Original Article

A Novel Ultrastructural Morphological Characterization of Platelet-Rich Fibrin among Diabetics

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Abstract

Background: The basic biology of the Platelet Rich Fibrin lies in the fact that they have platelets with enclosed granules in them and fibrin with the ability to release growth factors. **Aims and Objective:** The platelet rich fibrin electron microscopic structure has been characterized in the present cross-sectional novel study. This unique biologic material being vastly used since four decades is not microscopically analyzed amongst type II diabetes mellitus subjects, using light microscopy and transmission electron microscopy. The present novel work employs the transmission electron microscope use to reveal the characteristics of cells in order to correlate the growth factor release. **Materials and Methods:** Venous blood samples drawn were subjected to analysis of HbA1c, CBC, platelet indices, and PRF membrane preparation. Platelet rich fibrin membranes were prepared from healthy, well-controlled, and poorly-controlled diabetic individuals following the protocols for Advanced-Platelet Rich Fibrin+ (1,300 rpm, 8 min) and subjected to assessment of morphological analysis using a light microscope, transmission electron microscopy and growth factor release. **Results:** A denser network of fibrin with highest growth factor release was seen in the present study. **Conclusion:** Hence, with the highest growth factors release and a denser network of fibrin, this novel study finds promising biomaterial in diabetics.

Keywords: Diabetes mellitus, growth factors, platelet-rich fibrin, transmission electron microscopy

Introduction

Platelets, also known as thrombocytes, are minute discs of 1–4 μm in diameter and are colorless, nonnucleated, moderately refractive bodies. The normal concentration of platelets in the blood is between 150,000 and 300,000/μL. These platelets, when activated, release an elevated concentration of biologically active proteins, promoting tissue healing in conjunction with the regeneration of tissue. These activated platelets, with their unique structure along with fibrin, find their application in surgical procedures in various fields such as plastic surgery, dermatology, ophthalmology, and sports medicine, with a particular focus on dentistry.[1] Platelet-rich Fibrin (PRF) has a distinctive fibrin architecture that effectively functions as a cellular biodegradable scaffold or graft material with a complex architecture and concentrates all platelets and leukocytes from blood harvest. PRF acts as a biodegradable scaffold that favors the development of microvascularization and is able to guide epithelial cell migration to its surface. Furthermore, PRF may serve as a vehicle for carrying cells involved in tissue regeneration and seems to have a sustained release of growth factors over a period of time. Their autologous origin offers a great benefit and provides the highest level of safety in their application.[2] Transmission electron microscopes (TEMs) will give us an in-depth description of these cells and membranes.

Diabetes mellitus (DM) is a chronic endocrine disorder, characterized by hyperglycemia resulting from absolute (Type I DM) or relative insulin deficiency (Type II DM). The impaired metabolism of glucose, lipids, and proteins in diabetes produces alterations in macro and microvascular circulation.

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The number of people with diabetes is increasing due to the increasing prevalence of obesity, physical inactivity, population growth, and urbanization. Type II DM, however, accounts for approximately 90% of all diabetes worldwide. As of 2021, 537 million adults are living with diabetes, representing 9.3% of the global adult population. This number is expected to increase to 578 million (10.2%) in 2030 and 700 million (10.9%) in 2045. It is one of the fastest-growing global health emergencies of the 21st century.[3] Hence, addressing this population becomes of utmost importance as there are morphological alterations that can be elucidated by recent microscopes.

Wound healing in diabetic patients is impaired because of several factors, such as reduced growth factors at the local site and an angiogenic response. There is inadequate production of growth factors such as platelet-derived growth factor, vascular endothelial growth factor (VEGF), insulin growth factor (IGF-I, II), transforming growth factor-β, and fibroblast growth factor (FGF) in the local wound area in diabetics, which modulate the extracellular matrix formation. Diabetes patients, unless managed properly, will have microvascular and macrovascular complications, eventually leading to the loss of soft and hard tissues.[4] Numerous techniques for platelet concentrate production have been developed and applied in regenerative fields. Advanced-PRF Plus has a centrifugation speed of 1300 rpm for 8 min and has shown increase in the growth factor release for an extended period in comparison with PRF.[5] The morphological alteration in the form of change in structure because of diabetes seen in A-PRF+ of diabetic individuals has not been characterized using the TEM. Till date, there are no studies evaluating the morphological changes in A-PRF+ in individuals with diabetes. Hence, this novel study is undertaken to characterize A-PRF+ morphologically and biologically amongst Type II DM individuals.

