ORIGINAL ARTICLE
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UDC: 616.31-08::[615.242:615.45
DOI: https://doi.org/10.2298/VSP240117030G

Comparative evaluation of titanium-prepared platelet-rich fibrin with and without herbal extract: a histological study

Poređenje titanijumom-pripremljenog fibrina obogaćenog trombocitima sa i bez biljnog ekstrakta: histološka studija

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Abstract

Background/Aim. Injecting herbal extract into platelet concentrates is one of the newer treatment protocols, which enables platelet concentrates to act as sustained drug delivery (DD) systems. Histological analysis of titanium-prepared platelet-rich fibrin (T-PRF) injected with herbal extract, could help assess the appearance (pattern) and structural changes of T-PRF. The aim of the study was to evaluate the appearance of the fibrin network, cellularity, and fibrin border area of T-PRF alone and T-PRF injected with herbal extract. **Methods.** A total of 40 histological slides were prepared from 10 mL of blood from each patient, 20 with T-PRF alone and 20 with T-PRF+herbal extract. The slides were divided into a group consisting of T-PRF injected with neem gel (test group) and a group consisting of T-PRF alone (control group). The preparation protocol was made according to Bankroft's manual adapted for light microscopy. **Results.** Regarding the fibrin network features (dense vs. loose), no statistical significance was found among the studied groups ($p = 0.172$). A statistically significant difference was shown in the packeting ($p = 0.018$) and layered ($p = 0.028$) patterns of the fibrin network, and there was no statistically significant difference in the scattered ($p = 0.749$) pattern among the examined groups. Cellularity and cell pattern values were not statistically significantly different for both groups ($p = 1.00$, $p = 0.3111$, respectively). Moreover, the values determined for red blood cells, white blood cells, and platelets were not statistically significantly different ($p = 0.147$), as well as for the fibrin border area between cells and meshwork ($p = 0.206$). **Conclusion.** The obtained results could be useful for the development of a new treatment strategy in dentistry, by utilizing T-PRF with incorporated herbal extracts or antibiotics, as a local sustained DD system.

Key words:
drug delivery systems; histological techniques; periodontitis; plants; platelet-rich plasma.

Apstrakt

Uvod/Cilj. Ubrizgavanje biljnog ekstrakta u koncentrat trombocita jedan je od novijih protokola lečenja, koji omogućava da koncentrat trombocita deluje kao sistem za kontinuiranu isporuku lekova. Analiza histoloških preseka titanijumom-pripremljenog fibrina obogaćenog trombocitima (*titanium-prepared platelet-rich fibrin* – T-PRF) u koji je ubrizgan biljni ekstrakt, mogla bi biti korisna za procenu izgleda (obrasca) i promena strukture T-PRF. Cilj rada bio je da se proceni izgled fibrinske mreže, celularnost i granično područje fibrina samog T-PRF i T-PRF u koji je ubrizgan biljni ekstrakt. **Metode.** Od 10 mL krvi svakog pacijenta pripremljeno je ukupno 40 histoloških pločica, 20 samo sa T-PRF i 20 sa T-PRF i dodatkom biljnog ekstrakta. Preparati su podeljeni na grupu T-PRF u koji je ubrizgan *neem* gel (testirana grupa) i grupu samog T-PRF (kontrolna grupa). Protokol pripreme napravljen je prema Bankroftovom priručniku prilagodjenom za svetlosnu mikroskopiju. **Rezultati.** Nije bilo statističke značajnosti među ispitivanim grupama ($p = 0,172$) u pogledu izgleda fibrinske mreže (gusto vs. rastresito). Pokazana je statistički značajna razlika u obrascima fibrinske mreže „pakovanje“ ($p = 0,018$) i „slojevitost“ ($p = 0,028$), a nije bilo statistički značajne razlike u obrascu „rasipanje“ ($p = 0.749$) među ispitivanim grupama. Vrednosti za parametre distribucija

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ćelija i celularnost nisu bile statistički značajno različite za obe grupe (p = 1.00, p = 0.3111, redom). Takođe, ni vrednosti koje su utvrđene za crvena krvna zrnca, bela krvna zrnca i trombocite nisu bile statistički značajno različite (p = 0,147), kao ni za granično područje fibrina između ćelija i mreže (p = 0,206). **Zaključak.** Dobijeni rezultati bi mogli biti korisni za razvoj nove strategije lečenja u stomatologiji, korišćenjem T-PRF sa inkorporiranim biljnim ekstraktima ili antibioticima, kao sistema za lokalnu kontinuiranu isporuku lekova.

