Bioactive Fibrin Scaffolds for Use in Musculoskeletal Regenerative Medicine

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**HIGHLIGHTS**

- L-PRP and L-PRF represents sources of biomolecules capable of stimulating cell function.
- Viability after cell therapy is essential for success of regenerative medicine strategies.
- Fibrin clot of L-PRP and L-PRF can act as bioactive scaffold in regenerative medicine.
- L-PRP scaffolds show high cell viability potential in human mesenchymal stem cells.

**Abstract:** Autologous fibrin matrices derived from the Leukocyte and Platelet Rich Plasma (L-PRP) and Leukocyte and Platelet Rich Fibrin (L-PRF) techniques present great potential to act as a bioactive scaffold in regenerative medicine, contributing to the maintenance of cell viability, proliferation stimulus and differentiation. In contrast, there are few studies that characterize the bioactive potential of these fibrin scaffolds by considering the process of production. The objective of this work was to characterize the intrinsic potential of maintaining cell viability of different fibrin scaffolds containing platelets and leukocytes. In order to achieve that, blood samples from a volunteer were collected and processed to obtain fibrin clots using the suggested techniques. To characterize the potential for in vitro viability, mesenchymal stem cells from human infrapatellar fat were used. The scaffolds were cellularized (1x10\textsuperscript{5} cells/scaffolds) and maintained for 5 and 10 days under culture conditions with Dulbecco’s Modified Eagle Medium, without addition of fetal bovine serum, and subsequently subjected to analyses by Fourier transform infra-red spectroscopy, circular dichroism and fluorescence microscopy. The results demonstrated distinct intrinsic potential viability between the scaffolds, and L-PRP was responsible for promoting higher levels of viability in both periods of analysis. No viable cells were identified in the fibrin matrix used as controls. These results allow us to conclude that both fibrin substrates have presented intrinsic potential for maintaining cell viability, with superior potential exhibited by L-PRP scaffold, and represent promising alternatives for use as bioactive supports in musculoskeletal regenerative medicine.

**Keywords:** regenerative medicine; fibrin; scaffold; mesenchymal stem cells.
INTRODUCTION

Traumas, degenerative pathologies and congenital defects represent the main causes of damage to the tissues that constitute the musculoskeletal system (bones, tendons, ligaments and muscles), which can determine high levels of functional limitation and impairment of quality of life, especially in the economically active population [1].

Considering the biological aspects, the musculoskeletal tissues have the potential of self-repair responsible for the restoration of tissue structure and function. However, when damaged, these properties are not fully reestablished due to the intrinsic limitations inherent to the repair process, attributed mainly to the limited action of the reserve mesenchymal cellular contingent [2] and/or to the loss of matrix structure due to extensive structural damage (volumetric losses) [3]. In those cases, the repair process is characterized by a predominantly fibrocartilaginous response, which compromises tissue structure and function definitively, generating irreversible functional sequelae [4].

Regenerative medicine (RM) is a contemporary biological therapy approach that has shown promising results in the treatment of musculoskeletal injuries. Its role is to aid the regeneration of tissues and organs in an attempt to more effectively restore the normal structure and functions peculiar to them, using as main strategies biological supports, cell therapy and bioactive molecules [5-8].

Biological supports (scaffolds) are temporary three-dimensional structures that aid the development of repair. They are necessarily biocompatible, bioabsorbable and with physical and chemical properties that allow cell adhesion, proliferation and differentiation [9-11]. Among the biological scaffolds available for the RM application, fibrin plays a prominent role. Fibrin is formed from serum fibrinogen during activation of the coagulation cascade. Its polymerization of the origin to a nanoporous reticulated three-dimensional structure (fibrin clot) that exerts the hemostatic support function [12]. In addition to its hemostatic role, it acts as an endogenous support matrix for the development of the initial phases of the tissue repair process. For these reasons, it is the natural polymer most used as a structural component in regenerative medicine and tissue engineering applied to the musculoskeletal system [13,14]. This autologous biomaterial, associated with platelets (PLA) and leukocytes (LEU), has the potential to act as a bioactive carrier, since these cellular components are able to release proinflammatory cytokines and/or growth factors responsible for the orchestrated modulation of process of tissue repair, stimulating events such as angiogenesis, cell proliferation and differentiation, and the formation of extracellular matrix [15-18].

