



The effect of preparation method on the fibrin diameter of leukocyte- and platelet-rich fibrin (L-PRF).

SADJ May 2017, Vol 72 no 4 p193 - p197

Peck MT,¹ Hiss D,² Stephen LXG.³

ABSTRACT

Introduction

The use of leukocyte and platelet-rich fibrin (L-PRF) in regenerative surgery has increased exponentially in the last decade. That success has led various centres to have introduced the biomaterial as a routine inclusion in oral grafting procedures. Evidence suggests that the physical properties of fibrin play an essential role in homeostasis of blood clots. Only a limited number of studies have investigated the features of fibrin fibre diameter of L-PRF concentrates.

Aims and objectives

To investigate and compare the fibrin network and fibrin fibre diameter of L-PRF prepared by two different methods.

Methods

Blood was collected from a single volunteer using established protocols. The resultant L-PRF clots were then prepared and examined using scanning electron microscopy. The results were subjected to statistical analysis.

Results

L-PRF prepared using the modified method had larger diameter fibrin fibres than that prepared using the official protocol. The difference was statistically significant ($P=0.001$). There was also a larger amount of thicker fibrin fibres observed in the modified L-PRF group.

Conclusions

Preparation methods affect the fibrin fibre diameter of L-PRF. This may have consequences that influence the biological properties of the biomaterial.

INTRODUCTION

The use of leukocyte and platelet-rich fibrin (L-PRF) in regenerative medicine has increased exponentially in the last decade.¹ First introduced by Choukroun et al. in 2001,² this autologous, blood derived platelet concentrate that has been extensively used in oral surgical procedures, with clinical success in the fields of

ACRONYMS

A-PR: Advanced PRF.

L-PRF: Leukocyte and platelet-rich fibrin

SEM: Scanning electron microscopy

surgical implantology and regenerative periodontology.^{1,2} Because of its popularity, various centres have introduced the biomaterial as a routine inclusion in oral grafting procedures.¹ Although Choukroun had specified the original method of L-PRF preparation, this protocol had been modified over time, with several variants of L-PRF now being made available.^{3,4} The modification of the original protocol included updating the equipment and materials used, as well as varying the centrifuge time and force.⁴ This resulted in two further variants of L-PRF being introduced i.e. advanced PRF (A-PRF) and advanced PRF+ (A-PRF+).⁴ Both of these variants use a lower centrifugal force when compared to the original protocol and this is thought to have distinct advantages over the original preparation method.^{3,4}

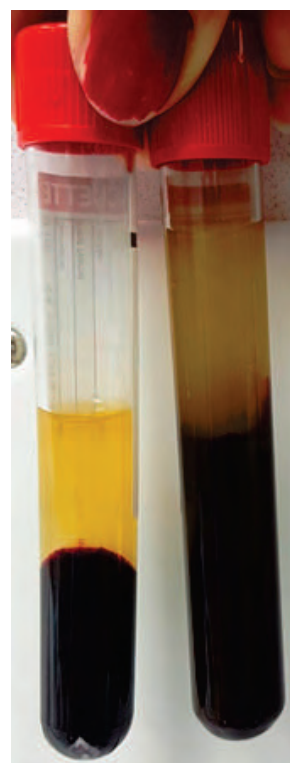


Figure 1: Modified L-PRF (left) and A-PRF (right)

1. **Mogammad Thabit Peck** BChD, MSc, MChD, MRD RCSEd, FDS RCSEd, MDTFEd Department of Oral Medicine and Periodontology, University of the Western Cape (mpeck@uwc.ac.za)
2. **Donavon Hiss** MSc, PhD (dhiss@uwc.ac.za) Department of Medical Biosciences, University of the Western Cape
3. **Lawrence Stephen** BChD, PhD (lstephen@uwc.ac.za) Department of Oral Medicine and Periodontology, University of the Western Cape

Corresponding author

Mogammad Thabit Peck Department of Oral Medicine & Periodontology, Faculty of Dentistry, University of the Western Cape, P/Bag X1 TYGERBERG 7505 South Africa Ph 021 9373186 Fax 0866187560 Email mpeck@uwc.ac.za

