Maria Adelia Faleiro Santana Silva

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Plastic tubes without additives as alternatives to glass and silica-coated plastic tubes in platelet-rich fibrin solid matrix production. Histomorphometric and ultrastructural study

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Uberlândia, 2021

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Orientadora: Prof.ª Dr.ª Paula Dechichi

Banca examinadora:

Prof.ª Dr.ª Paula Dechichi

Prof. Dr. Jonas Dantas Batista

Prof. Dr. Luis Eduardo Carneiro-Campos

Uberlândia, 2021

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Coordenação do Programa de Pós-Graduação em Odontologia
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ATA DE DEFESA - PÓS-GRADUAÇÃO

Programa de Pós-Graduação em:	Odontologia				
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Data:	Seis de Julho de Dois Mil e Vinte e Um	Hora de início:	09:00	Hora de encerramento:	10:35
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Reuniu-se em Web Conferência pela plataforma Zoom, em conformidade com a PORTARIA Nº 36, DE 19 DE MARÇO DE 2020 da COORDENAÇÃO DE APERFEIÇOAMENTO DE PESSOAL DE NÍVEL SUPERIOR - CAPES, pela Universidade Federal de Uberlândia, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Odontologia, assim composta: Professores Doutores: Jonas Dantas Batista (UFU); Luis Eduardo Carneiro Campos (UFF); Paula Dechichi Barbar (UFU) orientadora da candidata.

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“Uma longa viagem começa com um único passo.”

Lao-Tsé

SUMÁRIO

RESUMO

RESUMO

Os concentrados sanguíneos de Fibrina Rica em Plaquetas (PRF) obtidos em tubos de vidro e plástico, revestido ou não por sílica, têm sido amplamente utilizados em procedimentos cirúrgicos para favorecer o reparo tecidual. Recentemente, foi demonstrado que micropartículas de sílica, provenientes de tubos plásticos revestidos por sílica, são incorporadas em matrizes de PRF e podem levar a toxicidade local. No intuito de buscar alternativas, biologicamente seguras, na produção de membranas de PRF, o presente estudo avaliou a densidade da rede de fibrina, agregados plaquetários e a morfologia celular de três protocolos PRF, obtidos em tubos de vidro e plástico, revestido ou não por sílica. Participaram do estudo oito voluntários (n=8), sendo coletadas nove amostras de sangue de cada indivíduo. As amostras foram separadas em três grupos, de acordo com o material do tubo de coleta: vidro, plástico revestido e não revestido por sílica. Em cada grupo, as amostras foram centrifugadas nos seguintes protocolos: Fibrina Rica em Plaquetas e Leucócitos (L-PRF - 700*g*/12min), Fibrina Rica em Plaquetas e Leucócitos Avançado (A-PRF - 200*g*/14min) e Fibrina Rica em Plaquetas e Leucócitos Avançado *plus* (A-PRF+ - 200*g*/8min). As membranas obtidas foram analisadas em Microscopia de Luz (ML), para avaliar densidade de fibrina e plaquetas (histomorfometria), e Microscopia Eletrônica de Transmissão (MET) para análise qualitativa da morfologia celular. As membranas obtidas em tubos plásticos sem sílica exibiram densidade da rede de fibrina significativamente menor nos protocolos L-PRF ($p<0,019$) e A-PRF ($p<0,001$). Observou-se percentual significativamente maior de plaquetas em membranas produzidas em tubos plásticos sem sílica, independente do protocolo ($p<0,005$). A análise por MET revelou morfologia ultraestrutural normal com núcleos, organelas e membranas celulares preservados em todos os grupos, independente do tubo ou protocolo utilizado. O presente estudo mostrou que tubos plásticos sem sílica podem ser utilizados para a produção de membranas de PRF, considerando que as membranas resultantes apresentam características histomorfométricas e ultraestruturais semelhantes às obtidas em tubos de vidro e plástico revestido por sílica, independente do protocolo de centrifugação.

PALAVRAS-CHAVE: Fibrina Rica em Plaquetas; Tubos; Medicina regenerativa; Plaquetas sanguíneas; Centrifugação; Sílica amorfa

ABSTRACT

ABSTRACT

Platelet-Rich Fibrin (PRF) blood concentrates, obtained from glass and plastic tubes, coated or not with silica, have been extensively used in surgical procedures to improve tissue repair. It was recently demonstrated that silica microparticles from silica-coated plastic tubes are incorporated into the PRF and could lead to local toxicity. In order to explore biologically safe alternatives in the production of PRF membranes, the present study evaluated the fibrin network density, platelet aggregates, and cellular morphology of three PRF protocols, obtained from glass and plastic tubes, coated or not with silica. Eight volunteers participated in the study (n=8), and nine blood samples were collected from each individual. The samples were separated into three groups, according to the tube material: glass, silica-coated plastic and plastic without silica. In each group, the samples were centrifuged in the following protocols: leukocyte-platelet-rich fibrin (L-PRF - 700*g*/12min), advanced PRF (A-PRF - 200*g*/14min) and advanced PRF+ (A-PRF+ - 200*g*/8min). The obtained membranes were analyzed in Light Microscopy (LM), to evaluate fibrin and platelet densities (histomorphometry), and Transmission Electron Microscopy (TEM) for qualitative analysis of cell morphology. The membranes obtained from plastic tubes without silica exhibited significantly lower fibrin network density with L-PRF ($p<0.019$) and A-PRF ($p<0.001$) protocols. There was significantly higher percentage of platelets in membranes obtained from plastic tubes without silica, irrespective of protocol ($p<0.005$). TEM analysis revealed normal ultrastructural morphology that preserved nuclei, organelles and cell membranes for all groups, irrespective of protocol or type of tube. The present study showed that plastic tubes without silica could be used to produce PRF membranes, as the resulting membranes exhibited histomorphometric and ultrastructural characteristics similar to those obtained from glass and silica-coated plastic tubes, regardless of the centrifugation protocol.

KEYWORDS: Platelet-Rich Fibrin; Tubes; Regenerative medicine; Blood platelets; Centrifugation; Amorphous silica

INTRODUÇÃO E REFERENCIAL TEÓRICO

1. INTRODUÇÃO E REFERENCIAL TEÓRICO

O processo de reparo tecidual é complexo, uma vez que compreende eventos biológicos simultâneos, especificamente as etapas de hemostasia, inflamação, proliferação e remodelação (Lindley *et al*., 2016). Ao longo dos anos, muitas estratégias têm sido propostas no sentido de explorar o potencial de cada uma dessas fases, buscando favorecer o reparo tecidual. Assim, esse desafio direcionou esforços à investigações projetadas para o desenvolvimento de agentes facilitadores do reparo, capazes de atender critérios de qualidade e previsibilidade de modo satisfatório (Stejskalova & Almquist, 2017).

