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FACULDADE DE MEDICINA DENTÁRIA
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PLATELET-RICH FIBRIN MEMBRANES RESISTANCE TO TRACTION COMPARING A-PRF *VERSUS* A-PRF+

Dissertação apresentada à Universidade Católica Portuguesa para obtenção do grau de mestre em Medicina Dentária

Por:

Mara Simões Pedro

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Orientador: Professor Doutor Gustavo V.O. Fernandes

Coorientador: Mestre Nuno Bernardo Malta dos Santos

Viseu, 2021

“Se me perguntarem o que vim fazer a este mundo, eu, um artista, responderei: estou aqui para viver em voz alta”

Émile Zola

À minha mãe por me ensinar que o saber não limita o tempo, pelo contrário há de munir-me de como quero vivê-lo e vê-lo refletido na verdade de quem sou. Ao meu pai que sempre insistiu comigo para não poupar esforços à satisfação pessoal, por mais trabalhoso que seja, é o bem mais sólido e próximo da felicidade.

A todos os céticos que assumem sonhos como uma constante da vida, dedico-lhes também o meu trabalho e a opinião que absorvi ao realizá-lo. Sonhar é uma função exponencial ilimitada e cada um tem a capacidade intrínseca de definir as suas variáveis.

Agradecimentos

Aos meus pais e avós, por serem um pilar emocional essencial para completar esta jornada com a mesma determinação do primeiro ao último ano.

Aos meus orientadores Professor Doutor Gustavo Fernandes e Doutor Nuno Malta por assumirem um acompanhamento exigente deste projeto com total abertura, disponibilidade e boa disposição. À enorme amizade, acrescento o meu respeito e admiração pela conduta que mantiveram durante este período desafiante.

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Ao Laboratório de Química Orgânica da Universidade de Aveiro, em especial ao Professor Doutor Leandro Lourenço, pela ajuda inalcançável no esclarecimento de dúvidas e acesso a todos os meios, por si disponibilizados para a realização dos protocolos clínicos. Também à sua equipa de investigadores, por me fazerem sentir parte de uma realidade desconhecida, mas que se tornou uma experiência única e fascinante.

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Resumo

Objetivos: O presente estudo teve como objetivo comparar as propriedades mecânicas de resistência à tração e estrutura entre as membranas produzidas por A-PRF *(Advanced- Platelet Rich Fibrin)* e A-PRF+ *(Advanced-Platelet Rich Fibrin+).*

Materiais e métodos: Recorrendo à colheita de sangue de um dador saudável sem história de uso de anticoagulantes ou imunossupressores, realizou-se a preparação das membranas seguindo as indicações protocolares definidas na literatura para A-PRF e A-PRF+. De um N=16/grupo, 13 membranas de A-PRF e 12 de A-PRF+ foram submetidas ao teste de tração, para a obtenção de valores referentes à tração máxima e tração média. Os dados obtidos foram analisados estatisticamente com o teste t não pareado. Após avaliação desta variável, as membranas foram observadas em MEV (Microscopia Eletrónica de Varredura).

Resultados: Foram obtidos em relação à tração máxima, 0.0020 $N.mm^{-2}$ para o A-PRF e 0.0022 $N.mm^{-2}$ para A-PRF+. Relativamente à tração média, A-PRF obteve 0.0012 $N.mm^{-2}$, enquanto o A-PRF+ obteve 0.0015 $N.mm^{-2}$ ($p<0,01$). Nas observações de superfície com MEV, A-PRF+ mostrou ser o concentrado plaquetário mais poroso, com maior abundância de fibras e preservação celular.
Conclusão: Este estudo permitiu concluir que o protocolo A-PRF+ foi capaz de produzir membranas com valores de tração máxima superiores aos obtidos pelo A-PRF, sendo os resultados indicativo de que o protocolo apresentou membranas com maior resistência e capacidade elástica ao serem tracionadas por duas forças opostas. A este fenómeno acrescenta-se a arquitetura demonstrada na matriz de A-PRF+ e as propriedades biológicas otimizadas descritas na literatura.

Palavras-chave: “Fibrina-Rica em Plaquetas”, “Viscoelástica”, “Teste de tensão”, “Porosidade”, “Rotura”.

Abstract

Purpose: This study aimed to formulate a comparison of the mechanical properties of tensile strength and structural organization between membranes produced by A-PRF (Advanced Platelet-Rich Fibrin) and A-PRF+ (Advanced Platelet-Rich Fibrin+).

Materials and Methods: Blood was collected from a healthy donor with no history of anticoagulant or immunosuppressant use, the membranes were prepared following the protocol indications defined in the literature for A-PRF and A-PRF+. From an N=16 for each protocol, 13 membranes of A-PRF and 12 of A-PRF+ were submitted to the traction test, evaluating maximum and average traction. Data was statistically analyzed using the unpaired t test. Membranes were then carefully observed in SEM (Scanning Electron Microscopy).

Results: For maximum traction were obtained 0.0020 for A-PRF and 0.0022 for A-PRF+. Regarding the average traction, A-PRF scored 0.0012 while A-PRF+ obtained 0.0015 ($p=0.01$ unpaired t-test). Surface morphology observations with SEM, A-PRF+ showed to be the most porous platelet concentrate, with greatest fiber abundance and cell preservation.

Conclusion: This study allowed to conclude that A-PRF+ protocol was able to produce membranes with higher maximum traction results than those found for A-PRF, indicating that the protocol with low centrifugation time, presented membranes with better viscoelastic strength when they are stretched by two opposed forces. To this phenomenon is added the architecture demonstrated in the A-PRF+ matrix and the optimized biological properties described in literature. A-PRF+, by the view of the developed findings in this work, a better option compared to A-PRF.

Keywords: “Platelet-Rich Fibrin”, “Viscoelastic”, “Tensile Strength”, “Rupture”, “Porosity”, “Low-Speed Centrifugation Concept”.

.

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Abbreviations

PRF (Platelet-rich Fibrin)

RCF (Rotational Centrifugal Force)

CF (Centrifugal Force)

LSCC (Low-speed Centrifugation Concept)

GFs (Growth Factors)

PDGF (Platelet-derived growth factor)

VEGF (Vascular Endothelial growth factor)

EGF (Epidermal growth factor)

TGF-β1 (Transforming growth factor beta-1)

IGF (Insulin-like growth factor)

HGF (Hepatocyte growth factor)

bFGF (basic fibroblast growth factor)

PSGL-1 (P-selectin glycoprotein ligand-1)

GPIIb/IIIa (Glycoprotein IIb/IIIa)

MLCK (Myosin light-chain kinase)

ROCK (Rho kinase)

Introduction

1.1 Background

Platelet concentrates have been widely utilized in dentistry, maxillofacial, and many other fields of medicine owing to their rapid angiogenic stimulation ability and a vast potential for tissue regeneration.(1) Platelet-rich plasma (PRP) is a first-generation platelet concentrate, introduced by Marx et al. in 1998, but one of the main reported disadvantages of this method is the use of anti-coagulants or animal-derived thrombin for its activation, which interferes with host response and natural healing process.(2,3)

Seeking to minimize contamination risk, Platelet-rich Fibrin (PRF) was proposed as a second-generation platelet concentrate, first described by Choukroun et al. in 2000, named as Leukocyte platelet-rich fibrin (2700 rpm for 12 minutes, L-PRF). (4) Briefly, its production protocol comprises the collection of autologous patient's blood and immediate centrifugation to trigger platelets activation and fibrin polymerization. Centrifugation forms a complex three-dimensional fibrin-rich scaffold, where the main broad cellular spectrum components are centered, such as platelets; growth factors (VEGF; PDGF; TGF-β1; EGF; IGF-I; HGF - Table 1); leukocytes; cytokines, among others, which are known to play a fundamental role in the interaction with the regeneration and wound healing. (5,6)

PRF is viewed by some authors as a matrix that supports angiogenesis, proliferation, cell differentiation and chemotaxis of inflammatory cells within the fibrin, acting as a biomimetically reservoir for cells and cell's signaling. (7)

Therefore it is appliable as a resource in multiple dental and medical procedures such as periodontal regeneration (root coverage, gingival recession); alveolar ridge preservation; sinus lift; ulcers/skin necrosis; chronic wounds; plastic and reconstructive surgery, musculoskeletal lesions and tendon injuries. (5,6,8,9)