Subjects and Methods

This was a cross-sectional study undertaken at DAPM RV Dental College, J. P. Nagar, Bengaluru. The selection of the participants was based on inclusion and exclusion criteria.

After approval by the Ethical Committee at DAPM RV Dental College, clearance for the study was obtained in accordance with the Helsinki Declaration of 1975, as revised in 2013. The procedure followed was explained in detail to the individuals, and a written informed consent form for the procedure was obtained from each participant before the procedure. Ethical clearance was approved from the ethical committee Board of DAPM RV dental college, IRB No;270/VDL-2/2018, dated 16/1/2018.

G*Power software (latest ver. 3.1.9.7) was used in study to estimate the sample size. The 90 samples planned in this study were divided equally into three groups (30 samples per group). This study yielded 95% power to detect significant differences, with an effect size of 0.40 and a significance level of 0.05 the total.

The Group A, Group B, and Group C, comprising 10 from each group, i.e., healthy, well-controlled, and poorly-controlled diabetic individuals, respectively, were in the age group of 35–55 and on oral hypoglycemic medication for 5–10 years were taken up in the study. The inclusion criteria of glycated hemoglobin level (HbA1c) for Group A were <6.5; for Group B, were between 6.5 and 7.5: group C, were >7.5. Individuals with any systemic diseases, obesity, a history of smoking, alcohol consumption, pregnancy or lactating women, history of medications such as oral anticoagulants, anti-platelet drugs, or immunosuppressive drugs, any diagnosed malignancy, or leukemia, or any bleeding disorders, history of other medications such as oral anticoagulants, anti-platelet drugs or immunosuppressive drugs, any diagnosed malignancy, leukemia or any bleeding disorders were excluded.

Venous blood of 17 ml was withdrawn from the antecubital vein, from which 2 ml was sent for analysis of HbA1c and the complete blood count. The remaining blood was collected into uncoated vacutainers for three membrane preparations. Vacutainers were balanced and immediately centrifuged at 1300 rpm for 8 min using a Remi R-8c centrifuge machine, which is of swing rotor type.[6] A-PRF+ is thus obtained after separation from platelet-poor plasma and is formed into the membrane by gentle compression on the PRF box with drainage of the excess fluid. Further PRF membranes were subjected to analysis for biological properties (growth factor release) and morphological analysis (light microscopy, TEM).

All the recorded parameters were statistically analyzed using version 2.0 (IBM SPASS statistics, IBM Corp., released 2011) of SPSS (Statistical Package for Social Sciences, IBM, SPSS® Statistics, version 27). A comparison of blood parameters between the three groups was performed using a one-way ANOVA test followed by a Tukey *post hoc*. The growth factor release (VEGF, IGF-1, and FGF-21) between the groups was analyzed. Similarly, for light microscopy, $P = 0.05$ or less were considered statistically significant.

Analysis of biological properties

At desired time points, the following growth factors: VEGF, IGF-1, and FGF-21, were quantified using ELISA assays (Everon Lifesciences, Shanghai Korain Biotech Co., Ltd. [BT Bioassay]) according to the manufacturer's protocol. Kinesis kits with catalog no. E-0080Hu for VEGF, E-0103Hu for IGF-1, and E-1983Hu for FGF-21 were used for VEGF, IGF-1, and FGF-21, respectively.

Morphological analysis

Light microscopic analysis was done by the cell block cytology method. Using a light microscope at ×40, stained histological slide analysis was done at the Department of Oral Pathology, RV Dental College, Bengaluru. Light microscopy photographs were analyzed using the blood elements adhesion index (BEAI), and fibrin network density was classified as dense fibrin network or loose fibrin network.[7] TEM [Figure 1] was used for analyzing the ultrastructure of platelets at the Department of Biological Science, Indian Institute of Sciences,

Bengaluru. PRF samples were prepared according to TEM protocols using 2.5% glutaraldehyde, osmium tetroxide 2%, and ethanol at concentrations of 50%, 70%, 85%, 95%, and 100% before embedding in epoxy resin. The samples were then sectioned using the ultramicrotome (Leica EM UC7) to obtain ultrathin sections of an approximate thickness of 80 nm and placed on grids. The samples were then subjected to staining for contrast with uranyl acetate and lead citrate. The sections were examined using a TEM (Talos L120 TEM) at 20–120 kV.