Ključne reči:
lekovi, sistemi za isporuku; histološke tehnike; periodontitis; biljke; plazma bogata trombocitima.

Introduction

Periodontitis is a painless disease that is difficult to treat because of its multifactorial origin [1]. Various treatment strategies, including some non-surgical treatments such as scaling and root planing and local drug delivery (DD) systems, have been used [2]. Surgical treatments such as open flap debridement, guided tissue regeneration, and guided bone regeneration have been utilized. Various biomaterials, which were reported to achieve good results, like bone grafts, collagen membranes, and platelet concentrates (PCs), were also used for controlling postoperative recession and restoring lost periodontal tissues [3]. PCs are considered a boon to the medical field because of their autologous nature, sustained holding, and release of growth factors (GFs) at surgical sites [4]. Initially, leukocyte platelet-rich fibrin (L-PRF) was introduced by Choukroun et al. [5]. However, because of its shorter resorption time and possible silica cross-contamination, titanium-prepared platelet-rich fibrin (T-PRF) was introduced by Tunali et al. [6], where medical grade titanium tubes (Grade IV) were used for the preparation of PCs.

T-PRF had better fibrin meshwork, a thicker membrane, and better cellular entrapment. It demonstrates better hemocompatibility, and the titanium passivates itself into the titanium dioxide layer to activate platelets [7]. In the studies by Chatterjee et al. [8] and Yajamanya et al. [9], it was stated that T-PRF had a better fibrin meshwork, border, and thicker membrane pattern than L-PRF. As stated in the study by Bhattacharya et al. [10], both T-PRF and L-PRF shared a similar pattern, but T-PRF had thicker fibrin meshwork with a thicker structure in the mid-membrane region. Histological sectioning of human specimens was always a difficult task because gaining ethical permissions and patient acceptance was troublesome.

Ercan et al. [11] used doxycycline hyclate gel that was incorporated in T-PRF clot and compared it with collagen sponge incorporated with doxycycline hyclate gel and concluded that T-PRF+ doxycycline (Doxy) – (T-PRF+ Doxy) group had longer release than collagen sponge group during their kinetic studies. Furthermore, their microbiological analysis also depicted the best outcome with the T-PRF+Doxy group regarding the zone of inhibition, thus depicting the newer treatment modality of DD at the required periodontal site [11]. Various antibiotics have been tried, such as lincomycin, minocycline, and clindamycin, and neem gels were used as local DD systems in non-surgical and surgical treatments, and good concentration levels of the drug in the pocket were achieved [12].

Histological analysis is one of the major types of assessment for diagnosing a clinical case scenario by a possible clinical correlation. However, it is not possible to perform this in every situation. Histological evaluation of membrane clots using light microscopy (LM) would be an indirect evaluation of cells and fibrin structure, which would hold the GFs, which in turn help in the formation of lost tissue cells.

Considering that a small number of studies has been done on this topic, the present study aimed to evaluate the features of the fibrin network (including distribution pattern and arrangement), cellular entrapment (cellularity), and the border area between fibrin meshwork and cellularity in T-PRF with and without injecting herbal extract using LM.

Methods

Sample size

With 80% power, an effect size of 0.25, and an alpha value error of 5%, a sample of 20 slides *per* group was sufficient to conduct the study. A total of 40 samples were considered for the study. The entire calculation was performed on G* Power software 3.0.

Study design

The present study was a histological study using LM. A total of 20 healthy volunteers were recruited after getting informed consent from the outpatient Department. The study was conducted by the Department of Periodontology and Implantology, GITAM Dental College and Hospital, Visakhapatnam. The study was performed according to the 1975 Helsinki Declaration, modified in 2013. Prior to the conduction of the study, ethical approval was obtained from the GITAM Dental College and Hospital Ethics Committee (Nº 6908606s33523, from July 28, 2023). There were no problems at the patient blood drawing sites.