1.2 Advanced Platelet-rich Fibrin (A-PRF)

Over the past few years, modifications in the original PRF protocol, updating centrifuge time and force, have led to the emergence of advanced platelet-rich fibrin (A-PRF), develop by Choukroun, that introduces a decrease of the rotation speed while centrifugation time is increased.(10)

This achievement demonstrates improvement of mechanical and biological properties regarding L-PRF, and also better outcomes. The use of low G-forces for A-PRF has revealed in immunohistochemical studies a different pattern of cell distribution. A greater number of platelets and leukocytes are widely dispersed throughout the clot and this finding also correlates with the interesting increase of granulocytic neutrophils populations in the distal part of the collected supernatant, not detected following high centrifugation forces. (11,12)

Based on the current published scientific papers, the reticular and porous microstructure of A-PRF explains its elasticity. In turn, these inherent factors enable the idea of encapsulation capacity for more cellular components in the interfibrillar spaces of the membrane, which has already been proven.(13) Thereby, favors the release of GFs and influences directly tissue regeneration, comparing to PRP and PRF. (14) It is also reported increased migration and proliferation of fibroblasts and mRNA collagen levels. (15)

1.3 Advanced Platelet-Rich Fibrin+ (A-PRF+)

Recently research has studied the additional effect on rotational centrifugation force (RCF) and rotation times applied as key elements regarding the structure and composition of PRF-based matrices. Thus, a new variant known as Advanced platelet-rich fibrin + (A-PRF+) was suggested by Fuijoka-Kobayashi et al. by reducing the rotation time although maintaining the same force used in A-PRF variant. This is thought to have distinct advantages, despite the slight decrease in time, impacted positively the preservation of cells in the formed clot and, consequently, a further improvement of the characteristics of A-PRF+ compared to those mentioned above from A-PRF. (11)

The highest percentage of cells that can be collected has been already proven, as well as the higher uniformity of platelet distribution and porosity than what is described in the A-PRF. This happens in a way that it possible establishes a balance between trapped cells and chemotaxis, migration, proliferation, and degradability, closely linked to sustained release of growth factors. (16)

Authors validated the hypothesis that changes in the mechanical properties of A-PRF and A-PRF+, may indeed induce differences in the release of certain groups of growth factors. For example, significantly higher values of PDGF and TGF-β1 were released within the scaffold on day 7 and 10 of a limited period measurement studies; for EGF the maximum release range in both membranes happened only at early time point of 24h, being more significantly marked in A-PRF+; and finally, VEGF appears to be one of the most accumulated essential growth factor in A-PRF+, possible explained by the affinity of the VEGF with the amounts of fibrin and fibrinogen in the organized mesh. (7,13,16)

Table 1 - Essential Platelet Growth Factors Properties.

Growth Factors	Biological function
PDGF	• Stimulates mitogenic activity and promotes proliferation in mesenchymal cell lines. • Chemotaxis of neutrophils, macrophages, fibroblasts and other leukocytes, but the main target is osteoblasts, being useful to induce bone regeneration.(17) • Collagen synthesis and tissue remodeling by up-regulating pathways to break down the old collagen. (18)
VEGF	• Allows the formation and development of new blood vessels, through the migration and differentiation of endothelial cells, that restore tissue perfusion and microcirculation. The formed vessels will be responsible for blood supply to the wound site. • Induce bone tissue formation through the recruitment of macrophages. (19)

Table 1 - Essential Platelet Growth Factors Properties (Continuation)

EGF	• Even at low concentrations has a higher potential than bFGF to increase osteogenic mineralization in Dental Pulp Stem Cells (DPSCs) • Induces formation of the peri-implant junctional epithelium. (20) • Pro-inflammatory function that acts as a mechanosensitizer through the production of fibronectin. (21,22)
TGF-β1	• Low concentrations are sufficient to induce the proliferation of fibroblasts, osteoblasts and chondroblasts. • Involved in complex mediation roles such as chemotaxis, mitogenesis, differentiation, apoptosis, remodeling and immunoregulation. • Induces monocyte and neutrophil chemotaxis. (23) • Osteoinduction and progression of osteogenesis.(24)
IGF	• Proliferation and differentiation of various types of mesenchymal cells. Its intervention overlaps with that of TGF-beta. (25) • Synthesis of type I collagen and differentiation of osteoblasts, through an important mediator LARP6, verifying the mineralization capacity. (26)

Table 1 - Essential Platelet Growth Factors Properties (Continuation)

HGF	• Regulation of cell morphogenesis • Matrix deposition and degradation, playing an antifibrotic role that allows the reepithelization of the wound. • Under physiological conditions, interacts with the mesenchymal epithelium. (27)
bFGF	• Involvement and reinforcement in various proliferation processes of different types of cells, such as fibroblasts, mesenchymal stem cells and osteoprecursors cells. • Stimulate the expression of tissue metalloproteinase inhibitors (TIMP-1), partially decreasing high concentrations of type I collagen, thus hypothesizing its importance in the reorganization of collagen fibers, avoiding excessive deposits with unwanted effects. (28) • Treatment of periodontal disease, increasing the migration and proliferation of Periodontal Ligament Stem Cells (PDLSCs). (29)

1.4 Viscoelastic potential of Platelets in the fibrin mesh

Platelets are anucleate cytoplasmic particles, fragmented from megakaryocytes that are released into the blood with a size of 1-3 μm in diameter and a density that varies within the span of 1.04-1.08kg/l, increasing with age and related to size and metabolic activity. It is reported that its granule content is enhanced in low-density populations, because of the reported presence of higher amount of P-selectin, a glycoprotein stored in the α-granules of the platelets that is moved to the cell surface when a platelet is activated. Exposure to the extracellular medium allows P-selectin to bind to PSGL-1, which is in turn expressed on the surface of many hematopoietic cells such as neutrophils. (13,30)

In the human body, platelets are the first cells to present at the site of injury forming the primary hemostatic plug by contractile forces and interaction with fibrinogen, through the abundant number of receptors (GPIIb/IIIa) on a single platelet. (31) Both fibrinogen and fibrin are necessary to achieve hemostasis and improve clot strength. Lang T and his collaborates, revealed that even in thrombocytopenic patients, levels of fibrinogen and fibrin formation have improved the clot strength, making possible for platelets to aggregate. (32)

However, they are not the only mediators in the development of clot tension. Interactions in adhesion and aggregation by a phenomenon called isometric contraction (platelet-platelet association), creates orientation of platelets and fibrin strands simultaneously in the direction of force generation. After activation, platelet start to emit long pseudopods formed of microfilaments and microtubules that can extend to combine with other platelets and attach to strands of fibrin. Pseudopods then retract, compressing the fibrin fibers in a continuous process, even under conditions of maximal tension. Platelet-fibrin attachment and extension of platelets bodies dictate the sustaining of maximum tension, but for the purpose of stabilization of this cross-linking, Factor XIII is very critical to provide conditions to normal isometric contraction so that the fibrin doesn’t lose its integrity when subjected to tension by platelets. (33)

Understanding the dynamics that come from the strength and elasticity of a single platelet can help to evaluate the properties of the clot material after retraction and predict its stiffness. Previous reports support that the force in these

cells is caused by the non-muscular isoform of myosin IIA, which, when phosphorylated by MLCK or ROCK, forms bipolar filaments that then allow it to move along the actin filament. This tension created by sliding is transmitted to integrin receptors on platelets as a force. It is estimated that a myosin II molecule generates a maximum of 6pN of force. Therefore, large amount of these molecules in a platelet, predicts its capability of generating an average contractile force of 72nN. (34,35)

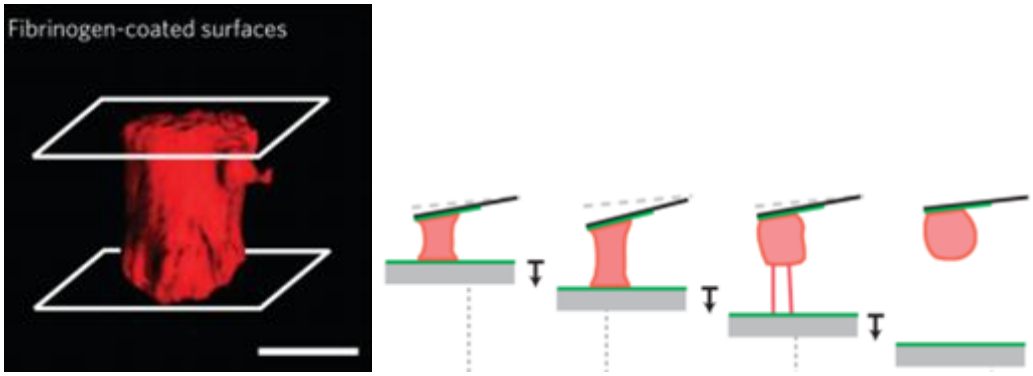


Figure 1 - Platelet elasticity and extensibility measurements. The elasticity and extensibility of individual platelets were assessed, as well as the adhesion strength across two surfaces or cantilever, coated with fibrinogen. To evaluate the reference parameters, the fibrinogen-coated surfaces were subsequently moved away for the platelet to experience attachment force and elongation until rupture. Rupture can happen either between the fibrinogen and the surface or between the platelet and the fibrinogen. (Adapted from Wilbor A. Lam et al.(35))

