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The *in vitro* release of cytokines and growth factors from fibrin membranes produced through horizontal centrifugation

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Abstract: Platelet-rich fibrin membranes are biomaterials widely used for therapeutic purposes, and canonically produced through the processing of peripheral blood with fixed-angle rotor centrifuges. In this work, we evaluate the *in vitro* stability and release of cytokines and growth factors when these biomaterials are produced with a horizontal swing-out clinical centrifuge. Membranes produced from the blood of 14 donors were morphologically evaluated by scanning electron microscopy and fluorescence microscopy, and their stability was assessed by photographic recording after incubation in culture medium for up to 28 days. The release of 27 cytokines and growth factors was monitored for three weeks through a multiparametric immunoassay. The fibrin membranes presented complex three-dimensional structure with a high density of nucleated cells. A large release of growth factors [platelet derived growth factor, fibroblastic growth factor (bFGF), and vascular endothelial growth factor] was detected in the first 24 h, followed by time-dependent decay, maintaining significant concentrations after three weeks. Both anti-inflammatory and pro-inflammatory cytokines presented different release peaks, maintaining high rates of elution for up to 21 days. Chemokines of relevance in tissue repair [RANTES, granulocyte colony-stimulating factor (G-CSF)] were also produced in large quantities throughout the experimental period. The present results demonstrate that blood-derived fibrin membranes with high structural stability and cell content can be generated by horizontal centrifugation, being able of a prolonged production/release of growth factors and pro- and anti-inflammatory cytokines. © 2018 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00B:000–000, 2018.

Key Words: wound healing, fibrin, growth factors, cytokines

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INTRODUCTION

Due to their high-regenerative potential, platelet concentrates have been widely used in clinical and surgical procedures that require tissue repair,[1,2] including treatments for chronic wounds, osteonecrosis, infra-osseal defects, and orthopedic procedures.[3–6] Among those materials, leukocyte- and platelet-rich fibrin (L-PRF) membranes belong to the second generation of platelet aggregates.[3] Favored by a rather simple protocol, its production is characterized by a single centrifugation of peripheral blood samples, circumventing the addition of anticoagulants of blood activators to trigger fibrin polymerization.[7] The confinement of platelets, leukocytes and growth factors in a high-density fibrin network allows it to be handled like a real solid material and contributes to structural stability for extended periods of time.[8] This feature turns PRF into a possible scaffold for tissue engineering, with high clinical applicability, simplified protocol, and low cost.[9]

The interesting clinical results of platelet-rich fibrin are directly related to the controlled release of platelet-derived growth factors[10] such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor. These factors promote regeneration through processes such as angiogenesis and stimulate the migration and proliferation of osteoblasts, fibroblasts and mesenchymal cells.[11] In addition to growth factors, several pro- and anti-inflammatory cytokines can also be produced by leukocytes present in the

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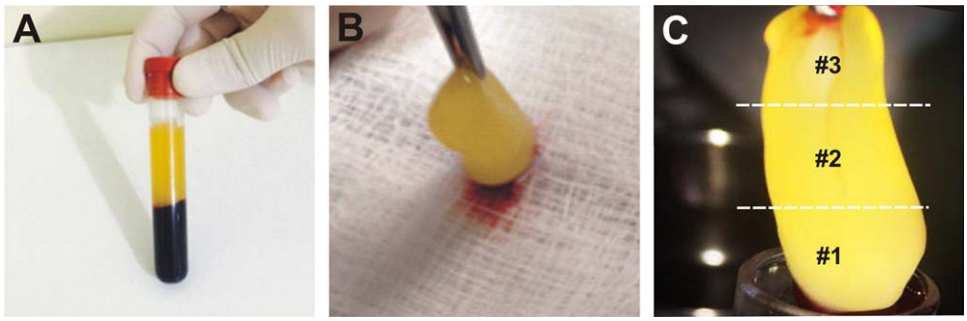

FIGURE 1. Fibrin membrane after centrifugation process. (A) Content in three layers: hematocrit, fibrin clot and acellular plasma—from the lower end to the top of the tube. (B) Fibrin membrane rich in platelets and leukocytes after being withdrawn from the tube. (C) Dashed lines indicate the points of sectioning for the cellular evaluation of the membranes: #1 (lower fragment, adjacent to the red blood cell fraction), #2 (medial fragment or main middle portion) and #3 (upper fragment, or the distal portion from the red thrombus).