The test group consisted of T-PRF injected with neem gel, and the control group consisted of T-PRF alone.

The study was performed based on the below inclusion and exclusion criteria.

Inclusion and exclusion criteria

Patients above 18 years of age, with a Plaque Index < 20%, with healthy periodontium, and patients with a range of platelets between 150,000 to 400,000/mm^3 were included in the study. Patients who were systemically ill, who were not interested in participating, lactating and pregnant women,

who underwent periodontal treatment in the last six months, and who were on medications that affect the status of periodontium were excluded from the study.

Procedure

T-PRF clots were prepared based on modified Tunali et al. [6], Bhattacharya et al. [13], and Mitra et al. [14] criteria. For the test group, the T-PRF clot was loaded with commercially prepared neem gel just immediately after clot retrieval from titanium test tubes, while for the control group, T-PRF alone in the clot form was used. With no further delay, these clots of both groups were immediately placed in 10% formalin solution (for fixing up to 24 hrs) and transferred to the Department of Oral Pathology and Oral Microbiology for slide preparation and LM analysis.

Neem gel preparation

Neem gel was commercially formulated from the PerioBiologics™ lab. The neem gel was prepared in the following manner. Aqueous liquid extract of *Azadirachta indica* was prepared by 20 g of neem leaves in 1 L of water. This was reduced to 100 g of neem extract. To obtain a minimum inhibitory concentration at 25 µg/mL, 20 g of Carbopol® 934P was added in 1,000 mL of water and soaked for 24 hrs to prepare the base gel. All the prepared ingredients were mixed, and 0.02% calcium chloride and 0.9% sodium chloride were added to prepare the neem gel [15]. These gels were loaded in µ syringe needles with the quantity of 10 U *per* syringe, which creates a concentration of 8 g of neem *per* syringe.

T-PRF clots preparation

Ten mL of blood was drawn from the antecubital vein and immediately transferred to medical-grade sterile titanium tubes, divided into 5 mL per tube. Figure 1 shows the titanium tubes, retrieval of T-PRF clots, and T-PRF injected with neem gel. Then, the tubes filled with blood were subjected to centrifugation using the modified Tunali et al. [6, 16] protocol [3,500 revolutions *per* minute (rpm) for 15 min]. Further, the upper layer of platelet-poor plasma was discarded, the middle layer, which was the fibrin clot with a buffy coat, was retrieved, and the bottom layer of red blood cell (RBC) layers was allowed to settle down at the bottom of the tube. The middle layer of T-PRF clots was retrieved, and those clots were placed on a sterile kidney tray.

Sample preparation for histological analysis

The considered samples were placed in a 10% formalin solution and fixed for a period of 24 hrs as a process of preventing the autolysis and maintenance of the intact structure. These clots were then retrieved and subjected to the process of desiccation in various concentrations of alcohol (25%, 50%, 75%, and 100%). Further clearing and paraffinization were continued, which takes up to 16 hrs. Later on, these clots were taken into blocks and made into thin sections using a microtome and transferred to a slide (Figure 2). In the

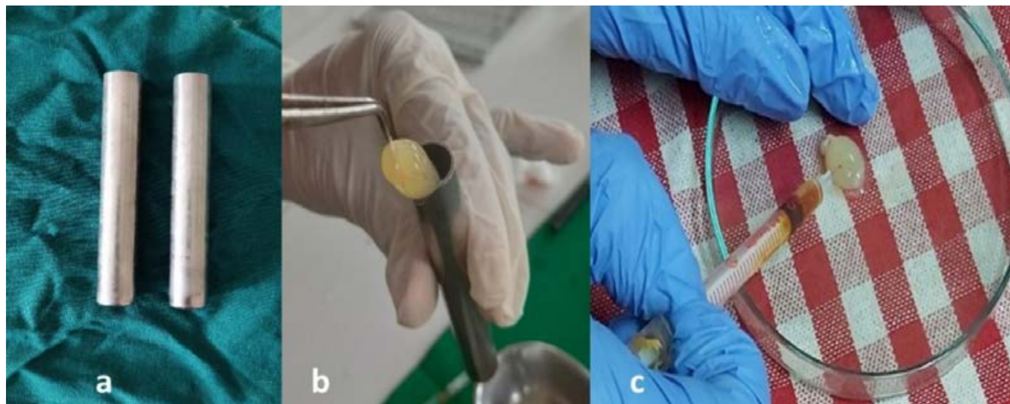

Fig. 1 – a) Titanium tubes; b) Retrieval of T-PRF clots; c) T-PRF injected with neem gel.
T-PRF – titanium-prepared platelet-rich fibrin.

