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Histological Preparation Technique of Blood Derivative Injectable Platelet-Rich Fibrin (I-Prf) for Microscopic Analyzes

Hugo de Almeida Varela*, Raimundo Fernandes de Araujo Jr, Roseane Carvalho Vasconcelos, Vinicius Barreto Garcia, Lelia Batista de Souza and Aurigena Antunes de Araujo

Department of Biophysics and Pharmacology, Centre of Biosciences, UFRN, Av. Senador Salgado Filho, S/N, Campus Universitário, Lagoa Nova, 59072-970, Natal, RN, Brazil

***Corresponding author:** Hugo de Almeida Varela, Department of Biophysics and Pharmacology, Centre of Biosciences, UFRN, Av. Senador Salgado Filho, S/N, Campus Universitário, Lagoa Nova, 59072-970, Natal, RN, Brazil, Tel: +55843215-3419; E-mail: hugo_varela@hotmail.com

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Abstract

Objective: The objective of the present study was to describe the histological preparation technique of blood derivative injectable platelet-rich fibrin (i-PRF) for microscopic analyzes.

Materials and methods: Blood samples were collected from 15 volunteers to prepare i-PRF samples. The difference between i-PRF and oral mucosa tissue experimental protocol for histological preparation was elaborated in Step 1 - fixation, with 01 hour for i-PRF fixation and 24 hours for oral mucosa tissue. The other histological preparation protocol steps used for the processing and staining of the specimens were similar to that used for oral mucosa tissue. Tissue and i-PRF samples were analyzed by immunofluorescence, immunohistochemistry and microscopic histology.

Results: In comparing i-PRF and oral mucosal tissue, it was observed that both had similarities in morphology and coloration of the visualized structures. The technique demonstrates immunostaining regulation of TGF-β from i-PRF. VEGF was fully detectable using the immunofluorescence technique applied to i-PRF. The method showed a fundamental advantage in reducing the fixation time, since the established fixation time in oral mucosa tissue is at least 24 hours. For the i-PRF, it was observed that the fixation was 01 hour, so the reduced fixation time shows an advantage in reducing the total working time without compromising the quality of the analyzed samples.

Conclusions: The method showed a distinct advantage in reducing the fixation time of i-PRF. These initial analyses revealed that the slow polymerization during i-PRF preparation generates a blood derivative with a special fibrin network rich in platelets and leukocytes, and the presence of immunostaining for TGF-β and immunofluorescence of VEGF.

Keywords: Platelet rich fibrin; Morphology; Histopathology; Human organism

Introduction

The search for ways to accelerate wound healing is constant in the medical area, and with this the influence of growth factors released by leukocytes and platelets on the biomaterials applied in the human organism has long been researched [1]. This evolution began in the late 1990s with the release of platelet rich plasma (PRP) [2], followed by the second generation of platelet aggregates, platelet rich fibrin (PRF) [3], and recently the production of platelet-rich fibrin for use in its liquid form (i-PRF) [4]. Traditionally, analyzes of cell constituents and growth factors present in PRP, PRF and i-PRF are performed through cell counts by flow cytometry and ELISA immunoassay. Due to its physical and biological properties (mainly solubility and high dispersion of the cellular components in the serum), the blood derivative called i-PRF requires an innovative protocol to be used by histopathological examination, and is not yet described in the literature. Histological processing of a blood derivative such as i-PRF opens up new opportunities for biomedical applications in diagnostic therapies from blood samples. The present study refers to the processing steps for histological, immunohistochemical and immunofluorescence analysis to perform i-PRF diagnosis. Once the use of i-PRF is microscopically well-characterized, it can be extended to other applications in plastic surgery, as well as orthopedic and oral surgery. Due to characteristics such as biocompatibility, simplified preparation techniques, the possibility of infiltration in several tissues, as well as association with other biomaterials, this platelet derivative presents a range of possibilities in the medical field. There are currently only a few studies describing the morphological and cellular aspects present in i-PRF [4-6]. The aim of this article was therefore to describe the histological preparation technique of blood derivative injectable platelet-rich fibrin (i-PRF) for histological, immunohistochemistry and immunofluorescence microscopic analyzes.

Material and Methods

All performed procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical Standards (Ethical Protocols Number 1.817.827). This study was reviewed and approved by an institutional review board. Peripheral venous blood (27 mL) was collected from all 15 healthy adult male volunteers (age range 25-38

years) upon signing an informed consent form. Smokers or patients with a history of using anti-coagulant or anti-inflammatory drugs in the previous 4 weeks were excluded from the study. Samples of oral mucosa from other patients were obtained from a database of histological parts for comparison purposes.