An important component of the biological advantages of L-PRF concentrates, is its specific structure which comprises a dense fibrin network, with high concentrations of platelets and leukocytes interspersed within the mesh of fibrin fibres.⁶ It is thought that this allows growth factors to be trapped, and released over a prolonged period thereby enhancing healing and regeneration.^{6,7} Recently, the role of fibrin structure has been researched for its role in blood clots.⁸⁻¹¹ Evidence suggests that the physical properties of fibrin fibres play an essential role in homeostasis of blood clots by affecting their mechanical and biological contribution properties.^{8,12} However, only a limited number of studies have investigated the features of fibrin fibre diameter of L-PRF concentrates.^{13,14}

With the increase in popularity of L-PRF, several authors have reported positive clinical results using L-PRF that is prepared using non-standardised equipment or materials.¹⁵⁻¹⁷ An example of this type of “modified” L-PRF has been clinically documented in previous publications by Peck et al.^{18, 19} This deviation from the proposed protocol may result in structural and functional changes to the consequences to the biomaterial that is being produced.²⁰⁻²² In this report, using scanning electron microscopy (SEM), we analyse and compare the fibrin network and fibrin fibre diameter of two different methods of L-PRF preparation i.e. our modified protocol for the preparation of L-PRF as previously described by Peck et al, and A-PRF+, prepared via the recommended protocol as previously outlined in the literature.^{4, 18, 19}



Figure 2: PRF Box with prepared L-PRF before compression

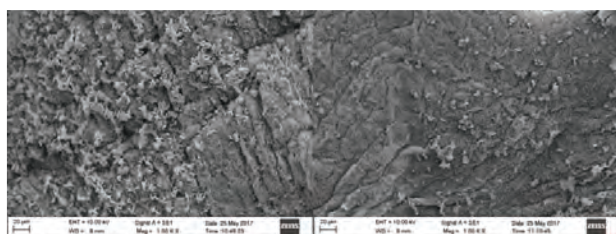


Figure 3: Modified L-PRF and A-PRF+ (Low magnification)

MATERIALS AND METHODS

The study was conducted at the Dental Faculty, University of the Western Cape, Cape Town, South Africa in 2017. Ethical clearance was obtained from the Research Ethics Committee of the university with the following reference number: BM 16/3/31

Preparation of the L-PRF samples

Thirty six milliliters of blood was obtained from a single 30-year-old healthy male volunteer via venipuncture of the left antecubital vein. Two methods were used to prepare L-PRF, i.e. :

1. Modified L-PRF: Blood samples were collected in 9ml blood collecting tubes that contain clot activator i.e., Vacuette® 9ml serum tubes with Z Serum Clot Activator (Greiner BioOne International AG, Germany). The blood samples were then centrifuged at 400g for 12 minutes in a standard benchtop centrifuge (PLC-03, Hicare International, Taiwan).
2. A-PRF+: Blood samples were collected in 10 ml A-PRF+™ tubes (Process for PRF, Nice, France), and centrifuged for 1300RPM for 8 minutes, using a dedicated tabletop centrifuge (PRF DUO™, Process For PRF, Nice, France).

After being centrifuged, the blood from both groups separated into 3 distinct layers (Fig 1). From previous reports, the layers could be distinguished as a topmost layer consisting of acellular platelet

poor plasma, Modified L-PRF/A-PRF+ in the middle, and red blood cells at the bottom of the test tube.^{5, 6} In order to standardise L-PRF thickness, the resultant L-PRF clots from both groups were removed from the tubes, and subjected to controlled compression, using a specifically designed tool i.e., the PRF Box® (Process for PRF, Nice, France) (Fig 2).²³ All the samples were then transferred for preparation for scanning electron microscopy analysis.

Preparation for scanning electron microscopy (SEM)

All the samples were fixed using 2.5% glutaraldehyde in phosphate buffered saline (PBS) for 1 hour. Each specimen was removed and washed in PBS for 5 minutes (twice) and thereafter distilled water for 5 minutes (twice). The specimens were then dehydrated serially with 50%, 70%, 90% and 100% ethanol, with each dehydration step taking 10 minutes. The samples were then transferred to the Electron Microscope Unit, Department of Physics, University of the Western Cape, for critical point drying, gold-palladium coating, and mounting for SEM analysis.