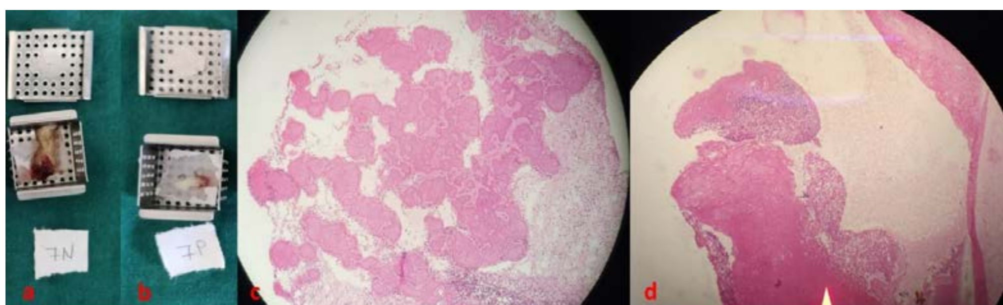

Fig. 2 – a, b) Paraffin block preparation of T-PRF incorporated with neem gel and plain T-PRF; Histological section of: c) plain T-PRF and d) T-PRF incorporated with neem gel (at 10× magnification, HE staining).
T-PRF – titanium-prepared platelet-rich fibrin; HE – hematoxylin eosin.

next step, deparaffinization was done by heating the slide at 55 °C and kept in xylene to remove any paraffin particles. Hence, as a part of the study, 40 slides (20 for each of the two groups) with proper coding and numbers were subjected to LM analysis. The entire process of histological analysis was performed according to Bancroft's manual [17] and observed under a penta head microscope.

Parameters assessed from the prepared slides

Based on the modified Tunali et al. [6] criteria, all the T-PRF slides, both with and without neem gel, were analyzed. In addition, it was noted whether the fibrin network pattern was loose or dense; the pattern was recorded as packeting, layered, or scattered. Concerning cellularity, presence and absence were scored. If present, the score of 0 was given when the presence of cellularity was < 25%; a score of 1 was given when there was 26–50% of cellularity; a score of 2 was recorded when there was 51–75% of cellularity; a score of 3 was given for > 75% of cellularity. Furthermore, the presence and absence of RBCs were assessed, including whether the border area between fibrin meshwork and cellularity was thick or thin.

The slides were examined by an experienced oral pathologist.

Statistical analysis

Entire data was noted in a Microsoft Excel spreadsheet and subjected to statistical analysis. Data analysis was done using Statistical Package for Social Sciences (SPSS) version 22, IBM Pvt Ltd, Chicago, Illinois, USA. Study data were presented as percentages of the frequency distribution for the following: fibrin network pattern; cell distribution; presence or absence of cellularity; presence of red and white blood cells (WBCs), platelets, and fibrin border area between cellularity and fibrin network. For these parameters, Fisher's exact test was used. The score was also noted and an unpaired *t*-test was used to demonstrate the cellularity significance. The value of $p < 0.05$ was considered statistically significant.