L-PRF membranes. As these mediators can impact both positively and negatively on the clinical outcome of its use in several treatments, they remain a controversial subject in the scientific literature.[12,13]

While presenting a rather simple production protocol, there is some controversy in the standardization of the manufacture of leukocyte and platelet-rich fibrin products. Initially developed as an open access technique, nowadays the production of L-PRF is strongly related to the only Food and Drug Administration (FDA)-approved Conformité Européenne (CE)-marked system with certified materials, marketed under the name Intra-Spin L-PRF (Intra-Lock, Boca Raton, FL).[3,13] This system uses a fixed-angle rotor centrifuge to process a 9 mL aliquot of peripheral blood for 10 min. A recent study investigated the vibration interference from different centrifugation patterns in the quality of PRF membranes obtained with three different models of commercially available table-top centrifuges when compared to the Intra-Spin system. The authors described a deterioration of cellular components and the abnormal formation of the fibrin network with the non-canonical fixed angle centrifuges.[14] However, the literature still lacks on reports of the impact of the production of leukocyte- and platelet- rich fibrin derivatives by the use of horizontal centrifugation with swing-out rotor centrifuges, which are very accessible and of extensive use in clinics and hospitals.

In this context, this study aimed to evaluate the morphological characteristics and *in vitro* structural stability of leukocyte- and platelet- rich fibrin membranes produced with horizontal rotor centrifuges, as well as their capacity of releasing growth factors, as well as pro- and anti-inflammatory cytokines, during the first weeks after production.

MATERIALS AND METHODS

The present work was developed according to the principles recommended for experimentation with human beings determined in the Declaration of Helsinki. The research protocol was approved by the Research Ethics Committee HUAP-UFF (CAAE: 22763513.6.0000.5243). The participants were informed about the procedures and objectives of the study and signed a consent form.

Preparation of platelet rich fibrin membranes

Peripheral blood was collected from 14 healthy donors, aged between 20 and 56 years, with no history of anticoagulant medication use. For the production of each membrane, a 9 mL aliquot of blood, collected in tubes without the addition of any substance (DryVacutube, Biocon, Brazil), was immediately centrifuged at 400 g in a horizontal swing-out rotor centrifuge (B-40, RDE, Brazil) for 10 min. After polymerization of the fibrin matrix, the material was collected, and a slight compression was applied with the aid of a sterile gauze, producing a resistant gelatinous structure, identified as the Platelet Rich Fibrin membrane.

Characterization of the platelet rich fibrin membranes

Scanning electron microscopy. Immediately after production, the membranes were laid over a glass surface, measured with a caliper gauge, and sectioned with a scalpel in three portions of equal length. The parts were identified as: #1 (lower fragment), #2 (medial fragment) and #3 (upper fragment) [Fig. 1(C)], corresponding to the previously described #1, #2 and #3 sections for microscopic evaluation of PRF.[15] The Region 1 was adjacent to the red blood cell fraction (RBC), Region 2 was the main middle portion, and Region 3 was the distal portion from the red thrombus.

Each portion was fixed with Karnovsky's solution and postfixed with 0.2*M* sodium cacodylate solution and 1% osmium tetroxide, and finally dehydrated in alcohol solutions (ranging from 15 to 100%) and hexamethyldisilazane. The materials were metallized with gold and observed at 15 kV with a scanning electron microscope (JEOL JSM-6490 LV, JEOL, Japan).