The most widely used methods for obtaining clot cells from fibrin come from techniques of platelet concentration by centrifugation. Ehrenfest and coauthors [19] classify the products of these techniques according to the form of platelet activation (spontaneous or induced) and the amount of leukocytes in the final product. Plasma rich in PLA and LEU (L-PRP) is composed of blood plasma with concentrations of PLA and LEU higher than baseline blood concentrations. In order to obtain the clot, the subsequent induction of platelet activation is indispensable. Fibrin rich in PLA and LEU (L-PRF) is composed of the fibrin clot formed during the centrifugation, which confines the PLA and LEU inside.

Another strategy adopted by RM in the treatment of lesions of the musculoskeletal system is cell therapy, which preferably uses adult mesenchymal stem cells (MSCs) as an autologous supplementary source of cells to contribute to the tissue repair process. Its use is beneficial considering its capacity of self-renewal, of differentiation in multilineage [20] and modulation of the repair environment (cellular niche), resulting from the synthesis and release of cytokines and growth factors [21]. In performing these functions, MSCs contribute to the modulation of the inflammatory process, to neoangiogenesis and to restocking by tissue specific differentiated cells, providing the restoration of extracellular matrix and tissue architecture [22]. In order for these benefits to be achieved, it is necessary to ensure the viability of these cells after the implantation process [23].

Review of recent literature shows the dearth of studies that characterize the intrinsic bioactive potential of cell viability of fibrin supports obtained from the L-PRF and L-PRP techniques in MSC. This study aims to establish the potential of these supports on the viability of MSCs derived from human infra-patellar fat (hIFPMSC) for future use in musculoskeletal regenerative medicine.

MATERIAL AND METHODS

Ethics committee

The protocol for this study was approved by the Research Ethics Committee (CEP-UNIARA), in accordance with the norms set forth in resolution 466/12 of the National Health Council. A written Consent Form was obtained from all participants.
Isolation and characterization of human infrapatellar fat pad mesenchymal stem cells (hiFPMSC)

Fragments of infrapatellar fat from a 35-year-old healthy man, extracted during an arthroscopic procedure for meniscal repair, were prepared according to the protocol described by Dragoo and coauthors [24]. They were subjected to the enzymatic digestion with collagenase type I (1 mg/mL) for isolation of hiFPMSC, expanded in Dulbecco's Modified Eagle Medium (DMEM / Vitrocell®) supplemented with 10% fetal bovine serum (FBS / Vitrocell®) and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL - Vitrocell®) and maintained in a cell culture oven (MOC -19 AIC-UV-Panasonic®) at 37 °C with 95% humidified atmospheric air and CO₂ (5%).

In order to characterize the mesenchymal profile of the isolated cellular contingent, an immunophenotypic characterization was performed, following the modified protocol described by Dernowsek [25], and the potential of osteogenic and adipogenic differentiation, according to the protocol described by Zuk [26]. Confirmation of the mesenchymal profile in the immunophenotypic characterization, performed by flow cytometry (FAC Scan Becton Dickinson - USA), was established by the presence of the MSC specific CD-73, CD-90 and CD-31 markers, and absence of CD-34 and CD-45 markers, hematopoietic profile indicators. The labeled monoclonal antibodies were purchase from Becton Dickinson and Company. For osteogenic differentiation, the cells were incubated with DMEM containing SBF (10%), 10 mM β-glycerophosphate, 0.1 mM dexamethasone and 50 mM ascorbic acid. After 28 days the cultures were stained with Alizarin Red to visualize the calcified matrix. For adipogenic differentiation, the cells were incubated with DMEM supplemented with SBF (10%), 0.5 mM isobutyl-methylxanthine (IBMX), 1 mM dexamethasone, 1 mM insulin, and 200 mM indomethacin. After 28 days the cells were stained with Oil Red for identification of fat droplets.