Scanning electron microscope observation

The surface microstructure of each specimen was analysed using the same scanning electron microscope (AURIGA Field Emission High resolution SEM, Carl Zeiss Microscopy GmbH, Jena, Germany). All measurements of the fibrin networks were carried out at 10 000 times magnification. At least 200 fibres were randomly selected from each group and measured for

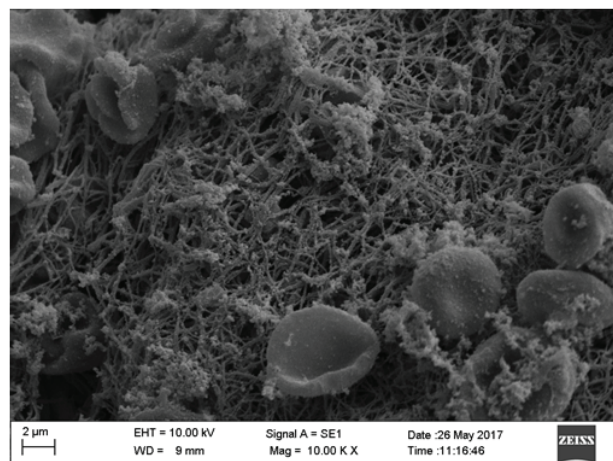


Figure 4: Modified L-PRF (High magnification)

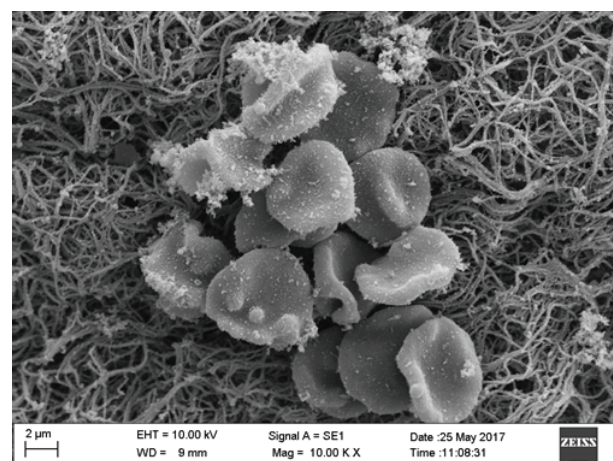


Figure 5: A-PRF + (High magnification)

analysis using ImageJ version 1.51o software developed by Wayne Rasband (National Institutes of Health, USA). The data was captured using Microsoft Excel 2010 (Microsoft Corporation, Washington, USA) and statistically analysed using the Mann-Whitney U and Wilcoxon W tests.

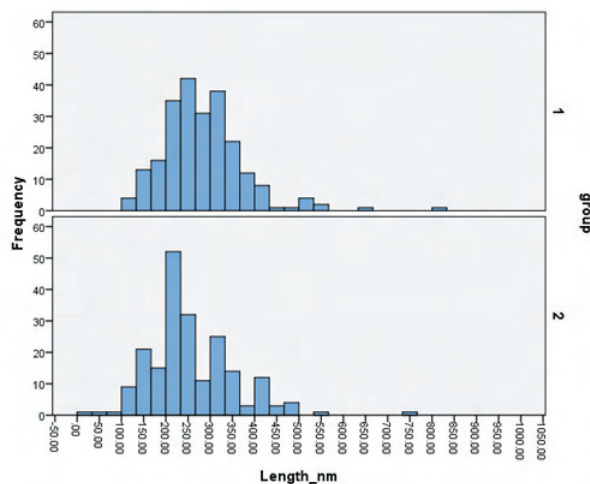


Figure 6: The distribution of fibrin fibre diameters for Modified L-PRF (group 1) and A-PRF+ (group 2) measured as length in nm