Fluorescence microscopy. The distribution of nucleated cells along the membranes was evaluated by fluorescence microscopy. Three samples divided into the previously described #1, #2, and #3 sections were fixed by treatment with 4% paraformaldehyde solution for 15 min. Cell nuclei were evidenced after treatment with 1:5000 4',6-diamidino-2-phenylindole (DAPI), diluted in phosphate buffered saline. The samples were observed with a 20× objective in an inverted fluorescence microscope (Axio.Observer A1, Zeiss, Germany). The microscopic quantitative analysis was

performed as described previously.[16] After centering the objective in the sample, ten aleatory horizontal (right or left) or vertical (up or down) movements were performed with the microscope stage. The resulting fields after each move were photographed with a digital camera (Axiocam Rev.3 MRc, Zeiss, Germany), and evaluated by cell counting with the help of the Image-Pro Plus 6 software (Media Cybernetics). Fine focus adjustments were performed to include cells above and below the initial plane in the counting. Cell density was represented by the mean nuclei count per field.

Elution and detection of cytokines and growth factors

Eight platelet rich fibrin membranes were cultured for up to three weeks after their preparation. For this, membranes were incubated in 6-well culture plates (TPP) in the presence of 4 mL of DMEM medium (Dulbecco's Modified Eagle's Medium, GIBCO), without antibiotics, in a humidified atmosphere at 37°C and 5% CO_2. Aliquots of the extracts were collected at the times of 1, 4, 7, 14, and 21 days of culture. At each experimental time, the culture medium was completely removed and stored in a freezer at −80°C, for later evaluation of eluted growth factors and cytokines. The remaining membranes were again immersed on 4 mL of fresh culture medium, and kept in incubation at 37°C and 5% CO_2 until the next experimental time.

The concentration of cytokines and growth factors was detected in the extracts through a multiparametric immunoassay based on XMap-labeled magnetic microbeads (LuminexCorp). A commercial kit (27-plex panel, Biorad) was employed, capable of quantifying IL-1β, IL-10, IL-12 (p70), IL-13, IL-15, IL-10, IL-10, IL- IL-17, CCL11, FGF-b, CSF3, CSF2, IFN-γ, CXCL10, CCL2, CCL3, CCL-4, PDGF, CCL5, TNFα and VEGF. Quantification of the magnetic beads and was performed with a BioPlex MAGPIX system (Biorad). Results were analyzed using Xponent v. 3.0 software (Luminexcorp).

To confirm the results obtained by the multiplex assay, one analyte (FGF-b) was also dosed in the 24-h elution samples by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Human FGF Basic ELISA KIT, AVIVA System Biology).

Membrane stability test

The evaluation of structural stability was performed by monitoring the evolution of the samples through photographic recording. Membranes were incubated in 6-well plates in the presence of 4 mL DMEM medium without antibiotics and humid atmosphere at 37°C and 5% CO_2. The photographic record was made in the periods of 1, 7, 14, 21, and 28 days. The photos were evaluated with Image Pro Plus 6.0 software, and the total length of the membranes at each experimental time was estimated.

Statistical analysis

The statistical analysis was performed using Graphpad Prism 6 software (Graphpad). One-way analysis of variance with *post hoc* Bonferroni test was employed to compare the cell content in the fluorescent assay and the mean length of membranes at each time point in the structural stability test. In the cytokine/growth factors assay, a paired *t* test was performed comparing the concentrations at each time point with the first elution (24 h). All statistical tests considered an alpha error of 5%.

RESULTS

After the centrifugation step, it was possible to observe subdivisions of the material inside the tube, consisting of a red blood cell's layer at the base of the tube, a fibrin clot in the intermediate region, and an obvious platelet poor plasma portion at the top of the tube [Fig. 1(A)]. The compression process of the membranes produced from the fibrin clot resulted in a resistant, yellowish and malleable material [Fig. 1(B)].