Obtaining L-PRF and L-PRP clots

To obtain L-PRF, following the protocol of Zumstein and coauthors [27], 5 mL of peripheral blood from the same donor, were collected in tubes containing clot activator and separator gel (Vacutainer®) and immediately centrifuged (400 G/12 minutes). In order to obtain L-PRP, 5 mL of peripheral blood were collected in tubes containing sodium citrate (Vacutainer®) and centrifuged (400 G/10 minutes). After centrifugation the corresponding platelet-poor plasma (PPP) and buffy coat were extracted, transferred to a dry tube and centrifuged again (700 G/17 minutes). The PPP was then removed, and 500 μL of this were used to resuspend the pellet of platelets and leukocytes, resulting in L-PRP. This, in turn, was transferred to a 48-well culture dish and activated by the addition of thrombin (10,000 U / mL) and calcium chloride (10%). As a negative control, a clot obtained from pure commercial fibrinogen (Beriplast® P) was used as described for L-PRP. 2 mL aliquots of whole blood and L-PRP were collected for platelet, leukocyte, and erythrocyte counts.

Characterization of fibrin clots

Fourier Transform Infrared (FT-IR)

Samples of L-PRP, L-PRF and commercial fibrin were prepared as described in the previous section and then stored in PBS under refrigeration for 5 days. The clots were then dried at room temperature and analyzed. The analyses were performed on a spectrometer (Perkin Elmer Spectrum 100 FTIR®) equipped with a diamond accessory for attenuated total reflectance (DATR). The spectra were acquired from 4000 to 650 cm⁻¹, 4 cm⁻¹ resolutions and 16 scans.

Circular Dichroism (CD)

Samples of L-PRP, L-PRF and commercial fibrin were prepared as described in the section entitled "L-PRF and L-PRP clots" and then stored in PBS under refrigeration. For the analysis, the wet clots were accommodated in 1 mm thick quartz cuvettes and analyzed (Circular Dichroism Spectropolarimeter - Jasco J-815®) with a wave number of 250 to 190 nm, at 100 nm.min⁻¹ and subjected to 8 scans.

Cell viability

The hiFPMSCs were cultured in culture bottles under the previously described culture conditions. The procedure for evaluating the bioactivity of L-PRP and L-PRF clots was started by sowing hiFPMSC (1x10⁶ cells/clot) onto clots in 48-well culture plates (KASVI®) containing DMEM without FBS. Cellular clots were maintained under culture conditions for 5 and 10 days. Cell viability was established by the Fluorescence method (ImageXpress/Molecular Devices) using the Metaxpress software. For these analyzes, the samples
were fixed with glutaraldehyde (2.5% / 40 minutes) and dehydrated for 30 minutes in increasing concentrations (50%, 70%, 90% and 100%) of ethanol. After fixation, the cells were labeled by immersion in 0.1 μM solution of DAPI (4', 6-diamidino-2-phenylindole), a specific fluorescent marker for identification of cell nuclei, for 10 minutes.

RESULTS AND DISCUSSION

Isolation, characterization and differentiation of hIFPMSC

After the period established for adhesion of the mesenchymal cell contingent, for the isolation of hIFPMSC, a homogeneous culture was observed by optical microscopy, constituted only by fusiform cells and dispersed homogeneously throughout the extension of the culture bottle. Such morphological characteristics were maintained throughout the cell expansion period.

The flow cytometry assay performed with hIFPMSCs demonstrated that a high percentage of cells constituting the analyzed sample expressed the surface markers compatible with mesenchymal stem cells (CD-90, CD-73 and CD-31) and did not express the markers (CD-34 and CD-45) indicating the successful isolation of hIFPMSC.

The results of osteogenic and adipogenic cell differentiation, which is indispensable for the determination of the mesenchymal MSC profile [28,29], confirmed the mesenchymal MSC profile of the isolated cell contingent. The staining with Alizarin Red was performed to identify the mineral phase of the extracellular matrix resulting from the osteogenic differentiation process. In Figure 1A, red areas represent the presence of a calcified matrix, demonstrating the occurrence of hIFPMSC differentiation in osteogenic cells. The staining with Oil Red was performed for the identification of typical fatty particles in adipocyte cultures [30]. In Figure 1B, the red areas represent lipid particles, indicating that the hIFPMSCs have undergone adipogenic differentiation. These results confirm that the extraction and isolation processes performed in this research provided the obtaining of a cellular contingent of hIFPMSC.