RESULTS

Descriptive analysis of Modified L-PRF and A-PRF+ Fibrin fibre networks

Under low magnification (1000 times) both samples show similar morphological characteristics. A dense mesh of fibres was evident with scattered cells seen on the surface for each sample (Fig 3). For the Modified L-PRF sample, more irregular bodies were present, possibly signifying the presence of platelets (Fig 3).²⁴ This may be characteristic of the region observed and may not be representative of the sample as a whole.⁶ No distinction in fibre architecture for the 2 groups was seen at this level of magnification. When observing the samples at higher magnification (10 000 times), the fibre mesh showed distinct characteristics (Figs 4 and 5). For both groups, the fibres were densely arranged in a netlike structure with clear spacing and crosslinking seen. Distinct differences could be noted with regards to fibre diameter, with both groups showing non-uniform fibre thickness. Thicker fibres were interweaved with thinner fibres and many of the fibres showed irregular surface characteristics indicative of platelet-fibrin fibre interaction.²⁴ The presence of cellular bodies could be noted for both groups. Distinguishing between both groups solely on the basis of SEM analysis, was difficult.

Table 1 a,b: Results of testing the difference in fiberfibre diameter (Length in nm) between both groups with the Mann Whitney test:

	Group	N	Mean Rank	Sum of Ranks
Length_nm	M-PRF	231	238.06	54992.00
	A-PRF+	206	238.06	40711.00
	Total	437		

Table 2

Test Statistics^a

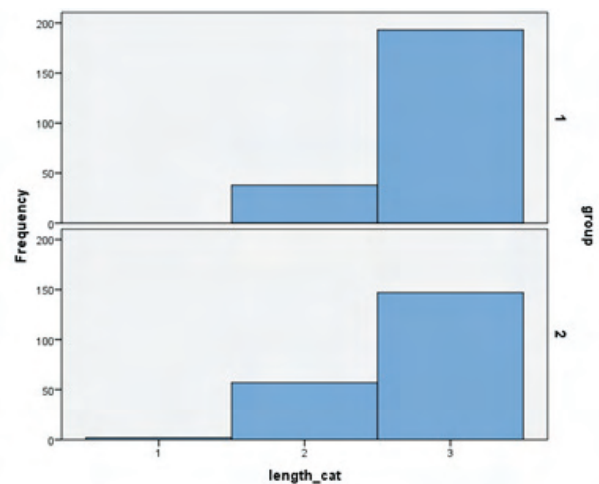
	Length_nm
Mann-Whitney U	19390.000
Wilcoxon W	40711.000
Z	-3.343
Asymp. Sig. (2-tailed)	.001

a. Grouping Variable: Group

Analysis of Modified L-PRF and A-PRF+ fibrin fibre thickness

A total of 437 fibrin fibre samples were analysed from both groups. The fibres were measured in nanometres (nm) with a range of 100-808nm and a mean of 281nm being observed for the Modified L-PRF group. For the A-PRF+ group, fibre diameters ranged from 43-742nm with a mean diameter of 256nm. For both the groups, statistical analysis revealed that the mean fibre diameter was not a

clear reflection of the various fibre groups present. It also showed that the fibrin fibre diameters were not equally distributed in each of the groups and that a wide range of fibre diameters were present (Fig 6). These fibre groups were present in both Modified L-PRF and A-PRF+, but were significantly different in their distribution for the two versions of L-PRF. Of the two groups tested, Modified L-PRF was characterized by larger diameter fibres and had a higher amount of thicker fibres when compared to A-PRF+ (Fig 6). Because of the wide range of fibre diameters noted in each of the sample groups tested, arbitrary size classes were selected to better illustrate the range of fibre diameters seen. This size selection was based on a previously published model which analysed human fibrin networks.²⁵ Consequently the fibre diameters were categorised into 3 groups based on "thickness" i.e. thin (lowest thickness to 85nm), intermediate (86 - 202nm), and thick (203nm to highest). This is represented by histogram A.



Histogram A: The distribution of "thin, intermediate and thick" fiberfibre of Modified L-PRF (group1) and A-PRF+ (group 2)

DISCUSSION

L-PRF in its various forms has shown promising results in both in-vitro and in-vivo studies.^{1, 15-19} This is attributed to the structural integrity of the biomaterial which results in a matrix that contributes to the prolonged release of various growth factors as well as provides a network for the migration of cellular components.²⁻⁶ The process of preparing L-PRF was initially documented by Choukroun in 2001.² However since the original protocol was introduced, several authors have attempted to replicate the platelet concentrate using non-standardized methods.¹⁵⁻¹⁹ Although clinically successful, the ultrastructure of these L-PRF variants has not been reported upon extensively.