After processing, the membranes were separated into upper, middle and lower portions [Fig. 1(C)], whose ultrastructure was observed by scanning electron microscopy (SEM), Figure 2 shows the formation of a dense fibrin network with overly closed frames, similar to gauze threads. The entrapment of cells between the fibers is quite evident in the lower segment #1 [Fig. 2(C)]. The analysis of the cell distribution along the membrane sections by fluorescence microscopy confirms the significant presence of nucleated cells in the fibrin structure (Fig. 3). These cells are present throughout the membrane, however with increasing numbers along its length, notably having a higher cell density at the buffy coat area, or region #1 [Fig 3(A)], as confirmed by the quantification of cells with DAPI-labeled nuclei [Fig. 3(D)].

Figure 4 shows the time-dependent structural stability of the membranes when immersed in culture medium, following their evolution from the first 24 h [Fig. 4(A)] to the 28th day of incubation (Fig. 4B). The estimation of the total length of the membranes at each time [Fig. 4(C)] shows that there is no significant change in membrane length over 28 days.

The culture supernatant was evaluated for the content of 27 analytes that could be eluted from the membranes in the culture medium. Table I shows that the detection of most of these molecules already occurred on the first day of elution. It can be observed that a range of pro-inflammatory and anti-inflammatory cytokines have been released, being accompanied by growth factors, with high concentrations of PDGF-BB and VEGF. Other molecules were detected at lower concentrations than previously described for L-PRF,[24] such as IL-2 ($<$4.5 pg/mL), IL-13 (6.5 ± 7.8 pg/mL), IL-15 (2.8 ± 0.3 pg/mL), IL-4 and RANTES (both at $<$2 pg/mL). To validate the results obtained through flow luminometry, the FGFb concentration was also evaluated by the ELISA method, detecting a quite similar to the concentration revealed in the multiplex assay (13.5 ± 3.3 pg/mL vs. 19.0 ± 3.1 pg/mL).

Figure 5 shows the changes in the release of these molecules over the subsequent three weeks of culture. It became evident that each analyte presents a peculiar release profile, most presenting an expressive increase of the concentrations, but at different moments of the elution process. In

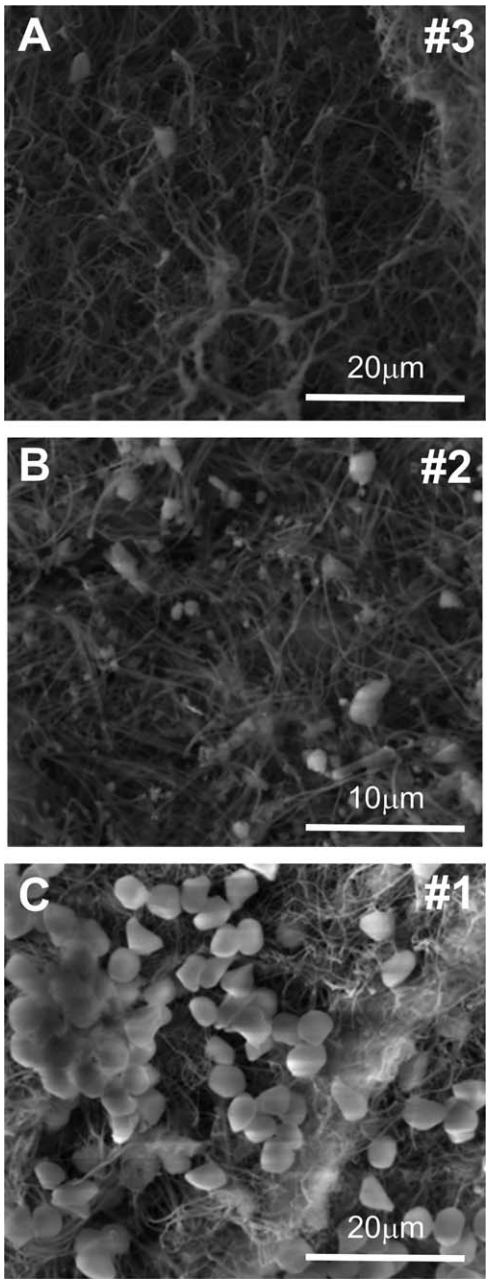

FIGURE 2. Micrographies obtained by SEM of the fragments of fibrin membrane rich in platelets and leukocytes. (A) Upper fragment, region #3. (B) Medial fragment, region #2. (C) Lower fragment, or region #1.