Figure 1. Differentiation potential of hIFPMSC. Osteogenic (a) and adipogenic (b) differentiation, stained with Alizarin Red and Oil Red respectively, after 28 days using specific differentiation culture media.

Stem cells derived from adipose tissue are widely used in regenerative medicine, due to the ease of obtaining them from liposuction fat, presenting minimal risks of complications for its acquisition and high maintenance capacity of its mesenchymal profile, even after being kept in cultivation for long periods [31]. The hIFPMSCs, obtained from arthroscopic knee procedures, exhibit chondrogenic, osteogenic and adipogenic differentiation capacity and are now an important source for obtaining MSC [32]. A relevant characteristic of these cells is to present a greater intrinsic capacity for chondrogenic differentiation when compared to stem cells obtained from the bone marrow [33], making them the preferred source of obtaining MSC for the treatment of articular chondropathies of traumatic and degenerative origin (osteoarthritis).

Obtaining L-PRF and L-PRP clots

The results concerning the counting of red blood cells, platelets and leucocytes of the whole blood and after the process of obtaining the L-PRP are presented in Table 1.
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Table 1. Concentration of blood constituents before and after obtaining L-PRP.

| Constituent     | Reference Values | Blood | L-PRP  |
|-----------------|------------------|-------|--------|
| Red blood cells | 4.5-6.1x10⁶/µL   | 4.9 x10⁶/µL | 0.07x10⁶/µL |
| Platelets       | 150-400x10³/µL   | 160x10³/µL | 1002x10³/µL |
| Leukocytes      | 4-11x10³/µL      | 4.0x10³/µL | 4.35 x10³/µL |

The determination of the cellular contingent in the L-PRF was not established with a view to the formation of the clot of fibrin during the blood centrifugation procedure, imprisoning the cellular contingent and making it impossible to quantify it. The results demonstrate compatibility between the values obtained from the blood analysis of the volunteer and the reference biochemical values. The centrifugation procedure provided, as expected, plasma with reduced erythrocyte concentration, leukocyte-like and high platelet count when compared to blood. The platelet concentration rate achieved by the L-PRP processing protocol used in this study was 6.26x, determining more than 1,000,000 platelets/µL. According to DeLong and coauthors [34], platelet concentrates are considered to be of high content when the platelet concentration efficiency ratio is between 4 and 6x the value corresponding to the basal concentration, which would correspond to a value between 750,000 and 1,800,000 platelets/µL depending of the basal values present in the blood. Most of the studies that have demonstrated the effective bioactive action of platelet concentrates are associated with platelet concentrations corresponding to these values. Research that used L-PRP with high platelet concentration values showed higher success rates in cases such as bone regeneration [35], tendinopathies [36], knee osteoarthritis [37], dermal lesion repair [38] and osteochondritis [39].

Structural, physical-chemical and morphological characterization of fibrin clots

Infrared Spectroscopy with Fourier Transform (FT-IR)

The FT-IR spectrum of fibrin clots from L-PRP and commercial fibrinogen activation exhibited a very similar profile with characteristic bands of protein structure in the regions of 1643 cm⁻¹, typical of C = O amide I, at 1535 cm⁻¹, typical of amide II NH, typical of amide III at 1235 cm⁻¹, as well as vibrations at the regions of 2930 cm⁻¹ and 2858 cm⁻¹ that correspond to the –CH2 bonds at 1730 cm⁻¹, corresponding to the COOH group and the band in the region of 3270 cm⁻¹ corresponds to the vibrations of the hydroxyl groups (Figure 2). The band corresponding to amide I, 1643 cm⁻¹, in both samples suggests a secondary structure, predominantly in α-helix. The L-PRP sample presents bands corresponding to chemical clusters present in protein, but without a typical peak of secondary structures. The typical vibration of group C = O is in the region of 1611, and the vibrations corresponding to the COOH group are found at 1704 cm⁻¹. Peaks in the regions of 1500 – 1250 cm⁻¹ are possibly related to the vibrations of amine groupings. Some peaks in the spectrum are displaced in relation to the peaks of commercial fibrin, suggesting the possibility of a chemical interaction of this protein with other components of the system. These displacements are clearly identified in the peaks relative to COOH (1704 cm⁻¹) and C=O (1611 cm⁻¹), which are capable of forming hydrogen bonds. The displacements of the peaks referring to the NH vibrations (1500 – 1250 cm⁻¹) also indicate the possibility of forming hydrogen bonds with system components. The O-H vibration band in the region of 3320 cm⁻¹ presents a different profile of the fibrin and L-PRP bands, indicating the presence of other components in the sample. This hypothesis is also indicated by the spectrum profile in the region below 1500 cm⁻¹.
Circular dichroism (CD)