The data from this study indicates that using both the A-PRF+ protocol as well as the modified protocol for the preparation of L-PRF that we used, may yield L-PRF clots that appear similar macroscopically. Both preparation methods resulted in separation of the centrifuged blood into 3 three distinct layers as previously described. When examining L-PRF clot size, the resultant L-PRF clots appeared smaller for the Modified L-PRF group, however this was not quantified statistically. We speculate this may be due to using a 9ml blood collecting tube as opposed to 10ml in the A-PRF+ group. However, the size of the collecting tube may not be the only factor affecting L-PRF size, since recent research indicates that using different centrifuges may affect L-PRF clot size.²⁰⁻²²

Compressed L-PRF clots from both groups appeared similar under low magnification (x1000), with a dense fibre network and large range of fibre diameters being seen. The density of the fibre networks seen in this study has previously been reported upon and is thought to be related to the compression of the clots in the PRF-Box™.²³ When the fibre diameters were analysed as thin, intermediate, and thick, the majority of fibres in both groups were classified as thick. This differs from the findings of Vieira et al.

2009,²⁵ who reported that the predominant fibre patterns were thin.

Previous studies that have examined the features of fibrin associated with L-PRF, have often used the mean diameter of the fibres to describe the characteristics of the L-PRF clot or are limited to a subjective description of the fibre diameter.^{13, 20} In the present study, statistical analysis revealed that using the mean diameter, did not reflect the range and frequency of the fibre types observed. Therefore when analyzing fibre diameters for Modified L-PRF and A-PRF+, we identified clear groupings (Fig 6). These were statistically significantly different for the two L-PRF groups, with the Modified L-PRF showing a higher number of larger diameter fibres as compared to A-PRF+ (Table 1). The exact reasons for this difference is not known, but is probably due to differences in the centrifugal force, centrifuge time, centrifuge make, agents present in the blood collecting tubes and shape of the blood collecting tubes.^{20, 26} Because all the samples were taken from the same individual in a 15 minute period, the above may serve as an adequate explanation for the differences in fibre diameters seen. However one must consider that for different individuals or in instances where blood is collected from the same individual at different times, that other factors such as pH, the presence of zinc, ionic strength, concentrations of calcium, polyphosphate, fibrinogen, fibrinogen binding proteins and thrombin, may also influence fibre thickness and network density.^{9,12}

The tubes used to collect blood to prepare Modified L-PRF, are coated with silica. Silica is a known procoagulant. It binds to plasma proteins and has the ability to cause reversible structural changes to these molecules.^{27,28} This is dependent on the size of the silica particle, with smaller silica particles demonstrating the ability to shorten coagulation time and increase the activation of factor X, whilst at the same time activate platelets.²⁸ The fact that silica coated tubes were used in the preparation of the Modified L-PRF, may further contribute to the morphological differences seen between the 2 groups.

The individual fibre diameters seen in the samples tested may have implications for the mechanical stability of each L-PRF clot.²⁹ Clots with thicker fibres tend to be less elastic than those with thinner fibres and may be more readily degraded by the fibrinolytic system.^{8, 29} This may have consequences for the biological behaviour of L-PRF since structural integrity and the controlled release of growth factors is thought to be a major contributing factor to its clinical success. Therefore, one might assume that because A-PRF+ has a lower amount of thick fibres than Modified L-PRF, that its rate of dissolution may be slower than that of the Modified L-PRF. As such, its ability to remain intact may be prolonged as compared with other platelet concentrates and may explain the extended release of growth factors as recently reported.^{3,4}

CONCLUSIONS

To the author's knowledge, this is the first presentation of the morphological fibre characteristics of an L-PRF clot prepared using a specific modified protocol as previously described by the authors. When compared to an established protocol, the resultant L-PRF clot appears morphologically similar to its A-PRF+ counterpart with a dense fibrin network interspersed with platelets and other blood cells. However, clear differences are noted for the fibrin fibre diameter with Modified L-PRF showing a higher proportion of larger diameter fibres. The reason for these differences is speculated to be associated with the different protocols used to prepare the platelet concentrates. One might therefore assume that changes in the protocol of preparation of L-PRF may directly affect its morphological structure. Whether these differences affect the clinical efficacy of these biomaterials is unclear, and warrants further research.