Results

In the present study regarding the fibrin network features, plain T-PRF showed a 60% dense pattern and 40% loose pattern, whereas, in the case of the T-PRF neem gel group, it was vice versa. No significant statistical difference was shown between the groups ($p = 0.172$). In the case of pattern, T-PRF had 50% of packeting pattern, 10% showed layered, and 40% had scattered pattern, while neem incorporated T-PRF showed 15% of packeting pattern, 40% showed layered, and 45% had scattered pattern. Their comparisons were significant for the packeting ($p = 0.018$) and layered ($p = 0.028$) patterns and non-significant for the scattered ($p = 0.749$) pattern. Regarding cell distribution patterns, both groups (T-PRF plain and neem gel incorporated T-PRF) reported non-significant differences ($p = 1.000$), i.e., 30% of narrow and 70% of wide patterns were detected. In the case of cellularity, 95% of plain T-PRF showed the presence of cells, whereas 100% of neem-incorporated T-PRF showed the presence of cellularity, which was not statistically significantly different ($p = 0.311$). Although the values were not statistically significant, 35 to 50% of both groups showed a range of cellularity greater than 50%, i.e., a score range of 2 and 3. Regarding RBCs, WBCs, and platelets, both groups showed 90 to 100% of presence without a statistically significant difference between the groups ($p = 0.147$) (Table 1).

Table 1

Frequency distribution percentage comparison of fibrin network, their pattern, cell distribution, cellularity cum scoring, presence of red blood cells, white blood cells, platelets, and the fibrin border area between cells, and fibrin meshwork of plain T-PRF and neem gel incorporated T-PRF

Parameters	T-PRF plain	T-PRF neem gel	*p*-value
Fibrin network			
dense	12 (60)	8 (40)	0.172
loose	8 (40)	12 (60)	
Pattern			
packeting	10 (50)	3 (15)	0.018*
layered	2 (10)	8 (40)	0.028*
scattered	8 (40)	9 (45)	0.749
Cell distribution			
narrow	6 (30)	6 (30)	1.000
wide	14 (70)	14 (70)	
Cellularity			
absent	1 (5)	0 (0)	0.311
present	19 (95)	20 (100)	
Red blood cells			
absent	2 (10)	0 (0)	0.147
present	18 (90)	20 (100)	
Platelets and white blood cells			
absent	2 (10)	0 (0)	0.147
present	18 (90)	20 (100)	

T-PRF – titanium-prepared platelet-rich fibrin; *statistically significant.
All values are given as numbers (percentages).

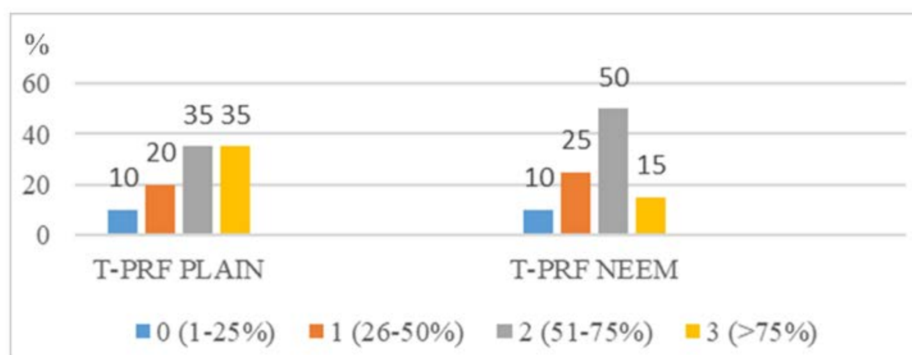

Fig. 3 – Score range criteria for plain T-PRF and T-PRF neem groups (*p* = 0.535). T-PRF – titanium-platelet rich fibrin.

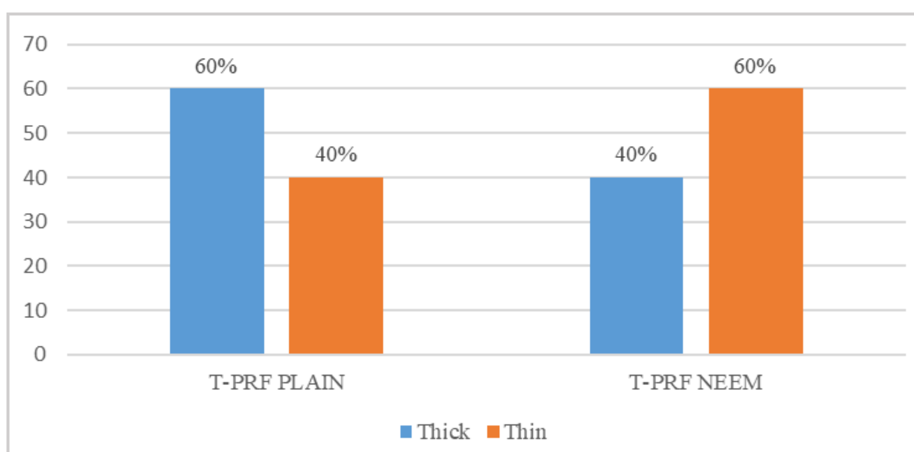

Fig. 4 – Graphical representation of the border area between fibrin meshwork and cellularity (*p* = 0.206). T-PRF – titanium-prepared platelet rich fibrin.