Preparation of i-PRF samples

For the analysis of i-PRF, 27 mL of blood was collected in three 10 mL glass-coated plastic tubes (Vacutainer; BD Biosciences, Allschwil, Switzerland) and immediately centrifuged at 400g (700rpm) for 3 minutes at room temperature using a table centrifuge specifically designed for this application (Intra-spin system, Intra-Lock, Boca-Raton, FL, EUA). The upper yellow fluid (i-PRF) liquid layer was collected immediately after centrifugation as close as possible to the red cells and processed as further described (Figure 1).

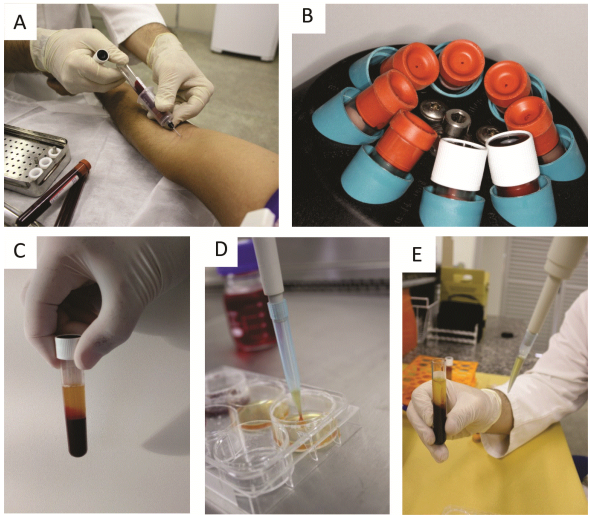

Figure 1: i-PRF preparation: (A) blood collection; (B) centrifugation machine; (C) aspect after centrifugation; (D,E) separation.

Histological analysis

Three samples of 2ml from i-PRF (total of three samples per volunteer) were transferred to separate wells in 6-well plates and left immobilized for 20 minutes for total fibrin polymerization. Then they were covered with 2ml DMEM and incubated in 5% CO_2 and 37% CO_2. All well material was collected in time intervals of 1 hour, 3 days and 7 days, washed 3 times in PBS and fixed in 10% buffered formaldehyde. The protocol used for the processing and staining of the specimens was similar to that used for tissues.

Step 1: Fixation - the isolated i-PRF clots were transferred into a medium sized perforated plastic cassette inside which a small chit was placed containing the patient number written with a lead pencil for the purpose of easy identification (Figure 2). The cassettes were transferred into a container containing 10% formalin where they were fixed for 30min. The aim of fixation is to preserve the biological tissues in a life-like state, thereby preventing autolysis or putrefaction. After this time the samples were longitudinally sectioned and again fixed for another 30 min. The purpose of sectioning is to allow penetration of the fastener throughout the interior of the sample. For oral mucosa samples the cassettes were fixed in 10% formalin for 24 hours.

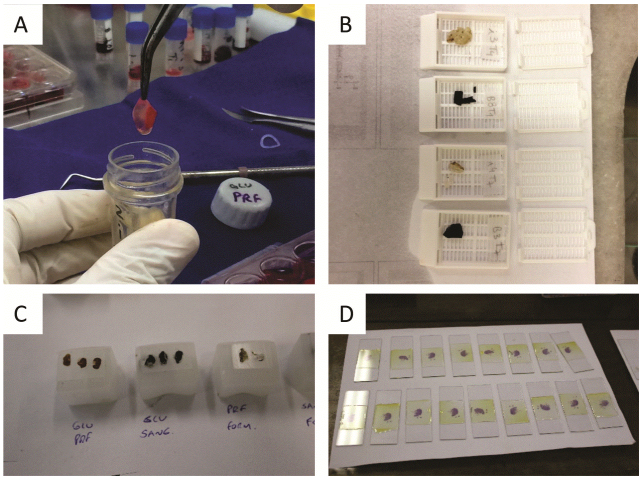

Figure 2: i-PRF clot slide preparation for histological analysis (A,B) fixation and labeling of i-PRF; (C) paraffin blocks; (D) prepared slide.

Step 2: Tissue processing - after the fixation process, the cassettes were transferred into a perforated plastic cylindrical container which were subjected to various steps such as dehydration, clearing, and infiltration of wax into the PRF clot as they passed through various processing solutions such as 10% formalin, 50%, 75%, 95% and 100% isopropanol alcohol, xylene (two changes) and paraffin wax in an orderly manner. Tissue processing was performed for 5 h using a manual process. The aim of tissue processing is to remove water from tissues and replace it with a medium that solidifies to allow thin sections to be cut.