Nesse contexto, o reconhecimento do potencial regenerativo das plaquetas sanguíneas impulsionou a introdução e o aperfeiçoamento dos concentrados sanguíneos no cenário multidisciplinar da bioengenharia tecidual moderna. Além de exercer função hemostática, as plaquetas são responsáveis pela liberação de uma série de biomoléculas que atuam diretamente no recrutamento, proliferação e diferenciação celular, estabelecendo assim um microambiente favorável à neoformação tecidual (Golebiewska & Poole, 2015). Tendo em vista esses princípios, concentrados sanguíneos foram desenvolvidos como biomateriais autólogos obtidos a partir de diferentes protocolos de centrifugação. Esses concentrados atuam como coadjuvantes no reparo de tecidos, considerando que são fonte de fatores de crescimento, citocinas, proteínas e enzimas capazes de estimular a atividade celular. (Prakash & Thakur, 2011).

A primeira geração de concentrados sanguíneos, o Plasma Rico em Plaquetas (PRP), foi elaborado como um concentrado autólogo de plaquetas compreendido em um volume de plasma reduzido (Marx, 2004). Entretanto, o método apresenta limitações consideráveis, uma vez que requer protocolos de preparo relativamente longos e incorpora substâncias anticoagulantes e coagulantes desfavoráveis ao processo de reparo. Além disso, a liberação de fatores de crescimento acontece predominantemente de forma precoce, em contraste ao perfil de liberação preferencial, lento e gradual ao longo de todo o período de reparo (Kobayashi *et al*., 2016). A segunda geração de concentrados sanguíneos, Fibrina Rica em Plaquetas e Leucócitos (L-PRF) proposta por Choukroun (Dohan *et al*., 2006a), revolucionou a aplicação dos concentrados sanguíneos, pois apresenta menor tempo de preparo e não utiliza fatores anticoagulantes e coagulantes. O

L-PRF, geralmente referido como PRF, é obtido por centrifugação de amostras de sangue coletadas em tubos de vidro a 700*g* por 12 minutos, obtendo-se uma membrana por tubo de coleta.

O PRF consiste em uma matriz autóloga de fibrina disposta em estrutura molecular tridimensional que retém plaquetas, leucócitos, células tronco circulantes (Choukroun *et* al., 2006) e fatores de crescimento que guiam o reparo de vasos e tecidos comprometidos (Dohan *et al*., 2006b). Além disso, citocinas leucocitárias são massivamente capturadas no arcabouço de fibrina e liberadas gradualmente, o que consiste em estímulo a mecanismos de defesa a patógenos e regulação inflamatória (Dohan *et al*., 2006c). O procedimento de obtenção do PRF é possível a partir do manejo simplificado do sangue biológico, mediante protocolos de centrifugação que não incorporam agentes exógenos. Originalmente, amostras de sangue eram coletadas em tubos de vidro e imediatamente submetidas a uma Força de Centrifugação Relativa (RCF - *Relative Centrifugation Force*). O contato do sangue com a superfície de vidro resulta na ativação plaquetária, desencadeando assim a cascata de coagulação. Dessa forma, o coágulo de fibrina é obtido na porção central do tubo entre uma camada superior de plasma acelular e uma porção inferior composta, essencialmente, por hemácias (Dohan *et al*., 2006a).

A morfologia e o potencial biológico do PRF são sensíveis aos parâmetros de centrifugação a que as amostras de sangue são submetidas (Ghanaati *et al*., 2014). A introdução do conceito de baixa velocidade de centrifugação, fundamentado na redução da RCF (Choukroun & Ghanaati, 2018), possibilitou o desenvolvimento de diferentes protocolos. Assim, podem ser produzidas matrizes sólidas avançadas, tais como Fibrina Rica em Plaquetas e Leucócitos Avançado (A-PRF - 200*g*/14min) e Fibrina Rica em Plaquetas e Leucócitos Avançado *plus* (A-PRF+ - 200*g*/8min), que exibem distribuição celular uniforme, aumento significativo na quantidade de células aprisionadas e liberação prolongada de fatores de crescimento (Fujioka-Kobayashi *et al*., 2017). Em sequência, foi proposto o PRF injetável (i-PRF- 60*g*/3min) que, apesar de exibir princípios biológicos homólogos ao PRF, não apresenta resistência (Miron *et al*., 2017). O i-PRF é produzido em tubos plásticos sem aditivos, cujas propriedades de superfície não aceleram a cascata de coagulação a ponto de estabelecer coágulos sólidos. Assim, o concentrado é

obtido inicialmente em consistência fluida, sendo que o processo de polimerização acontece de forma contínua, porém mais lenta, após a centrifugação.

A produção de tubos de vidro tradicionalmente utilizados para a confecção de membranas (matrizes sólidas) foi descontinuada por razões ecológicas, exceto por marcas especializadas na produção de PRF. Diante desse cenário, se deu a inserção de tubos plásticos alternativos revestidos por sílica, na prática clínica (Tsujino *et al*., 2019a). Essa substituição é fundamentada na hipótese de que micropartículas de sílica exibem propriedades semelhantes ao vidro, de modo a acelerar a cascata de coagulação e produzir concentrados de PRF com propriedades equivalentes à de matrizes obtidas pelo protocolo convencional. Entretanto, estudos demonstraram que micropartículas de sílica são facilmente destacadas das paredes internas dos tubos e incorporadas ao coágulo de fibrina, em quantidade significativa (Tsujino *et al*., 2019b). Além disso, a presença desses fragmentos pode modificar de modo considerável a distribuição de plaquetas e, possivelmente, influenciar negativamente a retenção e a liberação de fatores de crescimento (Tsujino *et al*., 2019a).

Naturalmente, micropartículas de sílica presentes nas matrizes são liberadas mediante a degradação fisiológica do coágulo de fibrina e podem apresentar risco de contaminação para células e tecidos adjacentes. Os efeitos biológicos derivados do contato com partículas de sílica são controversos, uma vez que a toxicidade do material parece ser estabelecida mediante interações complexas entre diferentes fatores, incluindo sua estrutura molecular, dimensão, quantidade e tipo celular em questão (Kim *et al*., 2015).

Nanopartículas de sílica são amplamente empregadas na engenharia de tecidos, atuando como ferramentas para a entrega de medicamentos e fatores bioativos (Eivazzadeh-Keihan *et al*., 2020), sendo que formas específicas apresentam até mesmo bioatividade inerente no reparo de tecidos (Chen *et al*., 2019). Contudo, trata-se de um agente com potencial citotóxico e carcinogênico (Fontana *et al*., 2017), além de possível indutor do processo inflamatório (Chen *et al*., 2018). Além disso, foi recentemente demonstrado *in vitro* que partículas de sílica provenientes de tubos de coleta de sangue são capazes de induzir a apoptose de células periosteais humanas, reduzindo significativamente a proliferação e a viabilidade celular (Masuki *et al*., 2020). Portanto,

é necessário o desenvolvimento de métodos alternativos de produção de PRF para diferentes necessidades clínicas, que não ofereçam possíveis riscos à saúde humana.