the case of the FGFb, VEGF, and PDGF growth factors, the large release found in the first 24 h was followed by a reduction in concentrations in the subsequent weeks, always significantly lower ($p < 0.05$) after 21 days. A similar pattern of release was obtained for inflammatory cytokines such as IL-12, IL-1β, TNF-α, and the chemokines GM-CSF and IP-10, although with very different rates of increase for each analyte.

The cytokines IL-1RA and IL-4, with potential anti-inflammatory activity, showed release peaks at the end of the first week, followed by a gradual reduction, but maintaining levels still higher than the first day of elution. A similar process was observed for the proinflammatory cytokines IFN-γ, eotaxin, and IL-6, the latter presenting a tenfold increase in the release at the 21st when compared to the first day of elution. RANTES, MIP-1a, and MCP-1 also had elution peaks within the first week, but with the maintenance of high release rates throughout the experimental time.

DISCUSSION

In the present work, membranes were produced based in the principles diffused by Dohan et al.,[13] i.e., the activation of the coagulation cascade due to the platelet shock against the walls of glass tubes in the absence of anticoagulants. Other derivate protocols, employing considerable changes in the G-force allowed the development of other platelet aggregate products, such as A-PRF.[15] Furthermore, for Kobayashi et al.,[16] the change in rotor angulation may impact in the platelet deposition during PRF production. According to those authors, as the rotor angle changes to a more horizontal position, the G force will focus on the tube so as to concentrate the platelet layer in a smaller area, without altering the cell distribution in the membrane, that is, maintaining the concentration of platelets mostly in the buffy coat.

In this context, the present study investigated a protocol employing a swing-out horizontal rotor centrifugal device, widely used in hospital settings such as hematology, and usually accessible in clinical and surgical procedures. Applying this methodology, while maintaining strictly the same G-force (400 G) proposed originally for PRF, the membranes produced presented high similarity in their basic constitution when compared to results from PRF studies.[17] The ultrastructure of the fibrin membrane evidenced by SEM is a dense and intricate gauze-like mesh, very characteristic of the PRF elastic and resistant membranes. It also presents similarity to collagen structures formed *in vivo* during the healing process.[18] The absence of alterations in the size of these membranes (Fig. 4) when kept in culture medium for up to 28 days after production provides a preliminary evidence of stability, similar to that of the PRF in an aqueous medium.[14,19] It is important to notice that further studies are necessary to confirm that this apparent evidence of stability by direct observation reflects the maintenance on the integrity of the fibrin network, employing techniques such as SEM. Such stability is also a desirable feature for scaffolds for tissue engineering,[20] supporting cell migration and proliferation for tissue remodeling purposes, as biodegradable barriers for guided tissue regeneration procedures.[21]

It is worth noting that cell entrapment is a central attribute of leukocyte-rich fibrin membranes. The present fluorescence microscopy assay was able to detect a high density of nucleated cells within the structure of the fibrin membrane, quite typical of PRF membranes, with an uneven distribution along its extension, and a higher cell density at the top portion.[22] The presence of apparently viable leukocytes, as well as a fibrin network which seems stable for weeks, could contribute to producing a reservoir for