Results

The mean values for age, gender, HbA1c level, platelet count, and white blood cell (WBC) count are shown in Figure 2. The one-way ANOVA displayed a statistically significant difference in mean platelet count ($F = 32.39$; $P = 0.001$), WBC (per mm^3) ($F = 13.59$; $P = 0.001$), plateletcrit (PCT) ($F = 35.41$; $P = 0.001$), mean platelet volume (MPV) ($F = 11.51$; $P = 0.001$), platelet to large cell ratio (PLCR) ($F = 15.76$; $P = 0.001$), and platelet distribution width (PDW) ($F = 3.6$; $P = 0.041$) between three groups [Table 1]. The poorly-controlled diabetes group displayed a statistically significant higher platelet count, MPV [Figure 3], PCT, higher PLCR, PDW level, and higher WBC (/cumm) count.

A statistically significant difference in mean VEGF, IGF-1, and FGF-21 levels among A-PRF+ at 1 day (F = 112.47, 13.886, 896.84; P = 0.001), 3 days (F = 138.05, 8.6, 409.2; P = 0.0017), and 7 days (F = 211.26, 9.345, 177.57; $P < 0.05$) with higher VEGF, FGF-21, IGF-1 level in poorly-controlled diabetes group [Figure 4]. The difference in BEAI scores [Table 2] between groups was not statistically

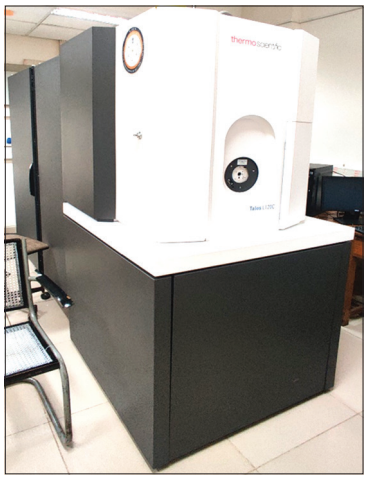

Figure 1: Transmission electron microscope

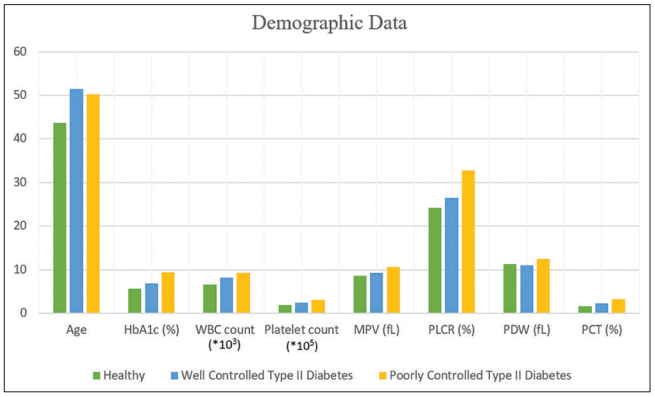

Figure 2: Demographic comparison between groups. HbA1c: Glycated hemoglobin, MPV: Mean platelet volume, PLCR: Platelet to large cell ratio, PCT: Plateletcrit

Table 1: Comparison of mean plateletcrit, mean platelet volume, platelet to large cell ratio, platelet distribution width among healthy, well-controlled diabetes and poorly-controlled diabetes