O processo de polimerização de matrizes fluidas obtidas em tubos plásticos sem aditivos pode ser acelerado após a centrifugação, por meio da ativação plaquetária proporcionada pelo contato com superfícies de vidro. Dessa forma, matrizes sólidas podem ser facilmente obtidas. Tendo em vista esse princípio, a hipótese do presente estudo é que não há diferenças morfológicas entre matrizes sólidas produzidas em tubos de vidro e plástico revestido ou não por sílica. Assim, o objetivo do estudo consistiu em avaliar a morfologia celular e a densidade da rede de fibrina e agregados plaquetários de concentrados sanguíneos L-PRF, A-PRF e A-PRF+, obtidos em tubos de vidro, plástico revestido de sílica e plástico sem revestimento de sílica.

CAPÍTULO I

2. CAPÍTULO 1

ARTIGO 1

Plastic tubes without additives as alternatives to glass and silica-coated plastic tubes in platelet-rich fibrin solid matrix production: A histomorphometric and ultrastructural study

***Artigo a ser enviado para o periódico "Materials Science and Engineering: C"**

Plastic tubes without additives as alternatives to glass and silica-coated plastic tubes in platelet-rich fibrin solid matrix production: A histomorphometric and ultrastructural study

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Plastic tubes without additives as alternatives to glass and silica-coated plastic tubes in platelet-rich fibrin solid matrix production: A histomorphometric and ultrastructural study

ABSTRACT

Platelet-Rich Fibrin (PRF) obtained from glass, silica-coated plastic and plastic without additives tubes has been extensively used in surgical procedures to improve tissue repair. It was recently demonstrated that silica microparticles from silica-coated plastic tubes are incorporated into the PRF and could lead to local toxicity. The aim of this study was to evaluate the fibrin network density, platelet aggregates, and cellular morphology of leukocyte-platelet-rich fibrin (L-PRF), advanced PRF (A-PRF) and advanced PRF+ (A-PRF+) obtained from glass, silica-coated plastic, and plastic without additives tubes. We collected nine blood samples each from eight volunteers and separated these into three groups according to tube material: glass, silica-coated plastic, and plastic without additives. In each group, the samples were centrifuged using different relative centrifugation force protocols (n=8): L-PRF (700g/12min), A-PRF (200g/14min) and A-PRF+ (200g/8min). The membranes were subjected to histomorphometric analysis and ultrastructural morphology was evaluated by transmission electron microscopy (TEM). The membranes obtained from plastic tubes without additives exhibited significantly lower fibrin network density with L-PRF ($p<0.019$) and A-PRF ($p<0.001$) protocols. A significantly higher percentage of platelets was found in membranes obtained from plastic tubes without additives, irrespective of protocol ($p<0.005$). TEM analysis showed normal ultrastructural morphology that preserved nuclei, organelles and cell membranes for all

groups. The present study revealed that plastic tubes without additives could be used to produce PRF solid matrices, as the membranes obtained from these exhibited histomorphometric and ultrastructural characteristics similar to those obtained from glass and silica-coated plastic tubes, regardless of the centrifugation protocol.

Keywords: Platelet-Rich Fibrin. Tubes. Regenerative medicine. Blood platelets. Centrifugation. Amorphous silica.

INTRODUCTION

Esthetic and functional rehabilitation in atrophic areas or areas of high tissue loss due to disease or trauma is challenging for clinicians. Various surgical techniques and biomaterials have been proposed to improve and accelerate tissue repair in order to restore patient health. In the last decade, special attention has been given to use of blood concentrates as an adjuvant therapy in the treatment of different surgical cases[1, 2].

Platelet-rich fibrin (PRF) has long been recognized as a noteworthy biomaterial in modern tissue engineering that utilizes the regenerative properties of blood cells and platelets. It consists of an autologous fibrin matrix arranged in a three-dimensional architecture, which retains platelets, leukocytes, circulating stem cells[3], cytokines, and a wide range of growth factors[4] that guide tissue regeneration after damage. In addition, PRF is known to stimulate inflammatory regulation and immune defense mechanisms[5]. Therefore, PRF has been increasingly applied in regenerative medicine and dentistry for the regeneration of soft and hard tissues[6-8].

The technique of PRF production involves a simplified preparation of blood samples by means of centrifugation protocols that do not include additives or exogenous substances. In fact, the morphology and biological potential of PRF depend on the parameters applied during the centrifugation process and on the tube material used for blood collection. The first PRF production protocol, proposed by Choukroun and collaborators in 2001, was for the leukocyte- and platelet-rich fibrin (L-PRF)[9], which has significant concentrations of cytokines and growth factors that are entrapped in the fibrin matrix during polymerization and released at a slow pace[4, 5]. However, the introduction of the low-speed centrifugation concept, based on the reduction of the relative centrifugation force (RCF), resulted in the production of advanced solid matrices, such as advanced PRF (A-PRF) and advanced PRF+ (A-PRF+), with enhanced regenerative potential[10, 11]. Furthermore, injectable PRF is prepared in plastic blood collection tubes, which activate the coagulation cascade at reduced levels. Consequently, a fluid concentrate is generated first, which polymerizes more slowly after centrifugation[12].

The production of plain glass blood-collection tubes has been gradually discontinued due to environmental reasons[13], except for manufacturers specialized for PRF production. In view of these circumstances, many clinicians have opted for the use of non-approved silica-coated plastic tubes in clinical practice, which are less expensive[14]. This modification in collection tubes was made based on the hypothesis that silica microparticles exhibit vitreous properties that accelerate the coagulation cascade and generate a similar concentrate to that obtained in glass tubes. However, it has been demonstrated that silica microparticles are easily detached from the inner walls of tubes and get incorporated into the resulting fibrin clot at significant levels[15]. Therefore, the

presence of these fragments has been reported to considerably modify the platelet distribution pattern and possibly influence growth factor retention and release[14, 16].

In addition, the biological effects of silica contamination on cells and tissues surrounding the implantation site are unpredictable. Although it remains a topic of discussion, silica nanoparticles have been reported to induce proinflammatory responses, in addition to demonstrating cytotoxic effects and carcinogenic potential[17-20]. It has also been shown that silica microparticles specifically released from blood collection tubes induce human periosteal cell apoptosis and significantly reduce cell proliferation and viability *in vitro*[21]. Therefore, it is necessary to develop alternative methods for manufacturing PRF that are not hazardous to human health.

The present study hypothesized that there are no morphological differences among solid matrices obtained from tubes of glass, silica-coated plastic, and plastic without additives. Therefore, the aim of this study was to evaluate the fibrin network density, platelet aggregates, and cellular morphology of L-PRF, A-PRF, and A-PRF+ blood concentrates obtained from tubes of glass, silica-coated plastic and plastic without additives.