The high contractile forces also cause stiffening of the fibrin fibers leading to a homogeneous increase in the elasticity and resistance to tension in the general network (36) The integrity of the plug is therefore enhanced by these biomechanical steps of platelet adhesion, clot aggregation and retraction. Platelets have a relevant role in the use of their cytoskeleton filaments and the generation of force by the actin-myosin reaction, to stabilize and prevent the clot from being dislodged from the area of interest or undergoing fibrinolysis. (37)

1.5 Principles of centrifugation in cell's segregation

Centrifugation is a widely used technique, which is based on the principle that the centrifugal force (CF), a force that pulls all objects outward from the axis of rotation, is much higher than the gravity, because it moves each cell away from the center of rotation, being often greater than the cell's own weight in gravitational field. Once applied, increases the gravitational acceleration, producing the effective gravitational force (EGF) a vector sum of CF and gravitational force (GF), causing the separation of biological mixture within a liquid phase. In addition, drag forces also influence particles migration along the tube, depending on their size, shape, density and the rotor speed. (38)

Due to the aforementioned differences between cells, their settlement process creates different levels, with denser particles migrating further away from the axis of the centrifuge, while less dense migrate to the center. These differences are responsible for particles to segregate distinctly, being arranged in bands that can go from dispersed to completely separate layers. (39) Platelets, for example, can be separated from red blood cells and leukocytes because they are much smaller. (30)

Konidena et al. investigated the interactions of hydrodynamic forces on particles of different sizes in a cylindrical tube, trying to predict the migration when they are submitted to drag, gravitational, centrifugal, and torque forces. They found that when the cylinder was in low rotation, the action of gravity on the particles became more predominant, causing them to fall through the fluid and heavy particles settle prior to the light particles, although a few from the upper light layer are dragged down with the rotation of the wall. On the contrary, as the rotational velocity increases, the influence of gravity decreases. Heavy and light particles remain completely dispersed through the fluid, forming distinct phases due to the balanced forces acting on them. This study established then the relationship between gravity and CF, as being responsible for the formation of different phases. (39) Imbalance of forces caused by the dominance of gravity or centrifugal parameters is therefore the fundamental reason for interferences on segregation. (13) By centrifugation at various speeds and times, particles of different sizes experience what is called as differential centrifugation, forming the

pallet and the supernatant. Thereby, underlying awareness of this phenomenon is relevant to develop a methodology that better separates cells of interest from other components, assuring the quality and quantity of these cells in order to contribute to the success of the final clinical results.

1.6 Low-Speed Centrifugation Concept

Leukocytes and platelets seem to require a reduction of RCF in order to preserve their source of GFs, since recent evidence demonstrates that the consequences of high RCF not only decreases the number of cells, but also influences negatively the ability to release GFs. (38,40)

Platelets recover from centrifugation speed during resting time but mainly larger and metabolically active ones, with prothrombotic potential, are removed in high speed centrifugation, which is shown by analysis of light transmission aggregometry (LTA), possibly leading to a decrease in aggregation.(41) TGF-β is an exception that isn't influenced by g-forces. (42)

Blood coagulation is a rapid process that initiates even before the tubes are exposed to centrifugation, and plastic tubes contain siliceous substances that consequently favor this clotting activation, so the recommended time between the blood drawing and start of centrifugation is around 2 minutes per 5 tubes, otherwise the size of PRF membrane is affected, suffering significant reduction. (43) Thereby, TGF-β is simultaneously released when platelets are activated and bonds to the newly formed fibrin-rich extracellular matrix, being protected from centrifugal forces. (42)

The considered LSCC used in A-PRF and A-PRF+, creates pores with higher diameter in the fibrin network allowing the invasion of cells, together with some vessels, to the outer scaffold regions. Histological analyses also showed higher number of cells penetrated the peripheral edges of PRF-low scaffolds. (44)

1.7 Purpose of this study

Some clinical studies have already demonstrated results on the mechanical properties of L-PRF and A-PRF, but none of them tested in isolation the differences in tension resistance parameter, between A-PRF and A-PRF+ after fixed angle centrifugation (IntraSpin®, Intra-Lock U.S.A). (7,44-49) Our study can be seen as the first one to measure the reach of both membranes.

Mechanical interest has been increasing in response to the unbalanced gap in the need of matrices with consistency and functional integrity that allows proper handling, suturing without breaking and enhancement of biological space for continued release of cells and GFs, over the time. (13,49) Even though blood composition is specific and individual, PRF-based matrices are reproducible systems with distribution independent of the donor's characteristics, making it possible to perform clots analysis under appropriate conditions for this study. (30)

The main purpose of our study is to test the viscoelastic stress, defined as the tensile stress at rupture of the fibrin matrices, following two protocols (A-PRF and A-PRF +). We propose to reproduce each of the membranes, according to previous validated methodologies to determine the tensile strength, assess the surface morphology and establish a meaningful explanation relating the results, in order to obtain the membrane with better properties. It is expected that these results allow us to produce a predictable outcome which can conduct clinicians who use PRF membranes, to rationale choosing, presenting itself as a tool for further knowledge of what could possibly be the behavior of these biomaterials in the oral cavity.

Are defined as study hypothesis: (i) Hipothesis 0: A-PRF has significant and better resistance to traction; (ii) Hipotesis 1: A-PRF+ proves to be more resistant than A-PRF.

Materials and Methods

2.1 Blood collection and Membrane Preparation

The design of this study and its consent forms for all procedures performed followed the Helsinki Declaration of 1975 as revised in 2013, and the study started after approval by the Ethics Committee n˚52/2020.

Peripheral blood was collected with a butterfly needle, from a single healthy donor, male, 23 years old and with no history of anticoagulants or immunosuppressors use, avoiding bias. Trials were conducted over 4 days (D1 to D4), spaced one week apart from D1 to D2 and one month later we performed D3 and D4, for donor's own recovery and comfort.

For each protocol, A-PRF and A-PRF+, it was collected 8 tubes (9mL silica coated tubes, BD Vacutainer®, UK) at a time. As blood was being drawn, the tubes were placed in the centrifuge (Intra-Spin, IntraLock®, FL, USA) in pairs opposed to each other to ensure an adequate balance (Figure 1A). The total period from harvest to the beginning of the centrifugation did not exceed 2 minutes, attempting to preserve polymerization and size outcomes. (31) The tubes were immediately centrifuged according to Dohan Ehrenfest et al. instructions: 1500 rpm for 14 minutes to produce A-PRF. In terms of the preparation of A-PRF+ the only difference was the centrifugation time: 1500 rpm for 8 minutes. After completing this step, the lid was opened, exposing the tubes to room temperature, and leaving them to rest for 15 minutes, to improve the thickness of the filaments. (32)

Careful removal of the clots from the first two tubes that had been placed in the centrifuge was carried out, matching the use for test with the collection order. The fibrin portion was then separated, leaving a small portion of red blood cells, a remnant called the buffy coat, attached to the removed A-PRF (Figure 1C).

After eliminating red blood cells, A-PRF was compressed for 5 minutes in a thin film using a compression device the Xpression box (IntraLock®).