When comparing the scores regarding the percentage of cellularity, there was no statistically significant difference between the tested groups (p = 0.535). The score range criteria for both groups, plain T-PRF and T-PRF neem, as well as values, are presented in Figure 3.

When it comes to the border area between the fibrin network and cellularity, plain T-PRF had a 60% thick border area, whereas neem incorporated T-PRF showed a 40% thick border area, but the values shown were not statistically significantly different between the groups (p = 0.206) (Figure 4).

Discussion

Autologous PCs always helped in the promotion of soft and hard tissue healing [18]. Initially, L-PRF was prepared in silica tubes or silica-coated glass test tubes. Silica within these tubes activates the platelets in the blood (particularly alpha and beta granules) and fibrin clots form as a result. The shorter resorption time of 7–11 days and possible silica contamination lead the researchers to search for better material [19]. Due to this quest, the researchers were attracted to titanium metal, which led to the introduction of T-PRF by Tunali et al. [6]. Animal studies on the rabbit model revealed that T-PRF had thicker fibrin meshwork and uniform distribution of cells over the fibrin [16]. Most clinical studies [7, 14, 20] checked for post-operative bone fill, decreased probing pocket depth, and gain in clinical attachment level from baseline to 6 or 9 months based on their respective criteria. Histological sectioning and surgical re-entry always raise a situation of ethical concerns. Hence, histological analysis of PCs will be an indirect way to assess and predict the possibility of periodontal soft and hard tissue healing.

Local DD of antibiotics like amoxicillin, metronidazole, tetracycline, clindamycin, etc., is one of the alternative ways of treating the periodontal disease non-surgically. Even some herbal extracts like neem, aloe vera, etc., have been tried as local DD systems and mouthwashes, and good results have been reported [21]. However, sustained drug release was one of the recent advancements. The drug was delivered at the surgical or inflamed sites that would control the post-operative infection and improve clinical parameters with constant timely drug release [22]. Most delivery systems were laboratory-prepared, such as carbo-polymers, collagen membranes, gel foams, etc. Although these have reported good results, because of their raised sensitivity reaction concerns and the cost of the material, researchers were made to search for better autologous biomaterial [23]. Because of this, in the study by Ercan et al. [11], T-PRF was used as a

sustained drug carrier system where doxycycline hyclate antibiotic liquid was incorporated. The results of their study suggested that T-PRF can be a good biomaterial that is able to withstand the drug because of its thicker fibrin meshwork and better GF holding capacity. The present study was an attempt to check whether injecting the neem gel into T-PRF led to any changes in the fibrin characteristics, cellularity, and intactness of fibrin meshwork and to compare results with plain T-PRF using LM.

Regarding the fibrin network, there was a numerically denser fibrin network for plain T-PRF than for neem gel-incorporated T-PRF, and it was statistically non-significant. Therefore, there was not much difference in both groups overall. Present plain T-PRF results were in accordance with previous studies done by Tunali et al. [6] and Chatterjee et al. [8], where a denser fibrin network in T-PRF was reported. The results of these studies were in contrast with the presented neem gel incorporated T-PRF. These variations of loose fibrin network in the test group might be due to the incorporation of neem gel into the fibrin clot, which lead to spaces in between the fibrin structure. Regarding the pattern, 50% of plain T-PRF had a packeting pattern, and the T-PRF neem gel group had a scattered pattern. This is contrary to the study done by Bhattacharya et al. [10], where a mostly scattered pattern was reported. Present study results were also in accordance with Tunali et al. [6] and Chatterjee et al. [8], where a packeting pattern and thicker fibrin network were reported. This might be due to the greater activation of platelets by the passivated titanium dioxide layer within titanium tubes, which led to intact fibrin mesh.