MATERIALS AND METHODS

Experimental design

This study included eight volunteers (four males and four females) aged 25–54 years who were healthy, non-smokers, and had not used anticoagulant, anti-inflammatory, or antibiotic drugs in the last three months. The above-mentioned criteria were adopted to reduce sample heterogeneity, as sex, age, and physiological condition

could affect the results[22]. This study was approved by the Human Research Ethics Committee of our institute (CAAE: 13857519.0.0000.5152). After reading and signing the written informed consent, each participant provided nine blood samples (~8.0 mL per sample), which were separated into three groups, according to the material of tube used: glass (Becton-Dickinson Vacutainer, Product code: 366430, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), silica-coated plastic (Becton-Dickinson Vacutainer, Product code: 367820, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and plastic without additives (Vacuette® Z No Additive, Greiner Bio-One GmbH, Kremsmünster, Austria). In each group, the samples were centrifuged using different RCF protocols: L-PRF (700 *g*/12 min), A-PRF (200 *g*/14 min), and A-PRF+ (200 *g*/8 min).

PRF preparation

Peripheral venous blood samples of each volunteer were collected using a clinically approved butterfly scalp in the respective blood collection tube. Immediately after collection, the blood was centrifuged using a table centrifuge (Spinlab®/ Spin 5000-8). The centrifugation process was initiated four minutes after blood collection started.

After centrifugation, the fibrin clots obtained from glass and silica-coated plastic tubes were separated from the bottom red fraction and softly compressed with a stainless-steel PRF compression device. The fluid material from the plastic tubes without additives was collected with a syringe/needle and transferred to glass recipients (4 mL), in order to accelerate the fibrin network polymerization process. A period of 15 min was expected for the generated membranes to be compressed and processed.

Each generated membrane was divided into two equal sections. One section was fixed in a solution of 10% phosphate-buffered formaldehyde for 24 h and embedded

in paraffin for light microscopy analysis. The other section was divided into small fragments, fixed in glutaraldehyde for 24 h, post-fixed in 1% osmium tetroxide solution for one hour, and then embedded in Epon resin for transmission electron microscopy (TEM) analysis.

Histomorphometric and Ultrastructural analysis

Six 5 μm-sections were obtained from the paraffin-embedded samples, which were stained using the modified Carstair's method (solutions: Harris' Hematoxylin, Picro-Orange G, Ponceau-Fuchsin, Phosphomolybdic-Phosphotungstic Acid Solution, Aniline Blue). Histological images were captured with an Aperio AT Turbo Scanner (Copyright © 2013 Leica Biosystems Imaging, Inc. All rights reserved) at 20x magnification. Thereafter, the images were visualized using Aperio ImageScope software (Copyright © Aperio Tecnologiches, Inc. 2003-2014. All rights reserved), and three regions of interest (ROIs) were delimited according to the staining pattern identified by visual inspection, considering the following cell and fibrin network density: low density (D1), medium density (D2), and high density (D3) (Figure 1A). The corresponding images were extracted and converted into TIFF format with a 500 × 500 area, in order to analyze them using the ImageJ software (1.52 version) (Figure 1B). The screenshots were converted to binary images for threshold execution, and fibrin network density (Figure 1C) and platelet aggregate density (Figure 1D) were quantified from each delineated ROI.

For TEM analysis, ultrathin 60 nm-sections were obtained (Reichert-Jung Ultra-cut 701701, Leica Microsystems®, Wetzlar, Germany) and double contrasted with uranyl acetate 3% and lead citrate 1%. The sections were analyzed using a transmission

electron microscope (HT7700, Hitachi®, Tokyo, Japan), and the cellular membrane, organelles, and nuclei integrity were assessed.

Statistical analysis

Statistical analysis was performed using Sigma Plot 13.1 (Systat Software Inc., San Jose, CA, USA). The results obtained were subjected to a Shapiro-Wilk normality test and two-way analysis of variance followed by Tukey multiple comparisons test, and results were presented as mean and standard deviation (SD). Differences were considered to be statistically significant at $\alpha < 0.05$.

RESULTS

Light microscopy analysis showed a delicate fibrin network with trapped leukocytes and platelet aggregates, irrespective of the tube material or protocol. Histomorphometric analysis revealed statistically significant differences in fibrin network and platelet aggregate densities between the tubes, considering each PRF protocol. Table I shows the means and standard deviation values for fibrin network density, and Table II shows the mean and standard deviation values for platelet aggregate density.

In terms of the fibrin network density obtained with different protocols, the membranes obtained from plastic tubes without additives exhibited significantly lower values for L-PRF ($p < 0.019$) and A-PRF ($p < 0.001$) (Figure 2A, B). For the A-PRF+ protocol, plastic tubes without additives showed lower fibrin network density as well; however, statistical differences were only observed in silica-coated plastic tubes ($p < 0.001$) (Figure 2C). Between glass and silica-coated tubes, there were statistical

differences only in the A-PRF+ protocol, and the glass tube showed a lower value ($p < 0.001$).

The analysis of platelet aggregate density revealed a significantly higher percentage of platelets in the membranes from plastic tubes without additives than in those of other tubes for all protocols ($p < 0.005$) (Figure 3). Between glass and silica-coated tubes, there were statistical differences only in the L-PRF protocol, and the glass tube showed a lower value ($p = 0.002$).

Under the transmission electron microscope, regardless of the protocol or tube material, undamaged cells with morphology similar to that of circulating blood cells were observed. The nucleus morphology from each leukocyte was characteristic and, in some of them, the euchromatin fraction was low. Most cells showed continuous cell membranes and into neutrophils and eosinophils the cytoplasm was filled with dark and dense granules. Occasionally, an increased space between nuclear membranes and some evident pores were observed. No ruptured or apoptotic-like cells were observed (Figure 4).

Platelets appeared as aggregates of heterogeneous shapes and sizes. It was possible to observe some vacuoles and eventually few mitochondria, as well as glycogen accumulation in platelets. Platelet aggregates and thick bundles of fibrin were linked in the cytoplasm (Figure 5).

DISCUSSION

The present study evaluated the effect of different blood collection tubes on the formation of solid PRF matrices obtained using distinct RCF protocols by histomorphometry and ultrastructural analyses. The hypothesis of the study was not

confirmed because the analysis showed significant differences for matrices obtained from distinct types of tubes, considering each PRF protocol. The data indicated higher platelet aggregate density in membranes from plastic tubes without additives, regardless of the protocol used. In addition, fibrin network density was comparable between the membranes obtained from the three types of tubes. Therefore, plastic tubes without additives may be a clinical alternative for silica-coated and glass tubes in the production process of blood concentrates for regenerative therapies.

It has long been known that human blood exhibits coagulant properties when exposed to glass and analogous surfaces by activation of the coagulation factor XII[23]. However, plastic surface contact triggers blood coagulation at reduced levels[24], such that the material isolated from blood samples after centrifugation in plastic tubes without additives is immediately obtained in fluid consistency. A solid clot might form in the plastic tube after a long time; however, this clot is quite fragile and no apparent consistent membrane can be formed. Considering these facts, it is possible to accelerate the polymerization of PRF obtained from plastic tubes without additives by maintaining the freshly prepared fluid material in intimate contact with a glass surface. This method was evaluated in the present study as a possible alternative to the method using silica-coated plastic tubes, which may pose health hazards.