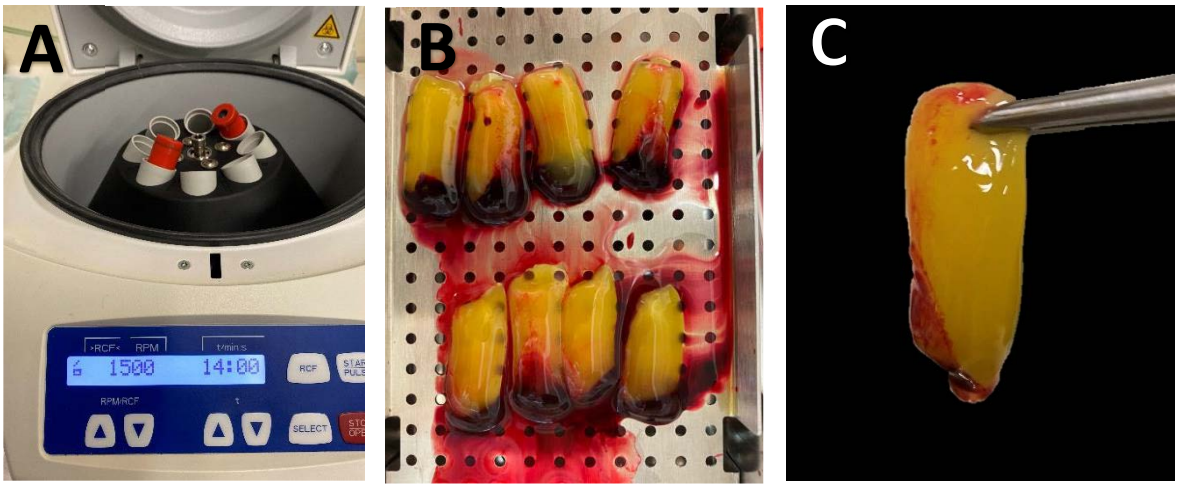


Figure 2- Visual representation of membrane production. A- Blood centrifugation; **B-** Visual demonstration of the clots placed in the Xpression box kit; **C-** Aspect of the obtained membrane after removal of red blood cells portion.

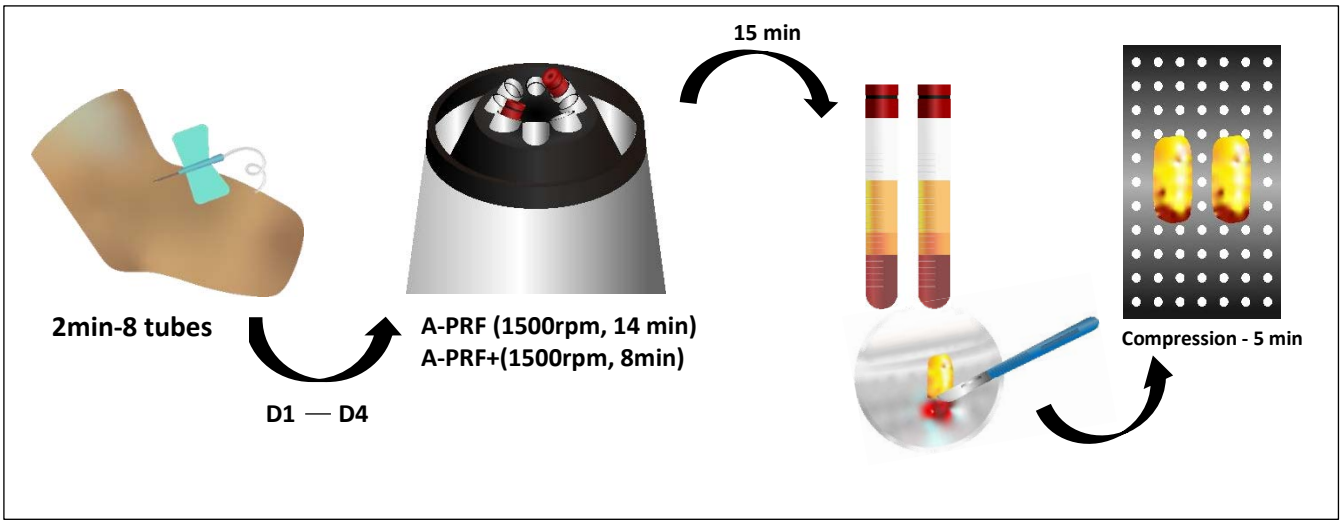


Figure 3- Illustrations of protocol steps regarding the preparation of test samples. This image reports the sequence repeated from D1 to D4, where is mentioned the respective times should be taken into consideration to accomplish ideal conditions.

2.2 Traction assay

A glass-mold was specially designed and manufactured to make the fibrin specimens identical in size, so all the membranes were cut following the dimensions of the mold (10x15mm), prior to traction test. (Figure 2A). The samples were gripped by clamps at each end such that the initial apparent gauge length (the distance between clamps) was set to 13 mm for all the samples tested. Traction test was performed with the Shimadzu MMT-101N equipment (Shimadzu Corporation; Japan) (Figure 2B) in collaboration of Centre for Mechanical Technology and Automation (TEMA) of the University of Aveiro. Maximum tensile strength and tensile strain at break, was measured in N=16 specimens for each protocol, applying divergent vertical forces. The maximum value for traction using in this equipment is set to 12 mm.

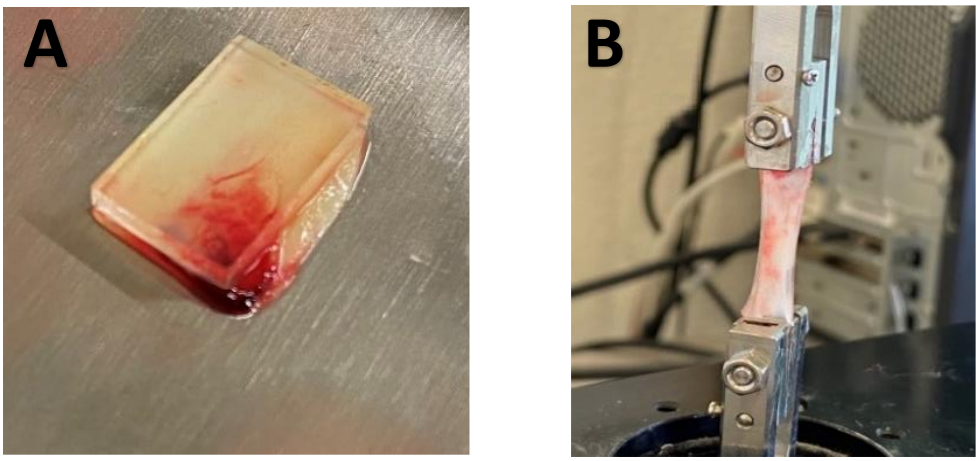


Figure 4- Membranes preparation for traction test. A- Glass-mold serving as a pattern to make the cuts at the edges of the membranes so that they can resemble each other. **B**- A-PRF+ membrane at maximum tensile strength on the traction testing machine.

2.3 Data and statistical analysis

Mechanical properties were plotted by the force applied to the membranes per section area (N.mm^2) and the tensile of the membrane, at a stretching speed of 1 mm/minute obtaining a stress-strain curve recorded until the rupture or until reaching the limit of the maximum traction of the machine. This gave us the maximum elasticity and tensile strength. Data were collected using Microsoft Excel and GraphPad Prism.

All statistical analyzes were performed using the GraphPad Prism software. Values are presented as mean ± S.D. Statistical comparisons included the unpaired t-test.

2.4 Scanning Electron Microscopy (SEM)

For SEM analysis, 6 additional membranes of A-PRF and A-PRF+ were used and it was also added 6 membranes of L-PRF function as our control group to better evaluate distinct higher and low centrifugation surfaces.

Right after preparation, PRF clots were sectioned with a scalpel in fragments of equal length (5x5mm), to have representative samples of the three layers of each membrane. The sections for microscopic evaluation were then distributed, starting with the removal of the lower fragment, adjacent to the fraction of red blood cells (RBC) encompassing the buffy coat; the second was from the middle segment of the membrane and the third from the upper-lower portion.

Each sample was fixed with 2.5% neutralized glutaraldehyde (2h, 4˚C) and postfixed with 0.2 M sodium cacodylate buffer solution and 1% osmium tetroxide (2h, 4˚C), and finally dehydrated in a series of ethanol solutions (ranging from 70 to 100%) and hexamethyldisilane. The materials were metallized with silver and observed at a voltage acceleration of 15kV using a scanning electron microscope (Hitachi SEM S4100), as previously described. (46)

Results

This study's purpose was to compare two preparations to evaluate differences in their mechanical properties. It was intended to evaluate primarily the traction of each membrane characterized by two parameters (1) maximum stress on the stress-strain curve and (2) stress at rupture. As shown in (Figure 5 A), it was important to determine the maximum strength of the membranes that could, in some cases, double the length that they were cut when subjected to traction, but also to generate a statistical representation of the average resistance, as shown in (Figure 5B).