The secondary structures of the samples, determined by the CD, are shown in Figure 3. The spectrum of the commercial fibrin scaffold, seen as a pure substance because it presented in its initial composition only fibrinogen, was used as the standard. Its spectrum indicates predominance of α-helical structural organization, presenting two typical negative bands in the ultraviolet (UV) region at wavelengths of 208 and 222 nm and a positive band in the region of 190-195 nm. The spectra related to the L-PRF scaffold showed a behavior similar to that of commercial fibrin, preserving as a priority the α-helix structure. The spectrum of the secondary structure of L-PRP exhibited a distinct pattern, characterized by the existence of the negative band at 230 nm, which does not correspond to any typical secondary structure (α-helix, β-sheet or Randon coil) normally identified by CD. The main hypothesis established for the occurrence of these results is related to the mechanism of platelet activation in L-PRP using thrombin and CaCl₂, responsible for the sudden and massive release of substances from platelet and leukocyte degranulation. Both the phenomenon of instantaneous polymerization of fibrin [40] and the intermolecular interaction between fibrin and the other proteins available therein could determine the conformational alteration of the fibrin molecules [41], modifying their secondary organizational structure.

Cell viability

Results related to fluorescence analysis, for the 5 and 10 day incubation periods, showed that viable cells (DAPI-stained nuclei) were present in the L-PRP and L-PRF clots in both periods of analysis. On the other hand, the clot belonging to the control group, characterized by fibrin matrix without platelets and leukocytes, did not present a potential for maintenance of cell viability (Figure 4), considering that the cell culture medium was not supplemented with FBS (source of growth factors and cytokines) during the experiment. These results suggest the bioactivity of the scaffolds, considering the maintenance of cell viability.
that would be associated with the release of bioactive molecules by the platelets and leukocytes. Zumstein and coauthors [42] have demonstrated that platelet and leukocyte growth factors, available from fibrin scaffolds or as injectable liquids, are capable of improving the repair process in cases of musculoskeletal injuries.

Although both scaffolds showed bioactivity in hIFPMSC, L-PRP demonstrated a greater intrinsic potential for maintaining viability in the two proposed analysis periods. Such difference can be explained by the distinct release profile of growth factors and cytokines, due to differences inherent to the activation processes. Different activation methods confer a profile of release of specific platelet and leukocyte growth factors [43]. L-PRP activated by thrombin and CaCl₂ has a sudden and massive release of growth factors and cytokines within the first hours post-activation, presenting a gradual decrease that lasts for up to 14 days [44,45]. In contrast, L-PRF shows a slow and progressive release of growth factors and cytokines, activating the platelets and leukocytes during the blood centrifugation process to obtain the clot [40].

Liao and coauthors [46] believe that L-PRF has medium- and long-term advantages when compared to L-PRP because its slow and gradual degranulation could maintain the bioactive potential of the scaffold for longer. However, according to the results presented in this research, the bioactivity of L-PRP scaffold in maintaining the viability of hIFPMSC was also superior in the longer period of analysis (10 days). This evidence determines that L-PRP would be the alternative of choice for the strategy of bioactive scaffolds in musculoskeletal regenerative medicine.

Figure 4. DAPI Fluorescent Staining from hIFPMSC cultured in control fibrin (a and b), L-PRF (c and d) and L-PRP (e and f) after 5 days (left column) and 10 days (right column) in culture.
CONCLUSION

It can be concluded, from the experiments carried out, that the fibrin scaffolds produced by the L-PRP and L-PRF techniques have demonstrated bioactive potential and were able to maintain the viability of hiFFPMSC. Furthermore, the L-PRP technique has shown greater bioactive potential resulting in a higher concentration of viable cells in the established periods of analysis.

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