The fibrin network density analysis revealed that membranes produced in plastic tubes without additives exhibited a slightly lower fibrin network density for each evaluated protocol compared to that of matrices obtained from glass and silica-coated plastic tubes. Fibrin formation has been characterized to be modulated by a series of factors, including environmental conditions, which are thought to affect the kinetics of individual polymerization steps, resulting in distinct clot structure and properties[25, 26]. As

centrifugation parameters remained constant in each protocol and only the type of blood-collection tube differed, we may infer that the latency in polymerization provided by the plastic surface influenced the fibrin network density.

Plastic tubes exhibit reduced coagulation properties as a hydrophobic procoagulant material. Upon contact with a plastic surface, coagulation factor XII activation is decreased due to the adsorption of abundant blood proteins[24]. This phenomenon leads to a delay in the blood clotting process and, consequently, in fibrin network formation, probably resulting in limited conversion of fibrinogen to fibrin; additionally, unreacted molecules are lost as they remain at the base of the tube at the end of centrifugation. However, it should be noted that this minimal difference might not result in clinically relevant effects, which requires further investigation. Nevertheless, this could interfere with the mechanical resistance of the clot, impairing its handling during production process of PRF membranes and plugs.

The quantification of platelet aggregates, represented by the accumulation of platelets formed after their activation, showed that the number of platelets was considerably increased for each protocol evaluated in plastic tubes without additives when compared to that in plain glass tubes or even silica-coated plastic tubes. This phenomenon can be explained by the fact that there is no artificial acceleration in the coagulation process when plastic tubes are used, which allow it to occur in a way that is more similar to the natural process.

This finding is clinically relevant as platelets play a key role in tissue repair and regeneration by providing an extensive range of biomolecules. Numerous growth factors, cytokines, and chemokines that regulate essential mechanisms related to the healing process have been identified to be stored and released from blood platelets[27, 28].

These molecules are responsible for stimulating angiogenesis, cell recruitment, proliferation, and differentiation, as well as contributing to the immune response and in maintaining the balance between cell death and survival[29].

It was further observed that for certain protocols, glass-prepared matrices exhibited lower fibrin network density (A-PRF+) or reduced platelet aggregate density (L-PRF) when compared to silica-prepared matrices. It has been previously proposed that differences in the mechanism of matrix formation modify platelet distribution depending on the type of tube used. In plain glass tubes, the coagulation process is thought to occur mainly on the distal wall, resulting in platelet accumulation on the distal surface of the matrices. In contrast, silica microparticles detached from silica-coated plastic tubes, which are found suspended in blood samples, create diversified activation spots for the coagulation cascade; therefore, fibrin formation, as well as platelet distribution, occurs homogenously[16]. This mechanism is more evident in high-speed centrifugation protocols and, besides modifying platelet distribution, may affect platelet aggregate and fibrin network densities.

The toxicity of silica particles has long been discussed in the scientific context. Despite its extensive application as nanoengineering tools, with specific forms acting as scaffolds for drug and bioactive factor delivery[30] or even demonstrating inherent bioactivity in tissue repair[31], the available evidence is not sufficient to accurately determine the extent of adverse biological effects related to exposure to this synthetic material[32]. It is well accepted that a variety of factors, including particle crystallinity, aggregation aspect, size, surface area, concentration, period and mode of exposure, as well as cell type, influence a material's toxicity potential[33, 34]. However, the underlying mechanisms are not fully understood.

Commercially available silica-coated plastic tubes were specifically developed for laboratory testing purposes. Therefore, they are not subjected to accurate quality control considering PRF manufacturing parameters, and information with respect to silica crystallinity is rarely disclosed. Amorphous silica has been reported as the most likely coating option because it is expected to be less harmful than other crystalline forms[15, 21].

Amorphous silica nanoparticles have been frequently described as inducers of cytotoxicity, genotoxicity, and immunotoxicity, and are considered inflammatory response stimulating agents[17-20]. In fact, silica microparticles that detached from silica-coated plastic tubes were identified to significantly reduce human periosteal cell proliferation and viability by inducing apoptosis in a recent *in vitro* study and could possibly impair tissue regeneration[21].

In view of the possible hazardous effects of silica-coated plastic tubes, research groups have focused on the development of alternative methods. Titanium tubes have been reported to effectively stimulate platelet activation and produce fibrin matrices with histological structures similar to that of L-PRF[35, 36]. However, its high cost implies considerable limitations. Additionally, the use of hybrid plastic tubes containing collagen I-like protein-based particles intended to induce platelet activation is another valid method for PRF-like matrices preparation; however, such material is not yet available for acquisition[37].

The method demonstrated in this study is a safe approach for PRF-like matrix manufacturing. It is a low-cost alternative that does not require the addition of synthetic agents and preserves the integrity of blood cells, as no cell damage was observed. Furthermore, it enriches blood concentrates with platelets, which play an essential role in

the tissue repair process. It should also be noted that the additional time required for fibrin network polymerization optimizes surgical time management and can be utilized as extra time for surgical field preparation. These facts support the use of plastic tubes without additives as a valuable strategy to obtain solid PRF matrices. However, further investigation is required to clarify the growth factor release patterns and intrinsic properties, such as membrane resistance.

COCLUSION

The present study showed that matrices produced in plastic tubes without additives exhibited a slightly lower fibrin network density, which might not be clinically relevant. However, platelet aggregate density significantly increased in blood concentrates obtained from plastic tubes when compared to that of concentrates from plain glass and silica-coated plastic tubes. Based on the results and considering the increasing concern regarding silica toxicity, plastic tubes without additives might represent an alternative to silica-coated plastic tubes.

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Table I. The means and standard deviation values of fibrin network density according to different protocols and tubes

Protocol*	Glass tube	Silica-coated plastic tube	Plastic without additives tube
L-PRF	72.9 ± 2.2*	72.3 ± 3.1*	70.2 ± 3.2**
A-PRF	71.9 ± 3.4*	73.5 ± 3.1*	67.8 ± 3.2**
A-PRF+	69.4 ± 4.1**	73.3 ± 4.5*	68.3 ± 4.5**

* and ** within a row indicate significant differences in fibrin network density ($p < 0.05$).

*Protocol: L-PRF (leukocyte - and platelet-rich fibrin), A-PRF (advanced platelet-rich fibrin), A-PRF+ (advanced platelet-rich fibrin +).