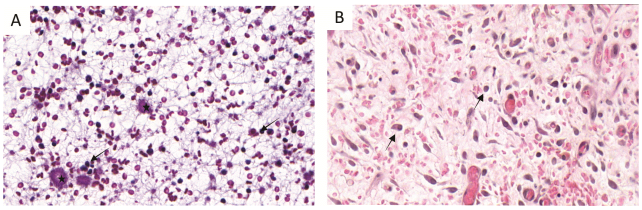

Figure 3: Representative microscopic findings of i-PRF (A) and oral mucosa (B). I-PRF images presents numerous leukocytes (lymphocytes)(arrows) of usual aspects and the presence of fibrin clumps (stars) associated with platelet aggregation. Sample of oral mucosa exhibiting leukocytes (arrows) (Scanner blades Panoramic Viewer – H&E, 400x).

Step 3: Embedding - Blocks were used as molds for embedding tissue using paraffin wax. Embedding grants support to the tissue section for sectioning and for producing a slide (Figure 2C).

Step 4: Tissue sectioning - sectioning was done using Leica microtome and 4µm-thick sections were sliced.

Step 5: Dewaxing - sections were first deparaffinized by heating the slides to about 55-65°C and then then were immediately dropped into

xylene to eliminate wax. The purpose of dewaxing is to allow the tissues to be stained.

Step 6: Tissue staining - sections were stained using hematoxylin and eosin stain. Staining is employed to give contrast to the tissue as well as highlighting particular features of interest (Figure 3).

Step 7: Histological slide analysis was carried out using a compound microscope at 400x magnification.

Imunohistochemistry

For immunohistochemical and immunofluorescence techniques (after step 4 as previously described) the sections were first deparaffinized by heating the slides to about 55-65°C for a period of 24 hours prior to preparation. In the exhaust hood, the slides are subjected to two 7-min dives in xylene. Thereafter, rehydration of the slides is carried out by immersions in decreasing concentrations of ethyl alcohol. Two dives should be performed for a period of 4 min in absolute ethyl alcohol (P.A.), followed by a dip for a period of 4 min in each alcohol solution (90%, 70% and 50%). After immersion in alcohol, the slides should be washed in distilled water twice.

There was no difference in the preparation of i-PRF samples when compared to tissue for immunohistochemistry and immunofluorescence analyzes. For immunohistochemical technique: Slides are immersed in proteinase K solution for 15 minutes for antigen retrieval, then washed with PBS for 3 minutes. Blocking of the peroxidase is performed through two incubations with 3% hydrogen peroxide, then washing with distilled water. Protein block (0.05% BSA in PBS) is then applied for 02 hours. The slides are washed in distilled water and then washed in PBS for further application of TGF-β specific primary antibody (at 1:400 dilution) for 18 hours. After this period, the excess of the antibody with PBS is removed and incubated with secondary antibody (LSAB+System-HRP) for 15 minutes. The slides are then washed in PBS and then the DAB is applied for 15 minutes, after which the HRB-Conjugate is applied for a further 15 minutes and the slides are washed in PBS. Thereafter, further incubation with DAB is carried out for 10 minutes, and the samples are washed in distilled water for further counter-staining with Mayer's hematoxylin for 5 minutes. The slides are subsequently washed in running water for 10 minutes, followed by dehydration through 1 bath in 95% alcohol for 10 seconds and three consecutive baths for 10 seconds each in absolute alcohol. Three washes of 10 seconds each are carried out in xylol for clarifying and posterior assembly of the blades in permount resin. Immunohistochemical staining for TGF-β antibody can be seen in Figure 4.

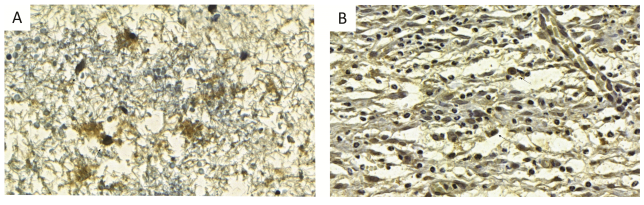

Figure 4: (A) The histological sections show i-PRF exhibiting immunostaining for TGF-β present in cytoplasm and nucleus of leukocytes (arrows). (B) Discrete immunostaining in cytoplasm and nucleus of infamous cells of oral mucosal tissue (arrows) (Scanner blades Panoramic Viewer, LSAB, 400x).