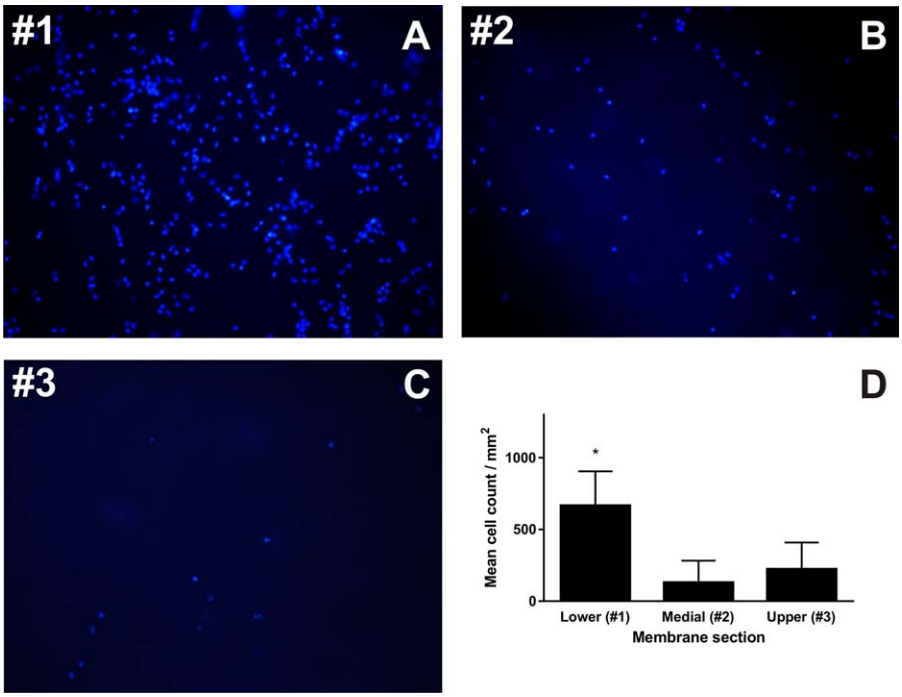

FIGURE 3. Fluorescence microscopy images with DAPI marker. (A) Lower fragment, or region #1 (Buffy coat). (B) Medial fragment #2. (C) Upper fragment, or region #3. (D) Cell/field count in relation to the membrane segmentation. *Significantly different from the other experimental groups ($p < 0.05$).

controlled release of growth factors and cytokines.[1,13] In fact, the literature reports the retention and release of these substances from one to four weeks after a surgical intervention with PRF in different tissues.[1]

The release of growth factors by cells trapped in the fibrin matrix has been pointed out as a vital element in the regenerative process.[12,23,24] However, there is controversy regarding the impact of the use of different centrifugation systems in the release of bioactive molecules by membranes produced with otherwise very similar protocols.[14] A widespread approach for the assessment of GF release is the *in vitro* analysis of elution, that is, the change in the concentration of these molecules in a liquid medium. With such methodology, diverse studies have identified the elution of the main growth factors, as well as relevant inflammatory cytokines in culture media. This release may start immediately after membrane production, and going as far as weeks of incubation of PRF.[23–26] One study[22] was able to identify a relationship between the concentration of growth factors and the region of the membranes, where the highest levels were evidenced in the buffy coat area, similar to the most cellularized fragment of the present work (Figs. 2 and 3), and evidencing the role of leukocytes in the production of growth factors.

In the present work, the *in vitro* elution assay shows that the release of several growth factors and cytokines is maintained in concentrations of biological relevance for weeks after the preparation of blood derived membranes through horizontal centrifugation. The evaluation of a broad range of molecules (27 analytes) was possible by the methodological choice of a multiparametric system based on XMap technology (LuminexCorp). This method allows analyzing multiple analytes in a single sample, increasing the reliability of the results concerning comparisons between