In the tensile strength test, from a total N of 32 collected tubes, only 25 were used, of which 13 membranes of A-PRF and 12 of A-PRF+ were prepared and used, giving rise to a waste of 22% due to loss of structure in protocol errors prior to traction test. Statistically significant differences were found between A-PRF and A-PRF+ regarding maximum traction, as the average traction between these two protocols were ($p<0.01$), (Figure 5B).

In reference to the maximum traction (Figure 5A), A-PRF obtained a value of 0.0020 $N.mm^{-2}$ while A-PRF+ scored 0.0022 $N.mm^{-2}$. When it was recorded the average resistance to traction, A-PRF obtained 0.0012 $N.mm^{-2}$ and A-PRF+ 0.0015 $N.mm^{-2}$. Superior resistance was obtained for A-PRF+ (Figure 5C).

Morphology characterization

Figure 5 shows several representative images from the surface microstructure of fibrin clots originated in each protocol, by scanning electron microscopy (SEM) observation. Based on SEM analysis, the A-PRF protocol showed a dense matrix (Figure 6a), composed of thin and elongated fibers that seem to follow a preferential and orientated direction (Figure 6d and c), in which platelets are well-adhered. Some porosity is also evident with large diameter of the interfibrous spaces (Figure 6b). It was detected few traces of silica microparticles lying on the surface, as shown in figure 6a, due to the tube of processing.

For the A-PRF+, the amount of fibers cross-linking is even more noticeable (Figure 6e-h), as the presence of some intact lymphocytes adhered to the surface of the mesh (Figure 6e) or entrapped together with clusters of platelets (Figure 6i). This matrix also showed great porosity, irregularity of the surface with exophytic portions of the fibers and low destruction of cellular findings.

Oppositely, control group (L-PRF) showed a compact surface with thick-fibers present in the interfibrous areas, and limited space for microvascularization (Figure 6l and m). In addition, severe destruction of red blood cells and leukocytes was clearly visible (Figure 6n).

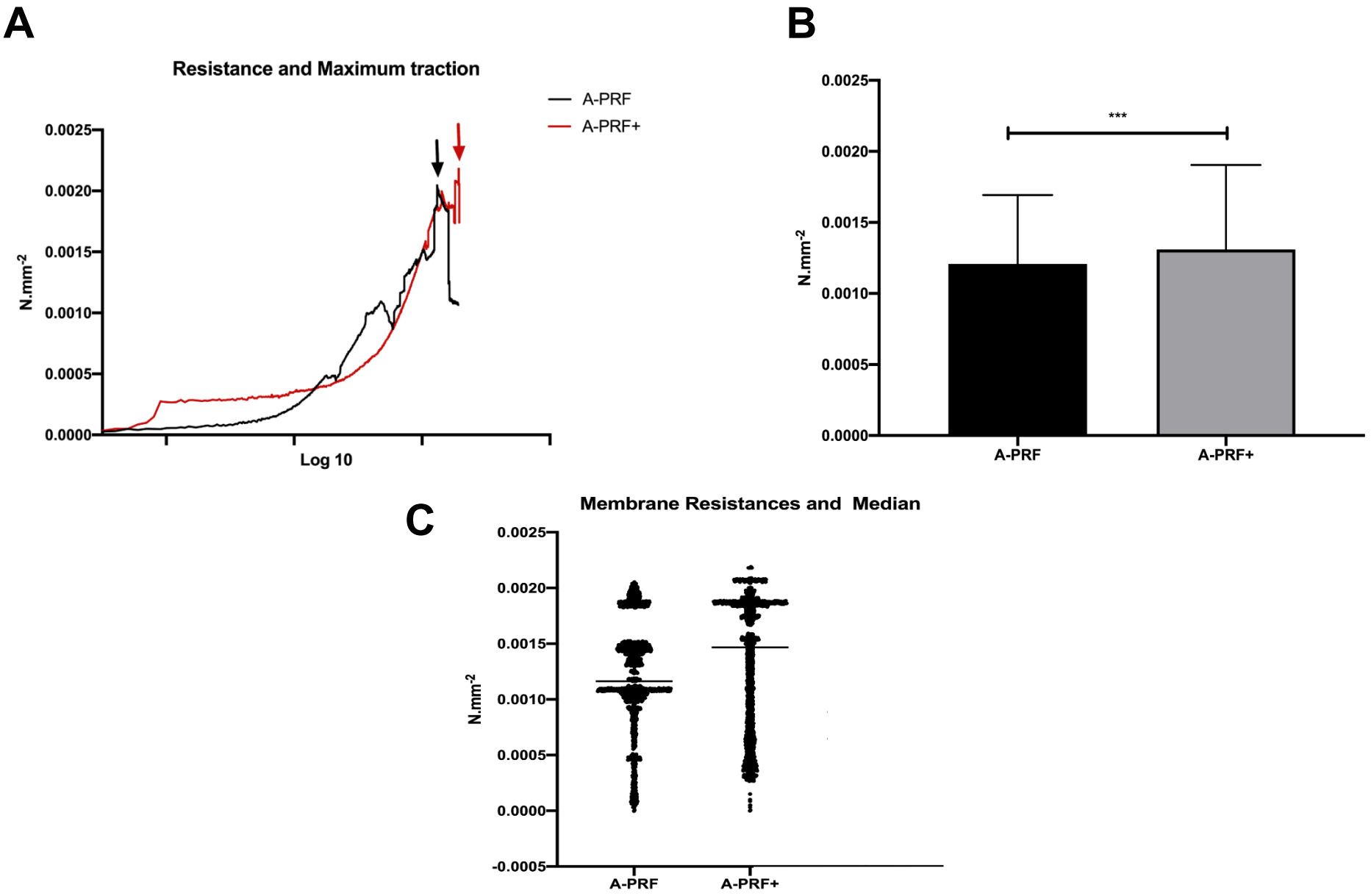
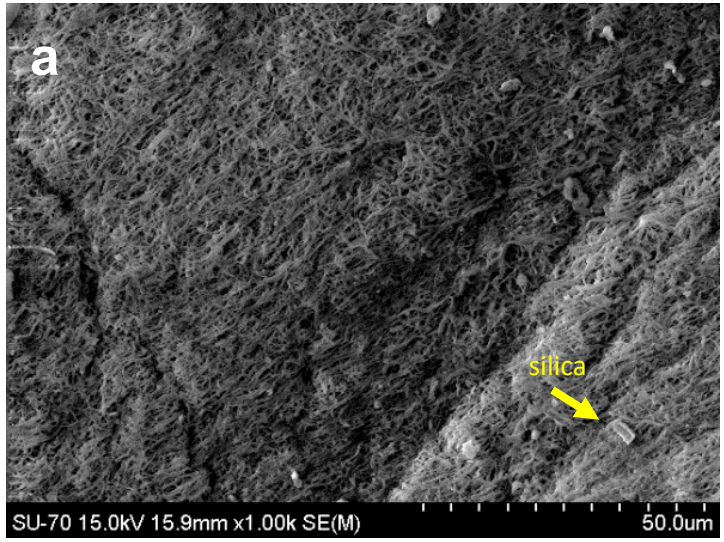
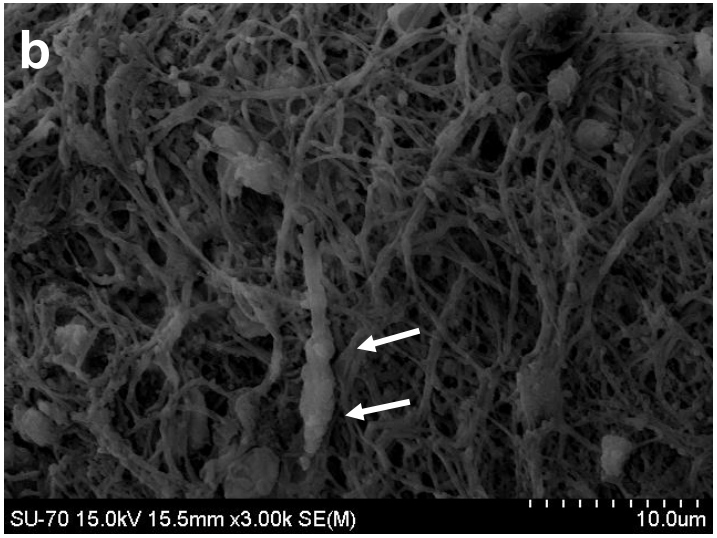


Figure 5- A- Resistance and maximum traction, where A-PRF+ reach the higher strength value comparing with A-PRF tested here; **B- Average and standard deviation (SD)** comparing both groups analyzed**; C-** Membrane Resistance and Median, shows the variability of results that was felt mostly on A-PRF and being more consistent in A-PRF+.