	One-way ANOVA				Tukey's *post hoc* analysis		
	n	Mean±SD	*F*	*P*	Multiple comparison	Mean difference	*P*
PCT							
Healthy (A)	10	0.16±0.03	35.41	0.001*	A versus B	−0.07	0.008*
Well-controlled diabetes (B)	10	0.22±0.05			A versus C	−0.17	0.001*
Poor-controlled diabetes (C)	10	0.33±0.05			B versus C	−0.10	0.001*
MPV							
Healthy (A)	10	8.62±0.87	11.512	0.001*	A versus B	−0.71	0.221
Well-controlled diabetes (B)	10	9.33±0.84			A versus C	−1.97	0.001*
Poor-controlled diabetes (C)	10	10.59±1.06			B versus C	−1.26	0.014*
PLCR							
Healthy (A)	10	24.20±3.43	15.763	0.001*	A versus B	−2.24	0.324
Well-controlled diabetes (B)	10	26.44±2.74			A versus C	−8.56	0.001*
Poor-controlled diabetes (C)	10	32.76±4.03			B versus C	−6.32	0.001*
PDW							
Healthy (A)	10	11.34±1.26	3.602	0.041*	A versus B	0.35	0.829
Well-controlled diabetes (B)	10	10.99±1.07			A versus C	−1.18	0.138
Poor-controlled diabetes (C)	10	12.52±1.62			B versus C	−1.53	0.042*

*Statistical significance set at 0.05. *n*: Number of samples, PCT: Plateletcrit, MPV: Mean platelet volume, PLCR: Platelet to large cell ratio, PDW: Platelet distribution width, SD: Standard deviation

significant (P = 0.078). The poorly-controlled diabetics displayed a denser fibrin network when compared to healthy individuals [Figure 5].

Pearson correlation analysis

Pearson's correlation [Figure 6] between HbA1c (%) and WBC count showed a statistically significant positive correlation, in all the groups but the level of HbA1c. FGF-21 growth factor had a strong positive correlation with HbA1c levels (r = 0.722; P = 0.018) in Group B [Figure 6]. A statistically significant strong positive correlation between HbA1c (%) and PLCR (r = 0.792; P = 0.006) and PDW (r = 0.827; P = 0.003) was seen in poorly-controlled diabetics.

The BEAI score difference between healthy, well-controlled diabetes and poorly-controlled diabetes was seen (P = 0.024) within A-PRF+, which was statistically significant. The Mann–Whitney Test displayed a statistically significant higher mean rank for those with poorly-controlled diabetes when compared to the healthy group (P = 0.015) [Table 2]. In comparison with healthy individuals, a denser fibrin network was observed in poorly-controlled diabetics [Figure 5].

Observations from transmission electron microscope

Healthy platelets were discoid in shape, representing the resting platelets, while some showed a few signs of activation as depicted by cell elongation and contained a number of distinguishable structural elements, including the plasma membrane, the open canalicular system (OCS), and numerous organelles, including the alpha-granules, dense-granules, lysosomes, and mitochondria. Abundant alpha granules were observed, which were characterized as having a spherical shape with a dark, electron-dense central core and an electro-lucent zone with the granule diameter ranging from 200 to 500 nm.

The platelets from A-PRF+ well-controlled diabetics showed a loss of integrity in the plasma membrane and pseudo-tubular form with alterations in the cytoskeleton, which aids in the shape changes. This is assisted by alpha and dense-granule fusion to the plasma membrane upon granule release. A few alpha-granules were partially broken, while others were fused together, and they contained a few tubular organelles with cross striations, indicating platelet activation.

Poorly-controlled diabetic patients demonstrate pronounced shape alterations, a pseudopodical stage showing numerous cell projections along with dilatation of the OCS indicative of strong platelet activation. Degeneration of plasma membrane with filopodia formation can also be seen in platelets. Dense areas of condensation of alpha granule subpopulations were detected at the center of the platelet or were localized to the periphery, denoting platelet hyperactivation.

Table 2: Multiple comparisons of blood element adhesion index score of A-platelet-rich fibrin + between healthy, well-controlled diabetes and poorly-controlled diabetes

	Multiple comparison	*n*	Mean rank	*P*
BEAI score (A-PRF+)	Healthy	5	4.6	0.18
	Well-controlled diabetes	5	6.4	
	Healthy	5	3.4	0.015*
	Poorly controlled diabetes	5	7.6	
	Well-controlled diabetes	5	4	0.072
	Poorly-controlled diabetes	5	7	

*Statistical significance set at 0.05. BEAI: Blood element adhesion index, PRF: Platelet-rich fibrin