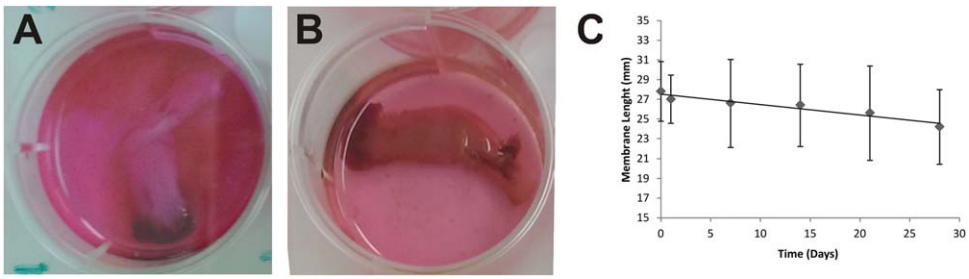

FIGURE 4. Monitoring the structural stability of membranes. (A) Sample photographed after 24 h of incubation. (B) Same sample of figure A, photographed with 28 days of incubation. (C) Evolution of the average length of the samples in millimeters over time. No significant statistical difference was found between experimental times ($p > 0.05$).

TABLE I. Concentrations of Released Mediators on Conditioned Medium After 24 h Elution

Analyte	Concentration (pg/mL) (mean ± SD, $n = 8$)
FGF-b	19.0 ± 3.1
VEGF	3075.5 ± 1341.7
PDGF-BB	22,0495.6 ± 4687.2
GM-CSF	3.8 ± 1.1
G-CSF	9.8 ± 1.1
IL-1β	77.8 ± 49.2
IL-2	<4.5
IL-6	15.5 ± 21.0
IL-8	12,441.6 ± 8074.9
IL-13	6.5 ± 7.8
IL-15	2.8 ± 0.3
IFN-γ	168.6 ± 15.7
TNFα	57.3 ± 17.5
IL-12p70	65.9 ± 3.4
IL-7	<4.5
IL-17A	<7.1
IL-9	<6.3
IL-5	3.5 ± 0.4
IP-10	4957.5 ± 1656.0
IL-10	9.7 ± 0.4
IL −4	1.2 ± 0
IL-1RA	81.6 ± 35.8
RANTES	1.2 ± 1
MIP-1α	27.8 ± 15.1
MIP-1β	594.5 ± 20.6
MCP-1	131.3 ± 38.8
Eotaxin	84.1 ± 20.4

the relative concentrations of the different analytes, and reduces the chance of conflicting results by experimental variability and manipulation errors.

The role of growth factors released by fibrin membranes on tissue regeneration is well known, either through the promotion of angiogenesis, migration, and proliferation of mesenchymal cells and fibroblasts and osteoblasts.[10] In this study, significant levels of released VEGF, bFGF, and PDGF-BB were identified in the first 24 h, similarly to reports on PRF membranes.[7,19,23] However, methodological differences such as the volume of medium used for elution, detection technique (ELISA, flow luminometry) as well as the parameters for normalization of results (e.g., membrane mass, initial volume of blood samples) prevent an adequate the comparison of the absolute concentrations identified from different reports. Nevertheless, a gradual reduction in the release of growth factors over time is confirmed in all of these studies, and in agreement with the present results. It is worth to note that, after 21 days, the released concentrations of molecules such as PDGF correspond to biologically relevant levels identified in previous studies.[27]

The interpretation of the impact of released molecules by the fibrin membranes and similar becomes more difficult when considering other molecules not identified as growth factors, such as the chemokine RANTES or CCL-5. A comparative study of different platelet derivatives[23] showed that the release of CCL-5 is much higher in PRP than in PRF in the first 24 h after its production. In fact, the present result for 24 h indicates a low release of this molecule but followed by a significant increase (more than 10-fold) from the 7th to the 21st day. While the possible effects of an increased release of RANTES in the clinical outcome of PRF remain undescribed, there is evidence in the literature of RANTES acting positively in angiogenesis, as well as potential effects of this molecule on the differentiation of osteoblasts during tissue regeneration.[28]