A-PRF


a
silica
SU-70 15.0kV 15.9mm x1.00k SE(M)
50.0um


b
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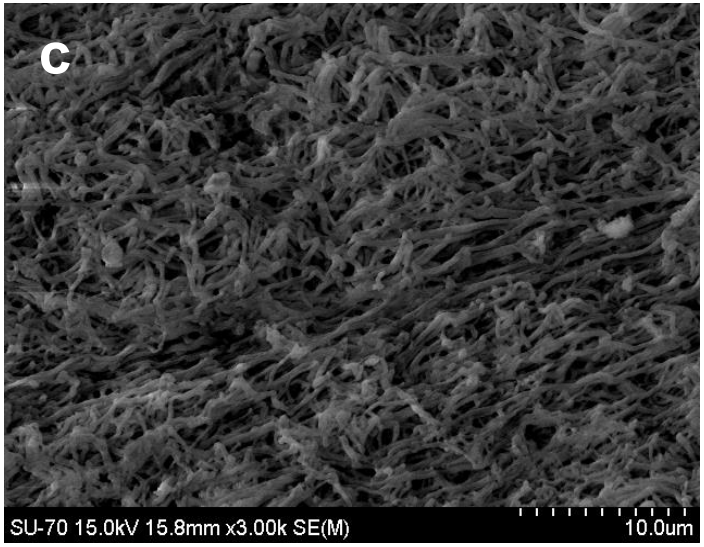
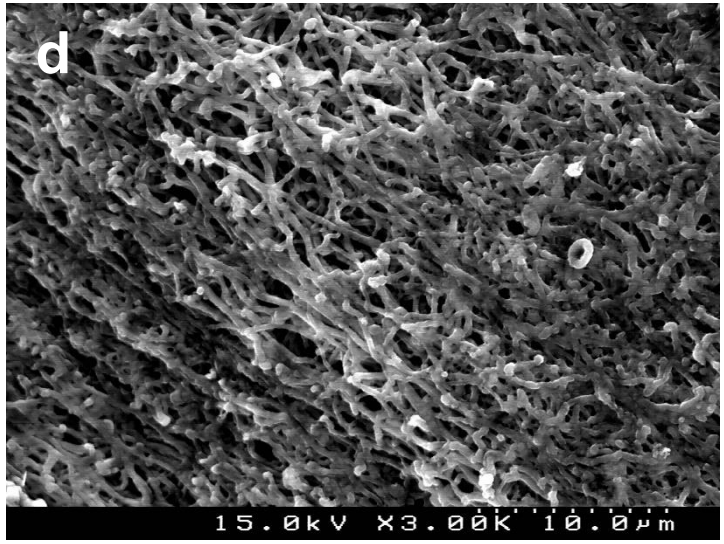
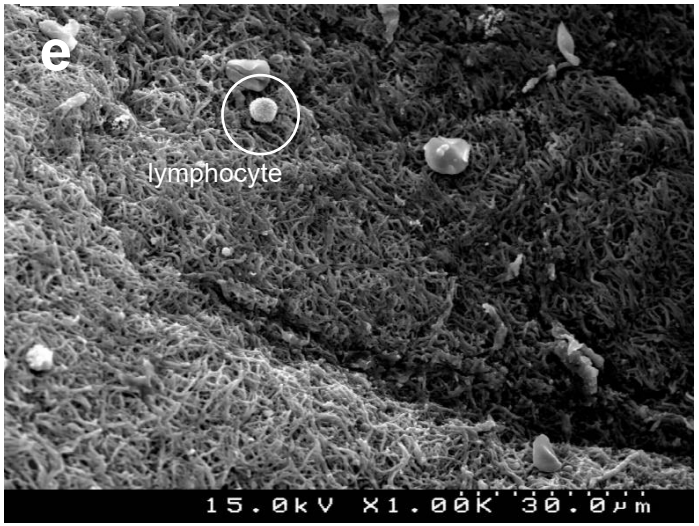
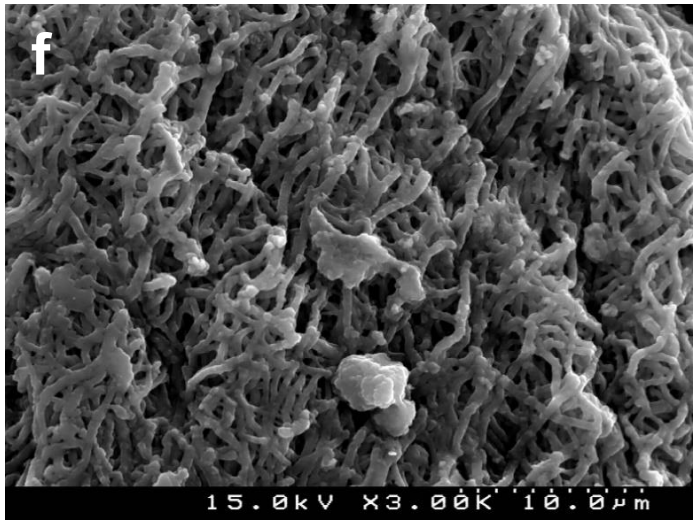
10.0um