Disclosure policy

The authors declare no conflict of interest regarding the publication of this paper. This paper forms part of the requirements of the partial fulfilment towards the degree PhD.

This paper forms part of the requirements of the partial fulfilment towards the degree PhD.

References

1. Miron RJ, Zucchelli G, Pikos MA, Salama M, Lee S, Guillemette V, Fujioka-Kobayashi M, Bishara M, Zhang Y, Wang HL, Chandad F, Nacopoulos C, Simonpieri A, Aalam AA, Felice P, Sammartino G, Ghanaati S, Hernandez MA, Choukroun J. Use of platelet-rich fibrin in regenerative dentistry: a systematic review. *Clin Oral Investig*. 2017 Jul; 21(6):1913-1927.
2. Choukroun J, Adda F, Schoeffler C, Vervelle A. Une opportunité en parodontologie: Le PRF. *Implantodontie* 2001; 42; 55-62.
3. Kobayashi E, Flückiger L, Fujioka-Kobayashi M, Sawada K, Sculean A, Schaller B, Miron RJ. Comparative release of growth factors from PRP, PRF, and advanced-PRF. *Clin Oral Investig*. 2016; 20; 2353-2360.
4. Fujioka-Kobayashi M, Miron RJ, Hernandez M, Kandalam U, Zhang Y, Choukroun J. Optimized Platelet-Rich Fibrin With the Low/low-Speed speed Concept: Growth Factor Release, Biocompatibility, and Cellular Response. *J Periodontol*. 2017; 88; 112-121.
5. Dohan DM, Choukroun J, Diss A, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part I: technological concepts and evolution. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* 2006; 101 (3); 37-44.
6. 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(4):546-55.
7. Choukroun J, Diss A, Simonpieri A, et al. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part IV: Clinical effects on tissue healing. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* 2006; 101 (3); 56-60.
8. Weisel JW. Structure of fibrin: impact on clot stability. *J Thromb Haemost*. 2007; 5 Suppl 1:116-24.
9. Wolberg AS. Plasma and cellular contributions to fibrin network formation, structure and stability. *Haemophilia*. 2010; 16 Suppl 3:7-12.
10. Pretorius E, Ekpo OE, Smit E. Comparative ultrastructural analyses of platelets and fibrin networks using the murine model of asthma. *Exp Toxicol Pathol*. 2007; Oct; 59(2):105-14.
11. Pretorius E, Briedenhann S, Marx J, Smit E, Van Der Merwe C, Pieters M, Franz C. Ultrastructural comparison of the morphology of three different platelet and fibrin fibre preparations. *Anat Rec (Hoboken)*. 2007; 290(2):188-98.
12. Henderson SJ, Xia J, Wu H, Stafford AR, Leslie BA, Fredenburgh JC, Weitz DA, Weitz JI. Zinc promotes clot stability by accelerating clot formation and modifying fibrin structure. *Thromb Haemost*. 2016; 115(3):533-42.
13. Bai MY, Wang CW, Wang JY, Lin MF, Chan WP. Three-dimensional structure and cytokine distribution of platelet-rich fibrin. *Clinics (Sao Paulo)*. 2017; Feb 1; 72(2):116-124.
14. Sam G, Vadakkettikal RJ, Amol NV. In vitro evaluation of mechanical properties of platelet-rich fibrin membrane and scanning electron microscopic examination of its surface characteristics. *Journal of the Indian Society of Periodontology*. 2015; 19(1); 32-6.
15. Anilkumar K, Geetha A, Umasudhakar Ramakrishnan T, Vijayalakshmi R, Pameela E. Platelet-rich-fibrin: A novel root coverage approach. *Journal of Indian Society of Periodontology* 2009 Jan; 13(1); 50-4.
16. Agarwal K, Chandra C, Agarwal K, Kumar N. Lateral sliding bridge flap technique along with platelet rich fibrin and guided tissue regeneration for root coverage. *Journal of Indian Society of Periodontology* 2013 Nov; 17(6); 801-5.
17. Kulkarni MR, Thomas BS, Varghese JM, Bhat GS. Platelet-rich fibrin as an adjunct to palatal wound healing after harvesting a free gingival graft: A case series. *Journal of Indian Society of Periodontology* 2014 May; 18(3); 399-402.
18. Peck MT, Marnewick J, Stephen L. Alveolar ridge preservation using leukocyte and platelet-rich fibrin: a report of a case. *Case Reports in Dentistry* 2011; 2011; 345048. doi: 10.1155/2011/345048. Epub 2011 Jul 3.
19. Peck MT, Marnewick J, Stephen LX, Singh A, Patel N, Majeed, A. The use of leukocyte- and platelet-rich fibrin (L-PRF) to facilitate implant placement in bone-deficient sites: a report of two cases. *South African Dental Journal* 2012 Mar; 67(2); 54-6, 58-9.
20. Dohan Ehrenfest DM, Pinto NR, Pereda A, Jiménez P, Corso MD, Kang BS, Nally M, Lanata N, Wang HL, Quirynen M. The impact of the centrifuge characteristics and centrifugation protocols on the cells, growth factors, and fibrin architecture of a leukocyte- and platelet-rich fibrin (L-PRF) clot and membrane. *Platelets*. 2017; 24:1-14.
21. Dohan Ehrenfest DM, Kang B, Del Corso M, Nally M, Quirynen M, Wang H, Pinto NR. The impact of the centrifuge characteristics and centrifugation protocols on the cells, growth factors and fibrin architecture of a Leukocyte- and Platelet-Rich Fibrin (L-PRF) clot and membrane. Part 1: evaluation of the vibration shocks of four models of table centrifuges for L-PRF. *POSEIDO* 2014 June; 2(2); 129-39.
22. Pinto NR, Pereda A, Jiménez P, Del Corso M, Kang B, Wang H,