Table II. The means and standard deviation values of platelet aggregates density according to different protocols and tubes

Protocol*	Glass tube	Silica-coated plastic tube	Plastic without additives tube
L-PRF	17.4 ± 2.7*	20.0 ± 4.1**	22.5 ± 2.4***
A-PRF	18.8 ± 2.9**	18.9 ± 2.9**	24.2 ± 4.3***
A-PRF+	21.6 ± 4.6**	20.0 ± 3.3**	24.2 ± 3.6***

*, **, and *** within a row indicate significant differences in platelet aggregate density ($p < 0.05$). *Protocol: L-PRF (leukocyte - and platelet-rich fibrin), A-PRF (advanced platelet-rich fibrin), A-PRF+ (advanced platelet-rich fibrin +).

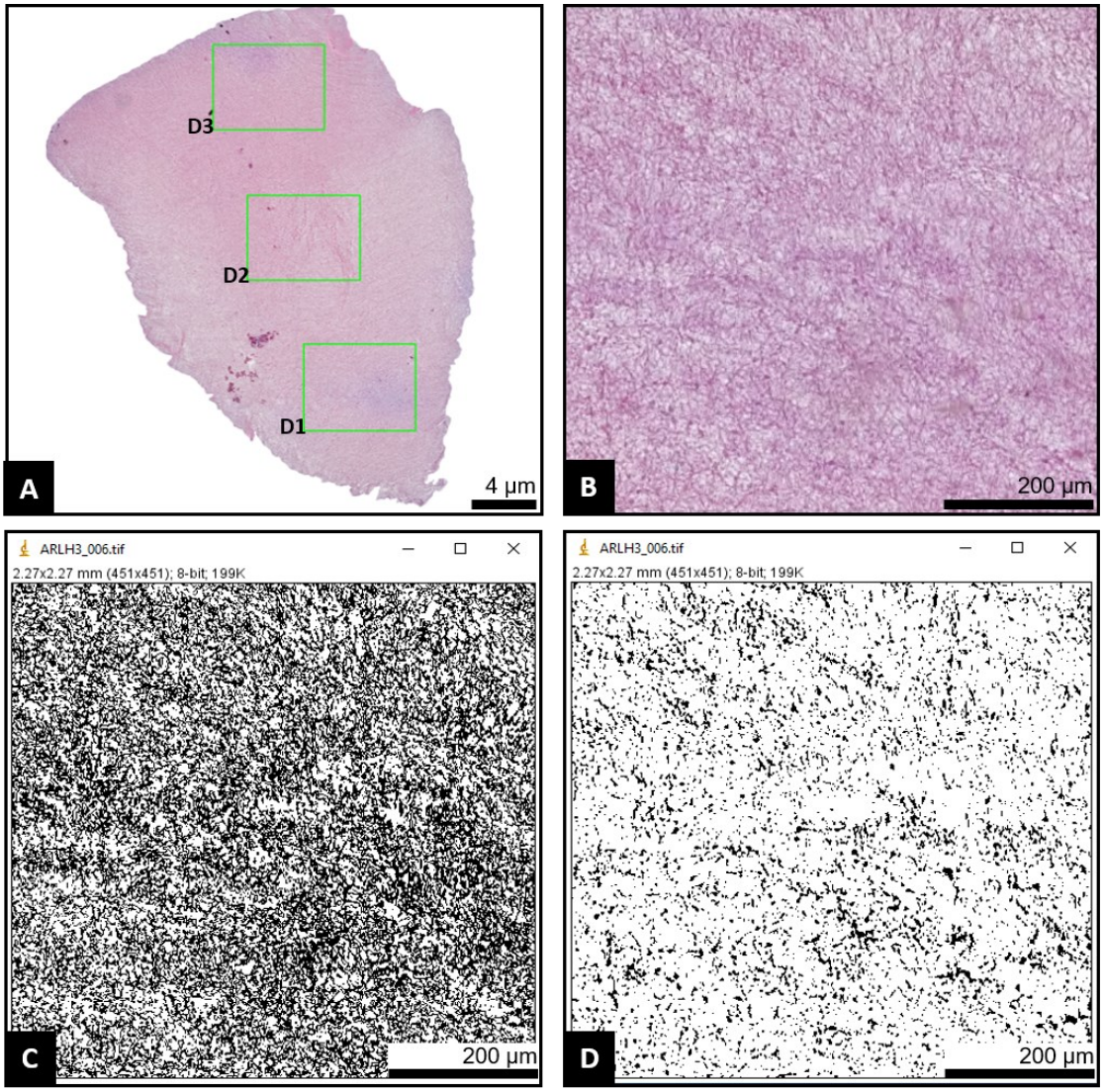

Figure 1. Image of a platelet-rich fibrin membrane stained using Carstair's method showing the three (D1, D2, and D3) regions of interest (A), one of the extracted areas (B), after conversion to a binary image for analysis of fibrin density (C) and platelet aggregates (D).

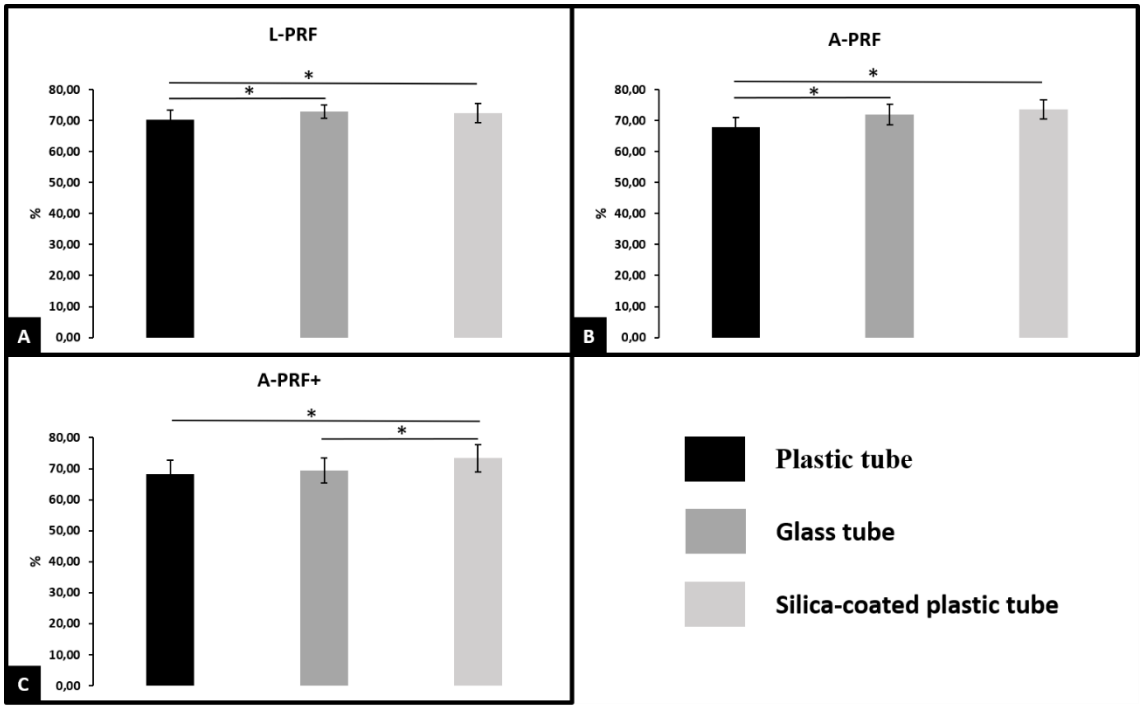

Figure 2. Fibrin network density percentage per tube material within individual protocols. *p < 0.05. L-PRF, leukocyte - and platelet-rich fibrin; A-PRF, advanced platelet-rich fibrin; A-PRF+, advanced platelet-rich fibrin+.