c
SU-70 15.0kV 15.8mm x3.00k SE(M)
10.0um


d
15.0kV X3.00K 10.0µm

A-PRF+


e
lymphocyte
15.0kV X1.00K 30.0µm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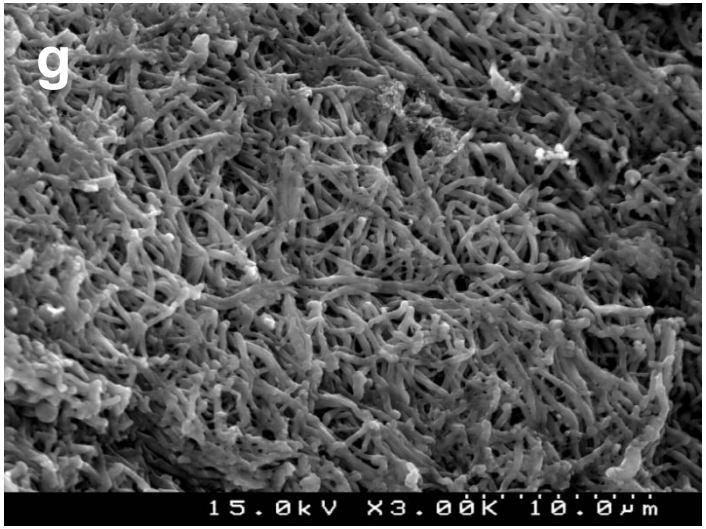
g
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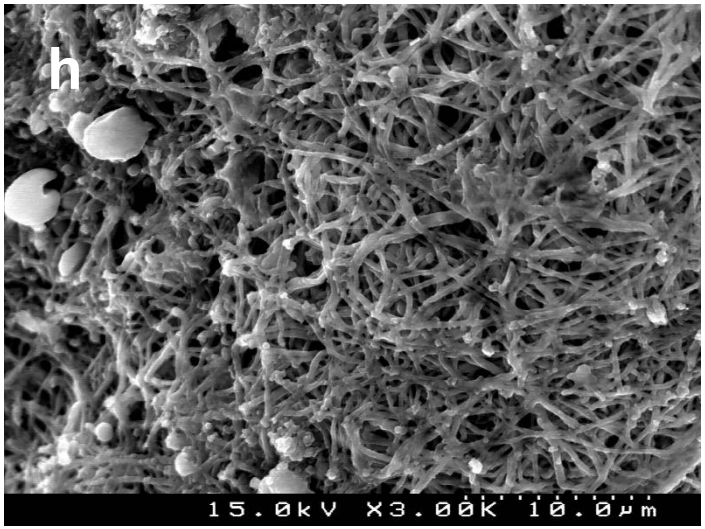
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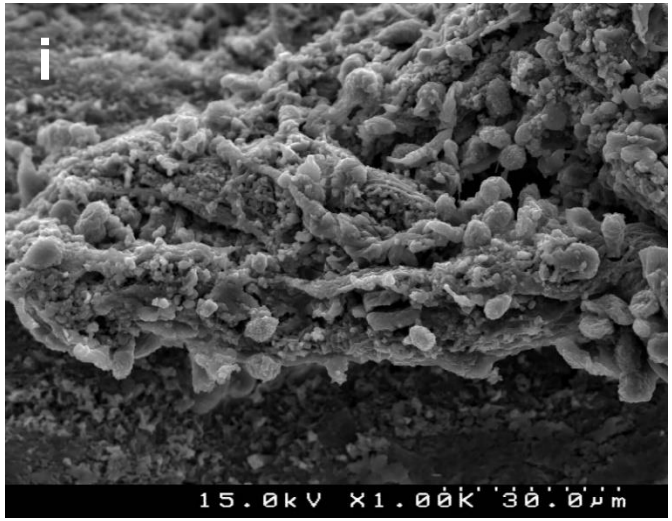
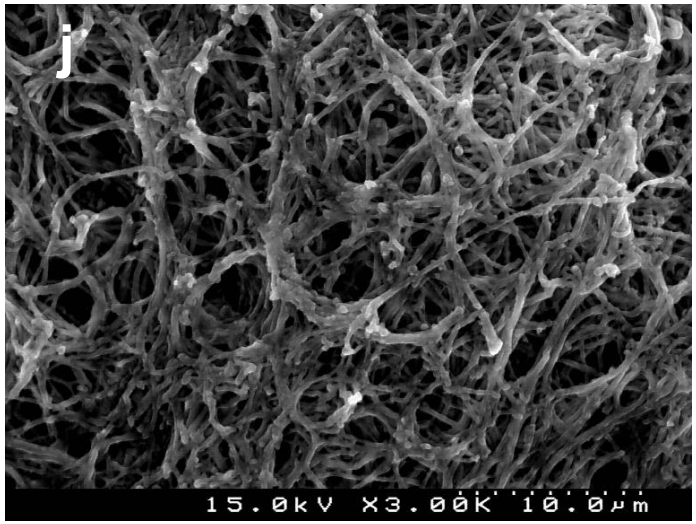
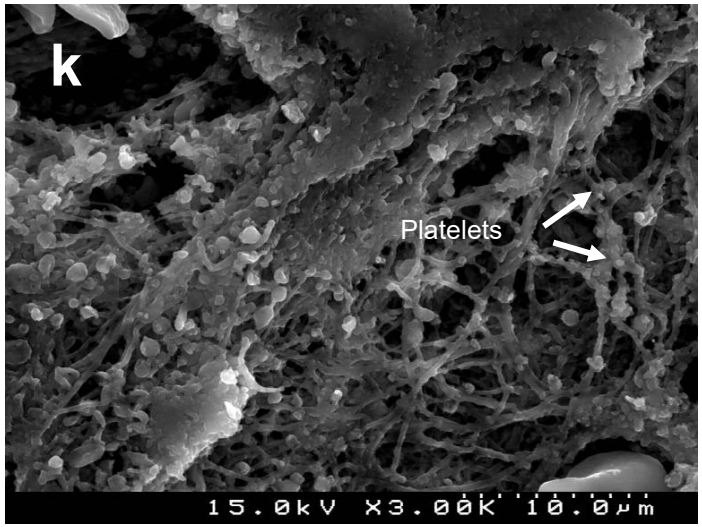
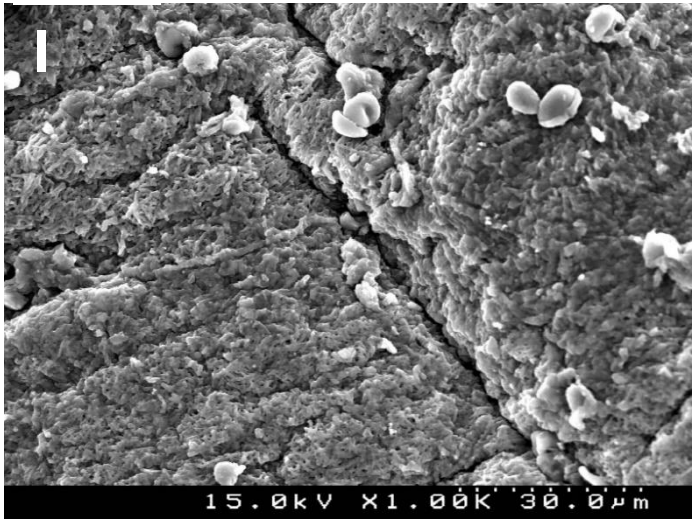
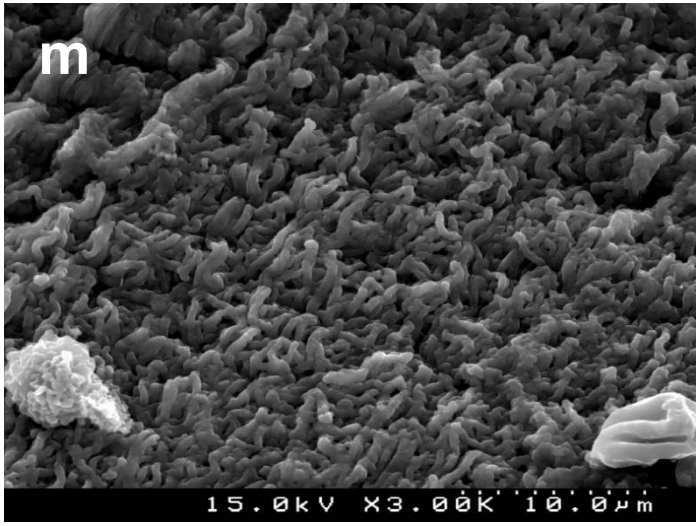

i
15.0kV X1.00K 30.0µm





L-PRF





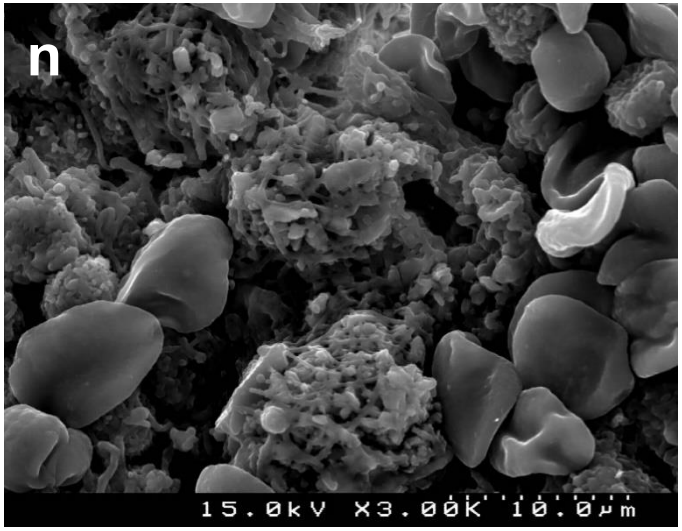


Figure 6- a-d A-PRF: 15000x, 3000x**; e-k** A-PRF+: 9000x, 3000x**; l-n** L-PRF: 9000x, 3000x.

Discussion

To the best of our knowledge, this is a pioneering study that reports the traction range of the membranes originated by two different A-PRF derivates, second generation of platelet concentrates. This study's results support that there are clinically relevant differences in mechanical properties between the produced membranes. Ideally, structural integrity of the biomaterial is a factor that suggest its contribution to the prolonged release of several GFs and should provide a non-immunogenic microenvironment that, through the properties of its network, enhances migration and cell adhesion. (13) Recent research by Lourenço et al (2020), confirms that the microenvironment created by PRF membrane architecture is directly related to its bioactivity, while degradation takes place. (51) The resilience of dense fibrin of the matrix in the final preparation allows a long and slow release of multiple molecules. (50)

In addition, it was accessed the quality of the respective fibrin meshes produced by each protocol in vertical angle centrifugation by IntraSpin® centrifuge. This work extends to the comparative analysis of the previous findings obtained by Pascoal et al, which refers to the higher traction ability of A-PRF membranes concerning L-PRF. (48) Thereby, the aim of this study was to amplify the validity of these results regarding A-PRF, giving introduction on the reliable evaluation of mechanical effects in the most recent protocol A-PRF+.