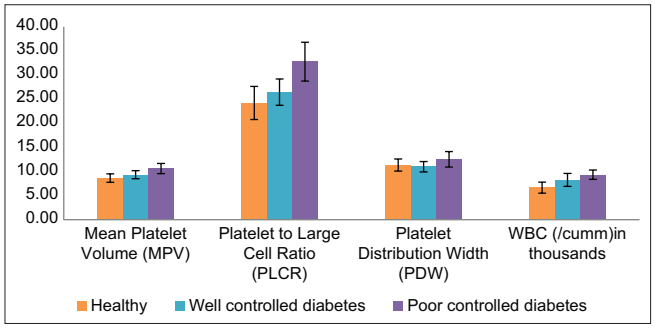

Figure 3: Platelet indices among healthy, well controlled diabetic, poorly controlled diabetic. MPV: Mean platelet volume, PLCR: Platelet to large cell ratio, PDW: Platelet distribution width, WBC: White blood cell

Discussion

The present cross-sectional *in vitro* study assessed and compared the morphological, and growth factor release amongst diabetics. DM is associated with impairments in wound healing and regeneration.[8,9] Thus, A-PRF+ with its morphological alteration releasing high growth factor can provide a promising therapy for diabetics. The nonobese, gender-, and age-matched individuals recruited in the present study were based on their glycemic control HbA1c as per the

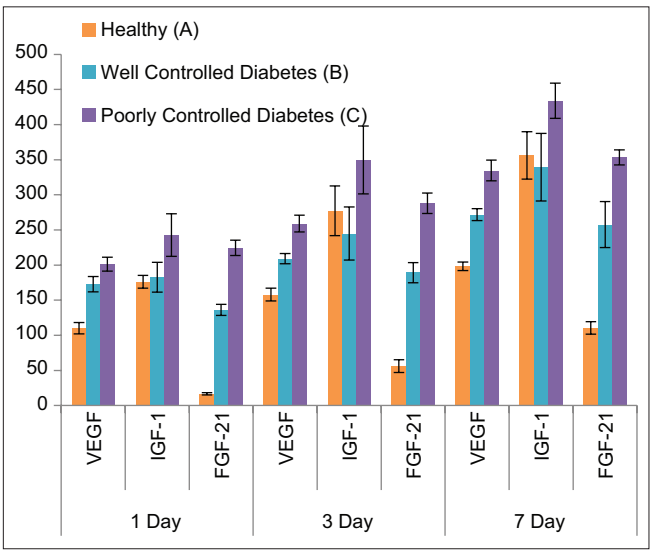

Figure 4: Comparison of mean growth factor release between healthy, well controlled diabetes and poorly controlled diabetes among A-PRF+. VEGF: Vascular endothelial growth factor, IGF: Insulin growth factor, FGF: Fibroblast growth factor