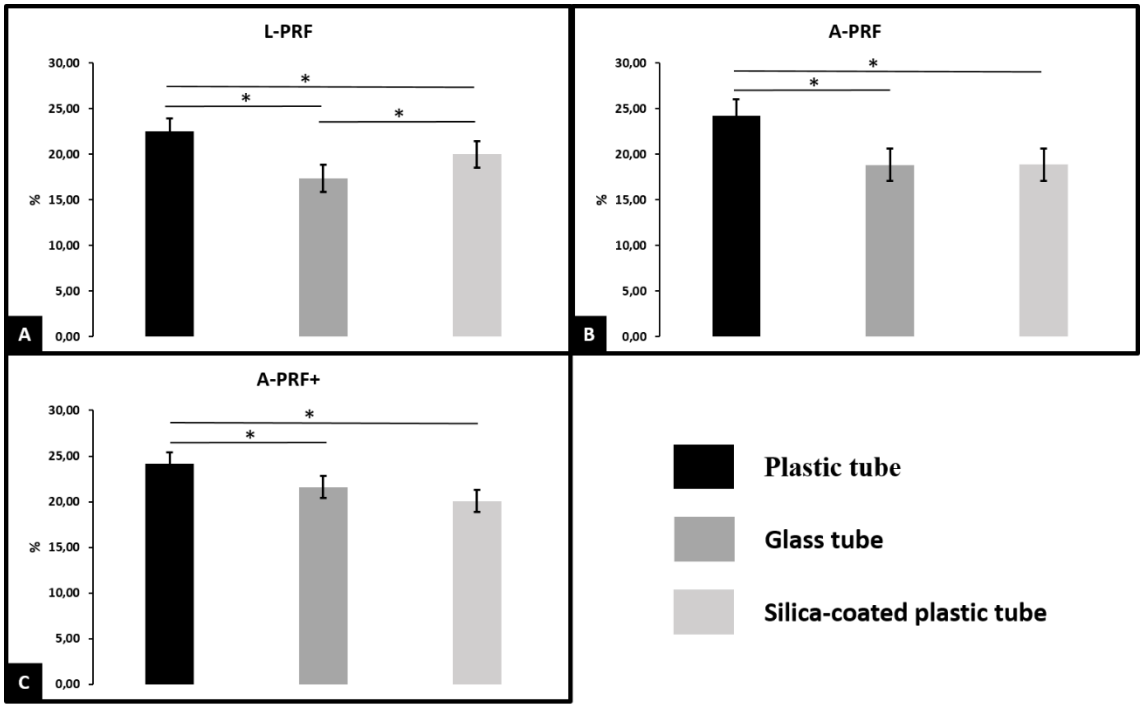

Figure 3. Platelet aggregates density percentage per tube material within individual protocols. *p < 0.05. L-PRF, leukocyte - and platelet-rich fibrin; A-PRF, advanced platelet-rich fibrin; A-PRF+, advanced platelet-rich fibrin+.

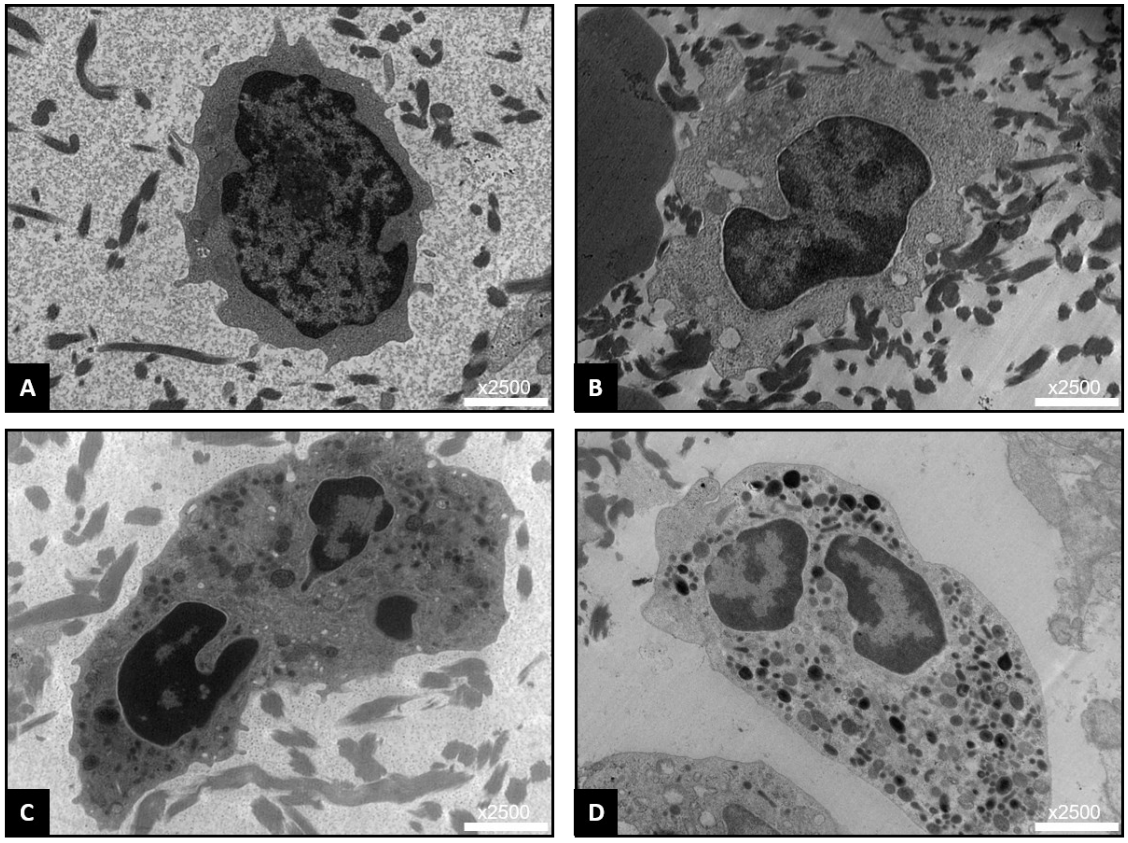

Figure 4. Gallery of transmission electron microscopy images of leukocytes exhibiting a characteristic aspect: lymphocyte (A), monocyte (B) and neutrophils (C, D) (2500x magnification).