In the tensile test, the overall evaluation tended towards the discovery of a quantitative order in the resistance parameter, demonstrating the maximum tensile strength results for A-PRF +, followed by A-PRF. Although Ravi and Santhanakrishnan, found extremely high values for A-PRF (362,565 ± 5.15 MPa), these values do not coincide with those presented in this study, which can be justified by different centrifugation settings. However, there was compliance with the scenario of a prominent increase in the elastic capacity when LSCC is implemented.(7) In the present work, A-PRF ranged the average of 0,0020 MPa, and A-PRF+ twofold this average to 0.0022MPa, depending on a much higher tensile force to suffer deformation or rupture, only by reduction of centrifugation speed and time. Although, both scored lesser than the obtained 0,2 ± 0,06 MPa resistance of L-PRF prepared by Khorshidi et al. (51) Results carried out by the methodologies adopted by Pascoal et al, recorded in A-PRF a value of 0.0752 MPa for maximum traction, higher than what is shown in this study but which still

surpasses the reach of membranes produced by L-PRF. (48) The motive for this result to be more elevated is the cut size of the membrane which was larger than the standardized measures in this study. The effect felt in A-PRF+ are approximately similar to A-PRF, however, they cannot be entirely compared with previously reported studies, since this study, as far as it's shown in literature, is the first to elucidate the inherent mechanical properties of this derivative using strictly standard protocol techniques, originally described. (11,12)

Lam et al, refer to the action of platelets on the clot, detecting a positive impact which in addition to fibrin, are a source of reinforcement of the adhesive properties. (35) This suggests that the significant increase in elasticity and tensile strength found in the low-RCF protocols performed, may also result from the more dispersed platelet distribution along the clot. Agreeing with previous reports by Aizawa et al, who came to the conclusion that the slight decrease on the centrifugation time, preserves largely the cellular contents, presenting an higher amount and an even more nearly homogeneously distribution of platelets. (13,52) Therefore, this suggests that A-PRF + could be most preferable to use as a scaffold for tissue repair when high tensile strength is an issue such as in wound closure, distribution of mechanical forces, and holding the suture.

Through SEM analysis, the morphology of the PRF membrane surface of each protocol was evaluated to understand the characteristics of fibrin fibers, which are essential for mechanical and biological clot homeostasis. Thus, it was found an abundant fibrin mesh in the A-PRF+, arranged in organized layers that, when magnified, may suggest the superior resistance to traction.

Great similarity, through microscopic analysis, was observed between A-PRF and A-PRF+ regarding porosity, formed by porous tangle spaced fibers, being notorious the high density of connections, directed longitudinally and laterally. The anatomy of the fibers in low-centrifugation protocols has revealed, commonly, a thin biotype, although A-PRF+ appears to have a higher polymerization maturity, despite its thin thickness.

Control group presents a matrix of dense and strongly polymerized fibrin but in contrast to its counterparts, less porous and thicker fibers. According to Li et al. (2016), the thickness of the fibers is suggestive of a weaker connection between the protofibrils, tending to a lesser modulus of elasticity. (53)

There is also experimental evidence that corroborates the lower value of the mechanical properties with the degradation process observed in the L-PRF (7). It can be assumed that a coagulum composed of many thin fibers is more auspicious to undergo slow lysis by the fibrinolytic system, leading to the belief and suggestion that A-PRF+ might have a longer dissolution rate than our control group and last approximately the same as A-PRF, according to its similar characteristics.

The key factor that provides membrane resistance is its structural integrity, which allows it to have a long sustaining mechanism in order to improve clinical outcomes. (13,46) This study's findings show increased porosity and strong fibrin architecture for the A-PRF+, may also help to explain the prolonged release of growth factors, as reported in other studies. (15,44) Concerning cell damage and destruction, it could be allocated to the vibrations inherent in the centrifugation step. (41,54) Of the three derivatives, A-PRF+ was the one that showed less damage without destruction of the content. Although more populations of lymphocytes were observed in the control, these had signs of their form altered or severely damaged, similar to what is found in A-PRF.

The findings of this study refute hypothesis 0 initially formulated, proving that A-PRF+ is more resistant than A-PRF, with a more porous microstructure in which its fibers have small physiognomic differences regarding those revealed in A-PRF, validating the reliability of the remaining hypotheses 1 of the study.

In the literature, the only article that opposed these findings was the experiment carried out by Dohan Ehrenfest et al., which noticed a disorganized fibrin network, with thin weak fibers and greater cell destruction in A-PRF, considering that it has a lower biological signature.(55) Nonetheless, empirical results reported herein must be seen in light of limitations that should be addressed in future research.

In the design of the current study, the first concern it still is unclear the use of plastic vacuum blood collection tubes coated with silica activators on the inner walls. Without anticoagulants, the formation of the fibrin clot is triggered immediately by contact with the silica particles. Possible risk of biological contamination has been associated with concentrates that are collected in glass or plastic tubes with this activator.(56) Small fraction of microparticles can inevitably detach from the walls of the tube during centrifugation and be

suspended or retained in the lower layer of fibrin. The consequences that this may have for the host organism are not yet fully known. Even in residual amounts, these microparticles are released as the membrane is degraded, raising questions about its possible help to develop cytotoxic or inflammatory effects.(56,57) Glass tubes or titanium tubes to produce the T-PRF, may be a more biocompatible alternative and so far indicate that they are capable of forming a clot that is clinically likely to that formed in glass or plastic tubes. (56) However, it is necessary to further investigation whether there is maintenance or improvement of the reported properties.

Other analysis validated the importance of the results presented in this study of tensile strength and stability created by the fibrin network.(7,45,46,48) The recognition of the properties that seem to be enhanced by the reduction of centrifugation, more precisely the increased resistance at rupture, the amount of microporous, and the flexibility of the membrane, has aroused a growing interest in several areas of medicine as a promising treatment in complex and delicate defects to repair.

In Medicine, examples of treatments that lack adequate biomechanics to repair of tympanic membrane perforations; regeneration of focal articular cartilage; tendon remodeling; repair of muscle, bone defects, or other soft tissue injuries. (51,55,58) In Dentistry, the application of these concentrates has shown favorable postoperative conditions to make their use routine. In a meta-analysis study involving periodontal surgery, PRF is currently a more beneficial and less invasive alternative than the connective tissue graft (CTG), which is considered the "gold standard" for root coverage of gingival defects, reducing discomfort for the patient.(58) It has also provided clinical results comparable to the use of bone grafts, with a gain of the attachment, resolution, and regeneration in two-to-three walls infra-bone defects, proving to be a biological safer, practical, and less expensive alternative.(59) In oral and maxillofacial surgery, the effects include the reduction of swelling and pain in third molar extractions; preservation of the alveolar ridge by decreasing bone resorption; reduction in healing time in sinus lift procedure and favoring optimal bone healing; acceleration of osteointegration in dental implants, are already strongly documented. (60)

Advanced experiment joins to the reviews above, managed by Xin and collaborators (2020), which were able to display a total repairment of the perforated Schneiderian membrane (SM) and a greater amount of new bone formed under the area, through the application of A-PRF. (61)

This study was, for the best of the authors knowledge, the first to evaluate biophysical modifications that occur in platelet concentrates and what thus that transduces in the final clinical choice of which protocol is more advantageous to adopt. It was noticeable that the A-PRF + gave rise to a more resistant environment. The decrease in time and the pull-down effect created by the centrifugation forces increase the total number of cells left and can thereby determine the degree of bonds between the molecules. (11,13) This resistance can be considered a gold factor that influences the performance of the membrane as a whole: in directing the differentiation of stem cells that are mechanosensitive to the surrounding environment; increasing the release of growth factors or other adhered nanoparticles with pharmacokinetic potential, and delayed speed of degradation since the mechanical deformation occurs in layers and is proportional to the rates of nutrient consumption by the cells.(16,49,62)

Further studies should be conducted in order to evaluate other parameters , as is the case of tube-rotor angulation by the use of horizontal centrifugation, less traumatic for cells and with better capacity for separation by densities.(63) The effects that it may have on PRF resistance are not yet known.

Conclusion

Despite its limitations, it is possible to affirm in the mechanical parameters that were tested, a significantly greater tensile strength in the membranes produced with the A-PRF+ protocol, making this type of membranes the most favorable to be sutured, handled, and promising in the gain of tissue volume.

A-PRF+ showed approximately superior resistance and an interesting surface morphology that balances the proportion of porosity with the arrangement and amount of fibers that may be more likely to contribute to membrane resilience than A-PRF, however more analyses should evaluate the direct impact of this parameter.

In conclusion, better mechanical properties are closely related with improved membrane support for regenerative treatments, guaranteeing functional integrity. However, findings presented in this study are only a prediction of what could be found on the application to the oral cavity. It is therefore necessary to carry out in vivo studies that evaluate the maintenance of these properties and how are they an advantageous interference on tissue regeneration in short- and long-term analysis, combining the suggested methodological considerations.

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