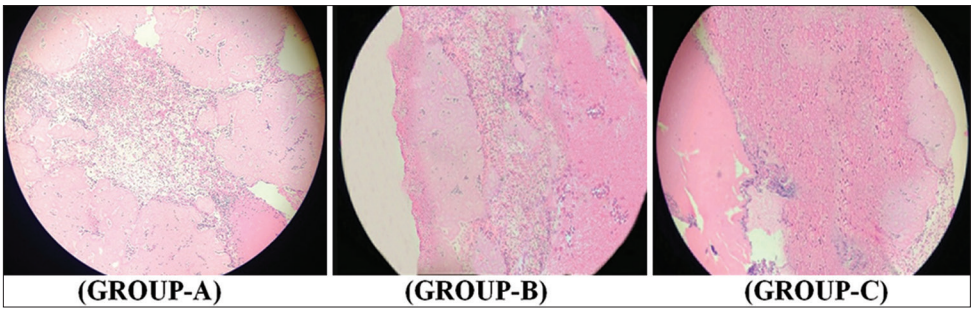

Figure 5: Light microscopy analysis - A-PRF+

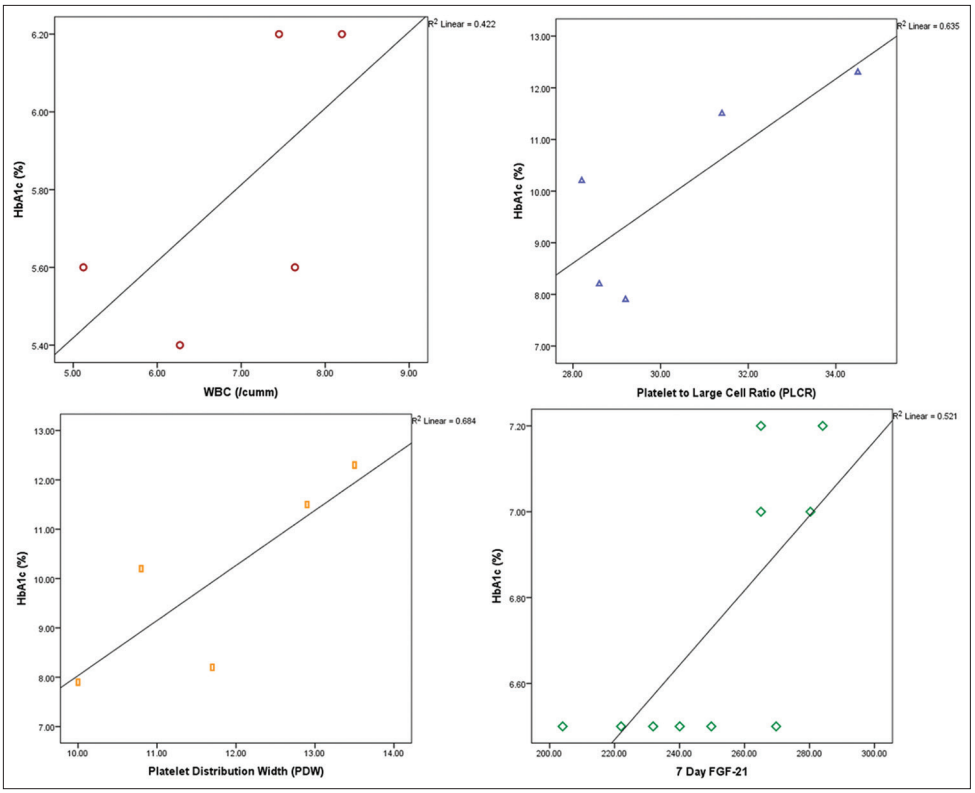

Figure 6: Pearson correlation of HbA1c levels, WBC counts, PLCR, PDW and pearson correlation of HbA1c levels and FGF-21. HbA1c: Glycated hemoglobin, WBC: White blood cell, PLCR: Platelet to large cell ratio, PDW: Platelet distribution width

WHO recommendation.[10] Increased activation of platelets at baseline and hyperactivation is multifactorial, associated with hyperglycemia, and insulin resistance, and could be attributed to an inflammatory state and oxidative stress.[11] Earlier studies have confirmed a significant alteration in platelet indices (MPV, PDW, PCT, and PLCR) in individuals with diabetes.[12] This study showed statistically significant higher values of platelet Indices such as MPV, PDW, PCT, and PLCR in diabetic patients, especially in poorly-controlled diabetics ($P < 0.05$) similar to previous studies. The results of this study are in accordance with the previous studies, which revealed similar results.[13-15] One of the plausible mechanisms could be due to osmotic swelling due to hyperglycemia, resulting in increased platelet volume and perhaps a shorter life span of platelets in diabetic patients, and platelet indices positively correlate with diabetic complications.[16]

Further elevated WBC and platelet counts are present in diabetics, especially poorly-controlled diabetics. This was in conformity with the findings by Zuberi *et al*. and Demirtunc *et al*. It is, however, in contrast with the findings of a study conducted by Hekimsoy *et al*.[17-19] In diabetics, increased thrombopoietin signaling leads to increased platelet synthesis as well as increased platelet turnover in the circulation.[20]

A significantly elevated growth factors release in A-PRF+ from all the groups, particularly more in poorly-controlled diabetic patients was seen in this study. These findings corroborate a previous study by El Bagdadi *et al*. Furthermore, as A-PRF+ shows a more porous structure, it may allow more space for trapped platelets and immune cells and therefore a higher and more sustained growth factors release, as demonstrated by Fujioka-Kobayashi *et al*., El Bagdadi