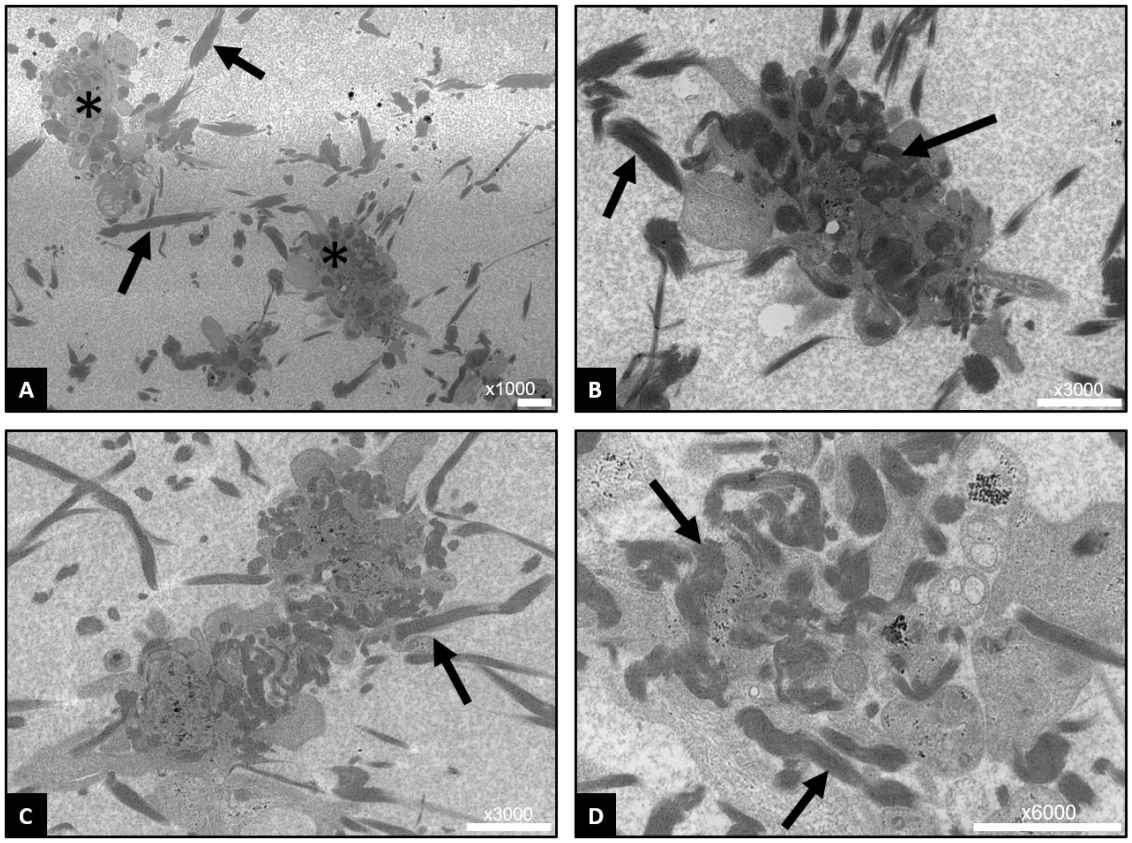

Figure 5. A. Transmission electron microscopy images of platelet aggregates (asterisks) (1000x magnification). B-D. Platelet aggregates associated with fibrin bunds (arrows) and glycogen accumulation. Magnification x3000 (B, C); x6000 (D).

CONCLUSÃO

3. CONCLUSÃO

O uso de tubos plásticos sem sílica para a produção de membranas de PRF se mostrou possível e promissor como ferramenta de enriquecimento plaquetário. Tal fato não descarta a necessidade de investigações aprofundadas em relação à liberação de fatores de crescimento, biocompatibilidade, atividade celular e propriedades mecânicas intrínsecas ao material.

Perspectivas futuram envolvem o uso de tubos plásticos sem sílica para a confecção de grandes membranas de PRF. O conteúdo fluido de diferentes tubos é incorporado em uma membrana única de maior extensão, possibilitando manejo tecidual otimizado durante o recobrimento de leitos extensos e redução do tempo cirúrgico.

Ressalta-se que o uso de concentrados sanguíneos como terapia adjuvante no processo de reparo tem sido extensivamente investigado ao longo dos anos, com o propósito de se obter protocolos aperfeiçoados. Entretanto, no cenário científico atual é evidente a disparidade entre achados laboratoriais e resultados clínicos efetivos. Tal fato reflete a necessidade de padronização de protocolos de preparo e aplicação clínica, somada a estudos adequadamente delineados e sem qualquer interferência de cunho comercial.

Além disso, trata-se de um método que requer entendimento biológico aprofundado em relação ao comportamento plaquetário. Aspecto muitas vezes negligenciado e que produz viés em potencial, refletindo na diversidade de resultados presentes na literatura.

REFERÊNCIAS

* De acordo com a Norma da FOUFU, baseado nas Normas de Vancouver. Abreviaturas dos periódicos com conformidade com Medline (Pubmed).

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ANEXOS

ANEXO 1

UNIVERSIDADE FEDERAL DE UBERLÂNDIA/MG

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: CARACTERIZAÇÃO MORFOLÓGICA DE CONCENTRADOS SANGUÍNEOS DE A-PRF. ESTUDO MORFOLÓGICO POR MICROSCOPIA DE LUZ E ELETRÔNICA DE TRANSMISSÃO

Pesquisador: Paula Dechichi

Área Temática:

Versão: 1

CAAE: 13857519.0.0000.5152

Instituição Proponente: Universidade Federal de Uberlândia/ UFU/ MG

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 3.347.524

Apresentação do Projeto:

Conforme apresenta o protocolo: Os concentrados sanguíneos consistem em uma matriz autóloga de fibrina rica em plaquetas e leucócitos, composta por uma estrutura molecular, com citoquinas e células tronco que favorecem o desenvolvimento da microvascularização e, consequentemente, a neoformação óssea e a regeneração tecidual. Podem ser utilizados de maneira isolada ou em associacao com enxerto osseo como material de preenchimento em alveolos pos-extracao ou tecnicas de levantamento do seio, sendo que com a sua arquitetura de fibrina e lenta liberacao de fatores de crescimento e glicoproteinas ao longo de varios dias, pode melhorar o reparo de tecidos moles e duros enquanto protege sítios cirurgicos e materiais enxertados.

METODOLOGIA

=>População: O estudo será conduzido no Laboratório de Biologia Celular localizado no Centro de Pesquisas Odontológico, Biomecânica, Biomateriais e Biologia Celular (CPbio) da Universidade Federal de Uberlândia (UFU).Os voluntários serão selecionados a partir de chamada através de cartazes e informativos no âmbito do hospital odontológico e faculdade de odontologia da UFU de 30/06/2019 a 30/10/2019.

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