Concerning cell distribution (26–75% of score range), RBCs, WBCs, and platelets, there was a non-significant, wider distribution pattern. RBC, WBC, and platelet presence was uniform, and this is in accordance with the study results by Bhattacharya et al. [10], Tunali et al. [6], and Chatterjee et al. [8], where a wider distribution of cellularity and various types of cells was reported. This greater cellular entrapment might be due to the modified Tunali et al. [16] centrifugation protocol (35,00 rpm at 15 min) utilized in the study, which could help incorporate RBCs, WBCs, and platelets.

In the present study, plain T-PRF had a thicker fibrin border area than the neem gel-incorporated T-PRF. This is contrary to the study done by Bhattacharya et al. [10] regarding the plain T-PRF group, where a thicker fibrin border area was reported for L-PRF compared to T-PRF. Their results favored neem gel incorporated T-PRF. In the study by Tunali et al. [6], plain T-PRF had a thicker fibrin border area, while neem gel incorporated T-PRF had a thinner fibrin border area. This variation in neem gel incorporated T-PRF might be due to the injected gel having disrupted the fibrin structure leading to much scattered and layered patterns with thin fibrin border areas. It can be thus indirectly understood that neem gel was incorporated into the T-PRF clot which might be washed out during the process of histological slide preparation and depicted as spaces within membrane meshwork. Apart from this, the inherent property of expansion of T-PRF clot might also have influenced the reporting of gaps in the T-PRF incorporation of neem gel.

In the present study, there was the presence of cells, i.e., RBC, WBC, and platelets, with a wider area of cell distribution and 51–75% cell percentage over the slide area in both, T-PRF alone and neem gel incorporated T-PRF. This is in accordance with the study by Ercan et al. [11], where doxycycline hyclate was incorporated into T-PRF, and a greater withhold capacity of the drug by T-PRF was reported. This might be due to the inbuilt capacity of T-PRF and it will make the addition of drugs into T-PRF a treatment protocol and give hope for a sustained DD system.

The incorporation of antibiotics into blood prior to centrifugation is always a concern for the centrifugation process, clotting mechanism, and fibrin structure quality. Studies done by Marzaman et al. [24] and Pillai et al. [25] used antibiotics and painkillers with PRF and concluded that there was no loss of structure, good anti-microbial efficacy was maintained, and post-operative pain after the third molar extraction was reduced. The present study utilized a similar pattern of incorporation of antibiotics after the process of centrifugation. In the present study, it was reported that there were gaps in the T-PRF incorporated with neem gel clots. However, there was no disturbance regarding the wider cell distribution, cell pattern, or presence of cells and layered pattern of fibrin meshwork. This might be due to the incorporation of 0.5 mL of gel into the clot. This was even supported by Polak et al. [26], where it was reported that PRF clots, incorporated with antibiotics beyond 0.5 mL, lost their structural integrity. Hence, 0.5 mL is the maximum amount of drug that can be incorporated into the clots.

A smaller sample size might be considered a limitation of the present study. Histological analysis of membrane clots may predict the healing to some extent, but further scanning electron microscopy, drug kinetics with spectrophotometry, GF release assessment for platelet viability, and animal or human clinical trial might help identify the efficacy of this treatment strategy of T-PRF incorporated with neem gel.

Thus, this might open a gateway for a newer treatment protocol of sustained DD system, utilizing T-PRF as a vehicle incorporated with herbal extracts or antibiotics in the form of gels or liquids.

Conclusion

Although neem gel incorporated T-PRF showed a thin fibrin network with scattered or layered pattern, wider distribution of cells (RBCs, WBCs, and platelets), and a 26–75% score range extended over the clot, it was not much significantly different from T-PRF plain which depicted thicker fibrin network, packeting pattern, and wider cellular distribution. Hence this may help deliver a newer treatment strategy to the dental fraternity utilizing T-PRF as a sustained DD system by incorporating herbal extracts or any antibiotics making it a part of the local DD system.

Acknowledgement

We are very thankful to all who have participated in the study.

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Received on January 17, 2024
Revised on February 16, 2024
Revised on March 6, 2024
Accepted on March 19, 2024
Online First April 2024