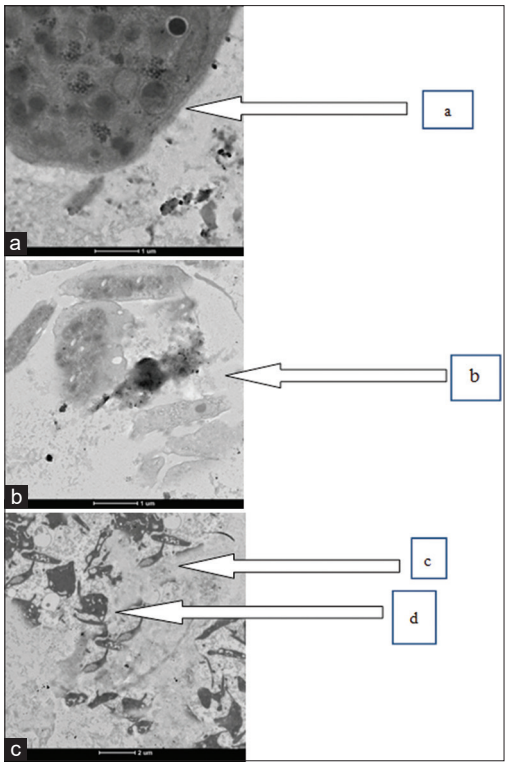

Figure 7: Transmission Electron Microscopy analysis. (a) Healthy (Group A) (b) Well-controlled diabetics (Group B) (c) Poorly-controlled diabetics (Group C). (a) Intact alpha granules, (b) Loss of membrane integrity, (c) Filopodia formation, (d) Formation of microaggregates

et al., and Pitzurra *et al*.[21-23] Poorly-controlled diabetics displayed statistically significant higher VEGF, IGF-1, and FGF-21, levels at days 1, 3, 7, and this can be attributed to the fact that worsening of hyperglycemia is related to the development of diabetic complications.[24,25] Diabetes is known to cause apoptosis in osteoblasts. Hence, IGF-1 through PRF may provide anabolic effects on the bone. A positive correlation between HbA1c levels and FGF-21 in diabetics in this study could be due to higher serum FGF-21 levels in poorly-controlled diabetics due to an increased hepatic production of FGF-21 as a result of the metabolic changes. However, hyperglycemia may cause resistance to the actions of FGF-21 thereby increasing its levels in the serum of diabetic patients. This is in contrast to the findings, which suggest that FGF-21 may stimulate bone resorption by directly stimulating osteoclastogenesis.[26]

A statistically significant higher crosslinking and denser fibrin network were noted among those with poorly-controlled diabetes when compared to the healthy group ($P = 0.015$). This implies that thicker fibers with a denser, more cross-linked structure found to be formed by diabetic subjects are more resistant to fibrinolysis, making them a more suitable barrier material for regeneration. This is in accordance with a study done by Abooj *et al*. This denser, less porous, and more rigid fibrin network seen in diabetics could be attributed to the glycation of the fibrinogen by AGEs.[27]

Ultrastructural analysis of platelets based on the TEM revealed that platelets from healthy A-PRF+ displayed [Figure 7] a discoid shape and intact plasma membranes, organelles, and various granules. However, some platelets did show some degree of activation, with the loss of their spherical shape indicating slight activation.[28] Unlike the platelets from healthy individuals, well-controlled diabetic individuals, presented with a loss of discoid shape with pseudopodia expansion among most of the platelets and also the fusion of a few granules, indicating early platelet activation.[29] Similarly, A-PRF+ obtained from poorly-controlled diabetics showed marked signs of platelet activation. Partial rupture of the platelet plasma membrane could be seen along with the signs of degranulation indicating hyperactive platelets.

Conclusions

The data from the present study revealed various morphological alterations and biological properties among A-PRF+ individuals that may have a further impact on the treatment outcomes. Significantly higher values were observed for platelet indices, platelet counts, and WBC counts in poorly-controlled diabetics. Interestingly, there was a high release of growth factor that was associated with the glycemic control of the individual, indicating a high release of VEGF, IGF-1, and FGF-21 from poorly-controlled diabetics as compared to well-controlled diabetics and healthy individuals. The elevated numbers of active platelets show signs of hyperactivation and degranulation. A denser fibrin network in diabetics can probably be correlated to the incorporation of more platelets and growth factors, with higher growth factor release immediately after A-PRF+ preparation as well as sustained release until day 7. With all these preceding results, the A-PRF+ membrane biologically with more growth factor could be the most suitable material for regeneration and healing in diabetic patients. With all the data collected from microscopy and TEM, the study gives a promising option for clinical studies to be undertaken in future directions for breakthroughs in treating diabetic patients.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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