- Quirynen M., Dohan Ehrenfest DM. The impact of the centrifuge characteristics and centrifugation protocols on the cells, growth factors and fibrin architecture of a Leukocyte- and Platelet-Rich Fibrin (L-PRF) clot and membrane. Part 2: macroscopic, photonic microscopy and Scanning Electron Microscopy analysis of four kinds of L-PRF clots and membranes. *POSEIDO* 2014 June; 2 (2); 141-54.
23. Dohan Ehrenfest DM. How to optimize the preparation of leukocyte- and platelet-rich fibrin (L-PRF, Choukroun's technique) clots and membranes: introducing the PRF Box. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2010; 110(3):275-8; author reply
24. Hantgan RR, Taylor RG, Lewis JC. Platelets interact with fibrin only after activation. *Blood.* 1985 Jun; 65(6):1299-311.
25. Vieira WA, Pretorius E, Oberholzer N, Auer REJ. Comparative scanning electron microscopy of platelets and fibrin networks of human and different animals. *Int J Morphol.* 2009; 27(1): 69-76.
26. Bonazza V, Borsani E, Buffoli B, Castrezzati S, Rezzani R, Rodella LF. How the different materials and shape of the blood collection tube influences the Concentrated Growth Factors production. *Microsc Res Tech.* 2016 ;79(12):1173-1178.
27. Kondo A, Oku S, Higashitani K. Structural Changes changes in protein molecules adsorbed on ultrafine silica particles. *J of Colloid and Interface Science.* 1991; 143 (1): 214-221.
28. Gryshchuk V, Galagan N. Silica nanoparticles :effects on blood coagulation proteins and platelets. *Biochem Res Int.* 2016 ; Article number 295941:1-6.
29. Li W, Sigley J, Pieters M, Helms CC, Nagaswami C, Weisel JW, Guthold M. Fibrin fibre stiffness is strongly affected by fibre diameter but not by fibrinogen glycation. *Biophys J.* 2016; 110(6):1400-10.

register online @ <https://sadacongress.co.za/register.html>

DENTAL & ORAL HEALTH
CONGRESS AND EXHIBITION

12-14 OCTOBER 2018 | Sun Time Square Arena and Conference Centre, Pretoria | SADA

BOOK YOUR SEAT FOR THIS EVENT

You are invited to join an **excellent line up of International and Local speakers** sharing **innovative technology and services** from within a melting pot of the **best in oral health care** that the world has to offer