In the present study, high concentrations of inflammatory cytokines such as IL-1β, IL-6, IL-5, IL-15, TNF-α, and IFN-γ were detected from the first to the 21st day, indicating extensive activation of immune cells in the material. In fact, the presence of immune system cells within the fibrin membrane, and the consequent release of inflammatory mediators has been a subject of controversy in the literature. Although proinflammatory cytokines such as IL-6 may affect the regeneration process of lesions,[11] they also play important immunological functions against possible infections and help to clean the perimeter of the wound. According to Dohan et al.,[7] the leukocyte-rich PRF membrane may represent an immune nodule capable of stimulating defense mechanisms. The high release of proinflammatory cytokines concomitant with growth factors could counterfeit a possible osteolysis[7] by the simultaneous induction of proliferation of osteoblasts, fibroblasts, and endothelial cells. Such duality may result in a rapid remodeling without the classic signs of the inflammatory picture (heat, flushing, pain, edema, and loss of function), as observed in clinical trials of PRF.[29] This may also be related to the long-term release of anti-inflammatory cytokines such as the antagonist of the IL-1 receptor (IL-1RA), which was consistently released in the present study, suggesting the possibility of induction of

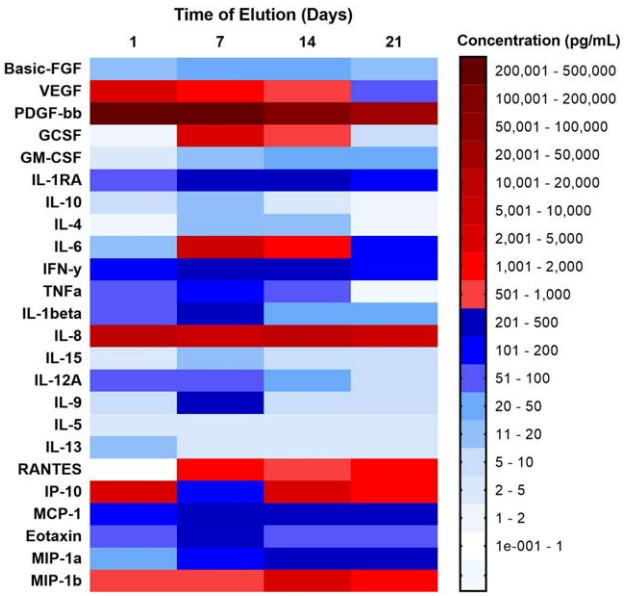

FIGURE 5. Heatmap of the variation of analyte concentrations in culture media from 1 to 21 days of incubation of fibrin membranes. The analytes IL-2, IL-7, IL-9, and IL-17 had undetectable concentrations.

a self-limited inflammatory process. Also, *in vitro* studies points to an active role for some proinflammatory cytokines in tissue repair, including evidence that IL-1beta released by PRF membranes may be associated with the migration of mesenchymal stem cells and human endothelial cells.[19] Furthermore, the high concentrations of G-CSF found in this work may also reinforce the potential for promotion of tissue remodeling, since effects on bone regeneration via revascularization and osteogenesis have been described this molecule.[30]

As recently stated by Dohan Ehrenfest et al.,[14] changes in the protocol of production of L-PRF, using centrifugation systems other than the CE/FDA approved Intra-Spin L-PRF system often result in the biological signature of the resulting material. In this context, the clinical and experimental results from the original L-PRF shall not be extrapolated to the material produced in the present protocol. Nevertheless, taken together, the results of the present work demonstrate that the structural stability of fibrin membranes generated by horizontal centrifugation allows the entrapment of nucleated cells that might contribute to a consistent release of modulatory molecules for weeks after their production. Therefore, further studies are needed to confirm whether this *in vitro* release is parallel to the physiological environment, as well as its actual impact on the clinical outcome of treatments involving these growth factor concentrates. Nevertheless, the present results demonstrate that blood derived fibrin membranes with high structural stability and cell content can be produced by horizontal centrifugation, which are able of a prolonged production/release of growth factors and pro- and anti-inflammatory cytokines.

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