Immunofluorescence

For the immunofluorescence technique: after the rehydration process, the slides should be immersed in citrate solution (0.15% and pH 6.0) and left in a water bath at a temperature of 90°C for one period of 30 min, then cooled at 22°C for a period of 20 min. After this period, the background reduction should be carried out by immersing the slides in 0.1% Sudan Black solution diluted in 70% alcohol for a period of 20 min. For removal of excess Sudan Black, the slides should be washed three times over a period of 4 min in a 0.02% Tween 20 solution diluted in PBS. After this procedure, the slides are permeabilized in 0.1% PBS-Triton-x-100 solution prepared with a volume of 20 mL of PBS with 180 mL of distilled water and 400 µl of Triton-x-100 (added while solution is stirred). The slides are immersed in this solution for three dives of 5 min each and then washed once with PBS for 5 minutes. The blocking solution, consisting of 2.5 ml of PBS, 1.25 ml of 5% BSA, 21.25 ml of distilled water and 75 µl of triton-x-100, should be added directly onto each slide. The slides should be allowed to stand for 2 hours in a humid chamber, and after that time washed in PBS for a period of 5 min for removal of excess blocking solution. Incubation of the anti-VEGF primary antibody diluted in 1% BSA in a 1: 500 ratio is performed and applied directly onto the slides, which remain in the refrigerator overnight. The slides are washed in PBS 3 times for 5 min each so as to remove the excess of primary antibody. Thereafter, the Alexa-Fluor 488 antibody is added at a 1:500 dilution. After incubation for a period of 2 h, the slides are washed in PBS 3 times for 5 min each. For assembly, the mounting medium is used with DAPI ab104139 (Abcam, Cambridge, UK). The slides were read in a Carl Zeiss Laser Scanning Microscope (LSM 710, 400x objective, Carl Zeiss, Jena, Germany). Quantitative analysis of the fluorescence intensity was determined from digital images of at least 3 different areas of each section (three samples per volunteer) at 400x magnification using Zeiss ZEN lite blue edition software. The labeling of the Alexa-Fluor 488 secondary antibody is observed in green and the nuclear labeling by DAPI is in blue.

Results

Histological analysis

From the HE staining, it can be seen that i-PRF presents a platelet-rich fibrin mesh surrounded by leukocytes (mainly lymphocytes) and some erythrocytes (Figure 3A).

Individually, the cells presented normal characteristics such as: Membrane integrity and normochromatism, demonstrating absence of atypia and preservation of morphological aspects. Fibrin was arranged in thin, tortuous, and eosinophilic filaments that sometimes formed eosinophilic clusters compatible with fibrin clumps and platelets. In comparing i-PRF with oral mucosal tissue, it presented similarities regarding the aspects of cellular staining by the HE technique (Figure 3B).

Immunohistochemistry analysis

After the culture interval for 7 days, i-PRF preserved its morphological characteristics. The findings demonstrate immunostaining for TGF-β factor (Figure 3C) from the i-PRF analyzes on the third day of the experiment, mainly at the cytoplasm and leukocyte nucleus. Immunoblotting was detected by the presence of moderatelyintense brownish staining in the nucleus and cytoplasm of leukocytes, demonstrating that i-PRF showed cellular

immunoreactivity similar to that observed in oral mucosal tissue cells (Figure 4).

Immunofluorescence

Cellular VEGF labeling (green) was fully detectable using the immunofluorescence technique applied to the iPRF (Figure 5). VEGF is fully detectable using the immunofluorescence technique applied to the i-PRF. The fluorescence labeling of the Alexa-Fluor 488® secondary antibody directed to the primary anti-VEGF antibody is shown in green. The leukocyte nuclear labeling with DAPI is observed in blue.

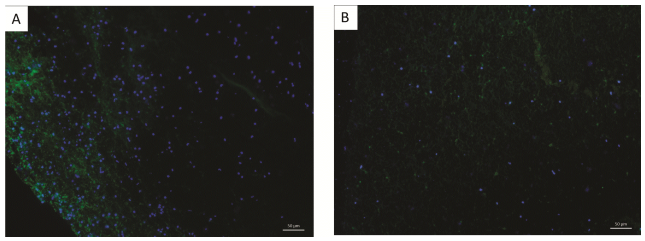

Figure 5: (A) VEGF is fully detectable using the immunofluorescence technique applied to the i-PRF. The fluorescence labeling of the Alexa-Fluor 488® secondary antibody directed to the primary anti-VEGF antibody is shown in green. The leukocyte nuclear labeling with DAPI is observed in blue. (B) Fluorescence labeling for VEGF in oral mucosa tissue (Zeiss ZEN lite blue edition software, 400x).

