzclaw D, Del Corso M, Ehrenfest DD. Introducing Choukroun's platelet rich fibrin (PRF) to the reconstructive surgery milieu. J Implant Adv Clin Dent 2009;1:21-32.
7. Shetty A, Vijaya C, Jayalakshmi VJ, Lekha MB. Comparison of mean platelet volume, platelet count, total leucocyte and neutrophil counts in normoglycemics, impaired fasting glusose and diabetic. Int J Sci Stud 2014;2:24-7.
8. Hekimsoy Z, Payzin B, Ornek T, Kandogan G. Mean platelet volume in type 2 diabetic patients. J Diabetes Complications 2004;18:173-6.
9. Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, *et al.* Platelet-rich fibrin (PRF): A second-generation platelet concentrate. Part I: Technological concepts and evolution. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;101:e37-44.
10. Bancroft JD. Theory and Practical of Histological Techniques. Ch. 6. Philadelphia, PA: Churchill Livingstone; 1990. p. 89.
11. Dohan Ehrenfest DM, Del Corso M, Diss A, Mouhyi J, Charrier JB. Three-dimensional architecture and cell composition of a Choukroun's platelet-rich fibrin clot and membrane. J Periodontol 2010;81:546-55.
12. Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, *et al.* Platelet-rich fibrin (PRF): A second-generation platelet concentrate. Part III: Leucocyte activation: A new feature for platelet concentrates? Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;101:e51-5.
13. Del Corso M, Sammartino G, Dohan Ehrenfest DM. Choukroun's platelet-rich fibrin membranes in periodontal surgery: Understanding the biomaterial or believing in the magic of growth factors? Letter to the editor. J Periodontol 2009;80:1694-7.

How to cite this article: Hemalata M, Jayanthi D, Vivekanand L, Swati, Mulla Z. Comparative Evaluation of Platelet Concentration and its Distribution in the Buffy Coat Region of Platelet Rich Fibrin in Non-diabetic and Controlled Diabetic Patients: A Light Microscopic Study. Int J Adv Health Sci 2016;3(1):6-10.

Source of Support: Nil, **Conflict of Interest:** None declared.