Discussion and Conclusion

The cell composition of PRF implies that this biomaterial is a blood-derived living tissue and must be handled carefully to keep its cellular content alive and stable [7]. The platelets and leukocytes in the i-PRF also corresponded to what has been reported in the literature [8]. It is believed that the difference between leukocytes, mainly between neutrophils and lymphocytes, is due to the difference between the molecular weights of these cell groups, with the centrifugation process causing the presence of lymphocytes in the i-PRF group [9]. This can also be found in papers comparing the cells between PRP, PRF and peripheral blood [10-14]. Regarding PRF in the literature, leukocyte populations have been pointed out several times [12,15-18]. The role of leukocytes is very important for understanding the biology of a complex biomaterial like i-PRF, but this is also a key issue for potential *in vitro* tissue engineering with i-PRF.

Macroscopic findings demonstrate that although i-PRF is initially in liquid form, it initiates a fibrin polymerization process by acquiring a gel form after about 20 minutes. We performed the cell block histological method to study morphological aspects of i-PRF, the fibrin networks, and the efficacy in their ability to entrap the platelets and WBCs; according to our knowledge, this is the first time this has been tried to histologically study i-PRF to date. This method was performed using hematoxylin and eosin stain, and proved to be satisfactory in identifying the fibrin network pattern and distribution of platelets and leukocytes entrapped within them. The histological findings show uniform leukocyte (mainly lymphocytes) and platelet conglomerate distribution throughout the analyzed specimen, unlike the histological findings of PRF [12,13,19,20] where the distribution of these cell groups is demonstrated in conglomerates and non-uniformly. This preliminary study allowed us to define the main cell and matrix characteristics of i-PRF, as a clear definition of the i-PRF composition was an essential prerequisite to guarantee technique reproducibility and to allow future investigations on a clearly identified and reproducible standardized protocol. When comparing the i-PRF with oral mucosal tissue, which represents a material with an already established protocol for analysis by HE, according to the histological aspects it was observed that both had similarities as to the morphology and coloration of the visualized structures. Likewise in immunohistochemistry and immunofluorescence techniques, similar leukocyte immunoreactivity was observed to that detected in inflammatory cells of the mucosal tissues, demonstrating the feasibility of using these techniques to analyze i-PRF.

The method showed a fundamental advantage which is reduced fixation time, since the established fixation time in conventional tissue is at least 24 hours for the i-PRF, and herein it was observed that the fixation time of 01 hour was similar to that found in the 24 hour time, so the reduced fixation time shows an advantage in reducing the total working time without compromising the quality of the analyzed samples. We believe that this is due to the penetration capacity of 10% formaldehyde in the iPRF being higher, because its composition of fibrin and blood cells presents a lower complexity when compared to a tissue that presents more complex architecture.

Another interesting finding was the fact that while blood clots slowly dissolved over time, i-PRF formed a small clot, likely as a result of fibrin components that acted as a dynamic gel. It is therefore hypothesized that i-PRF would retain its properties even following 10 days, as the blood clot had basically entirely dissolved after 10 days.

Although most studies have primarily focused on the effect of growth factors, the three-dimensional fibrin matrix also plays key roles in tissue repair and regeneration. Fibrin acts as a scaffolding biological material for adherent cells to concentrate at the site of tissue regeneration [21-23]. Additionally, fibrin is a carrier of growth factors in a well-controlled release system that sustains proper bioactivity over the healing period [24,25].

The main limitation of our study is the artificial *in vitro* system used for culturing the concentrates. *In vivo*, a physiological environment of the tissue would influence the behavior of platelet concentrate in terms of structure, cellular crosstalk, exposure to degradation enzymes, and release of growth factors. However, i-PRF characterization *in vitro* remains an important step toward understanding its effects *in vivo*. For example, the knowledge of i-PRF histology could provide important guidelines for the choice of tissue and/or injury in future preclinical and ultimately clinical studies.

Declaration Statements

Ethical approval and consent to participate

The study protocol was approved by the Ethics Committee of University Hospital, Federal University of Rio Grande do Norte (UFRN) protocol N. 1.817.827 and all procedures performed in this study were in accordance with the ethical standards of the institutional research committee. Informed consent was obtained from all individual participants included in the study.

Consent for publication

Written informed consent was obtained from all participants included in the study for publication of this report and any accompanying images.

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