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EVALUATION OF INFLAMMATORY INFILTRATE AND MESH COMPRESSION PRESENT IN THE LEUKOCYTE- AND PLATELET-RICH FIBRIN (L-PRF) NETWORK IN WISTAR RATS

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ABSTRACT

The purpose of study was to verify if the variation in the drainage time of the L-PRF clot promotes changes in fiber density and in the number of leukocytes, from the drainage times of 1', 2', 3' and 5'. An experimental model with Wistar rats, submitted to cardiac puncture to obtain blood was performed. After centrifugation, a PRF clot was obtained. The clots were drained for different times according to the group: 1' (GII),2' (GIII),3' (GIV), 5' (GV).The control group (GI) was composed of clots that did not undergo drainage, were removed from the tube and taken directly for fixation. Taking the clot as a whole, there is no statistically significant difference in the number of leukocytes between the groups, but the fibrin mesh density is higher in GIV samples compared to groups I, II and III, without any statistical difference to group V. Depending on the desired application in each case, clinicians should consider that the duration of drainage of blood clots will significantly affect thedensity andclot cell composition, even though there is only a difference of 1'between the compression times.

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INTRODUCTION

The use of autologous fibrin has been reported widely, with several applications in health since its three-dimensional structure becomes an ideal scaffold and also because of the large capacity to stimulate collagen, vascularization and even bone tissue.(Gülşen, 2017, Vieira *et al*. 2017)However, further studies are needed to better understand the best methodologies to harvest, best source between animal models, ideal blood volume, adjustment of protocols used, quality of the fibrin mesh formed, and assess the possible interference of the methodologies used on the structural changes and quantification of the cellularity, present in the structure of the network.(Agrawal, 2014)
Fibrin is the activated form of a molecule called plasma fibrinogen having a decisive role in platelet aggregation during hemostasis. Autologous platelet-rich fibrin (PRF) is a new generation of platelet concentrate, more specifically a second generation of concentrate,

characterized by centrifugation-based processing and absence of biochemical additives in the blood sample.(Mosesson, 2006)The action of this biomaterial is osteoconductive, stimulating autologous cells and leading to regeneration. It is also important in the activation of platelets and their subsequent degranulation with significant release of cytokines such as TGFβ-1, PDGFs and IGFs.(Agrawal, 2014, Dohan *et al*, 2006). The clinical applications of L-PRF in Dentistry are diverse, ranging from the maxillary sinus elevation in combination with bone grafts, through preservation of the alveolus after dental extraction or avulsion to enhancement of palatal wound healing after free gingival graft, among others.(Almeida *et al*. 2016) Therefore, it is relevant to study the three-dimensional fibrin mesh, especially its structure and cellularity post-processing and drainage, in order to better understand the L-PRF, leading to advances in techniques and enabling the development of new biomaterials.(Dohan *et al*. 2006, Almeida *et al*. 2016).

METHODS

The study was approved by the Committee on Ethics in Research and Use of Animals (CEUA)/UFJF (Opinion no. 046 /2017). An animal model-based clinical trial with Wistar rats (*Rattus norvegicus*) from the UFJF was developed. Thirty animals (N = 30), with an average age of 3 months and 250g to 300g weight were used. The animals were housed in 0.15 m^2 cages in an environment with an average temperature of 22 °C and alternating photoperiod (12 hours light/12 hours dark), relative humidity maintained around 40 - 60%; water and food (pellets) *ad libitum*. The animals were randomly divided into 5 groups (N = 6) and submitted to the same treatment consisting of euthanasia and cardiac puncture for blood collection, which was immediately transferred to vacuum-filled tubes without additives (Vacuttainer®) and centrifuged at approximately 400*g* for 10 minutes in a Montserrat® fixed angle rotor bed centrifuge. Euthanasia was performed by anesthetic deepening with ketamine hydrochloride, 60-80 mg/kg and chlorpromazine hydrochloride, 1.6-2.0 mg/kg. After loss of consciousness and with the heart still beating, cardiac puncture was performed by the same operators, using a 5 mL syringe (figure 1). Each tube was immediately centrifuged at 400*g*. After centrifugation, three phases were observed; Platelet-Poor Plasmain the upper part, Red Blood Cells in the lower part and the clot in the intermediate part. The clots were drained according to the following groups: 1' (GII), 2' (GIII), 3' (GIV), 5' (GV), and the control group (GI) was the only one in which clots were not drained, being removed and directly sent for fixation.

Drainage of the PRF clot and preparation of histological slides: Each blood clot was carefully removed from the tube and the bottom layer (red blood cells) separated, remaining only the L-PRFclot, which was weighed and drained. Drainage consisted of the use of FibrinBox®, a stainless steel instrument, consisting of a drainage grid, collection reservoir, glass plate for drainage and cover. The clots were individually arranged on the grid and a 77g glass plate was carefully superposed and centralized to allow drainage according to the periods for each animal in each group. The fixation was performed with 10% buffered formalin for 24 h; after this period, the samples were submitted to posterior fixation in paraffin and the samples were sliced in a microtome calibrated for 4 μm slice thickness in the longitudinal direction in relation to the clot body, allowing an analysis along the sample in a reliable way. Then a routine histological processing was carried out with hematoxylin and eosin (HE) dual staining.

Slide Morphometry: The analysis of the slides was performed in a semiautomatic way with the same operator obtaining all the data through a microscope camera (ZEISS Axiocam Microscope Cameras) and ZEN blue capture and measurement software. To evaluate the images, the samples were assessed by areas, an external portion (80 μm thick layer, measured by the software, along the whole extension of the clot body) and internal portion (internal area 80 μm thickness from the edge/outer portion). Ten representative images of each area were randomly captured along the body of the clot. The captures were carried out at two distinct times and all the images for the internal portion were captured at one time and all the external portion at another time, to avoid distortions and bias in the capture method.

Stastical analysis: The results of the survey were tabulated in the software *GraphPad Prism 5* and submitted to statistical analysis. The tests used for the analysis of leukocytes were applied to certain areas in the blood clots as external portion, inner portion and both (external portion + inner portion). The tests applied were the Kolmogorov-Smirnov (KS), D'Agostino & Pearson and Shapiro-Wilk normality tests. The graphs were constructed by applying the Kruskal-Wallis tests and Dunn's multiple comparison test. For the analysis of the fiber density the areas considered were the same under the same previous parameters and the tests applied were KS, D'Agostino & Pearson and Shapiro-Wilk normality test. The graphs were obtained by applying the Kruskal-Wallis tests and Dunn's multiple comparison test. Correlations between groups were obtained from the Pearson correlation test. The level of significance was set at 5%.

RESULTS

The measurement of the density of fibers was performed with the aid of the Zen blue semi-automatic analysis software (Figures 1 and 2), and the cell count was performed with the ImageJ Software (Figure 3). Both analysis, fiber density and leukocyte count, were conducted at different moments and different areas considered in the study. The results were obtained separately by area and alsotaking the PRF clots in their entirety.
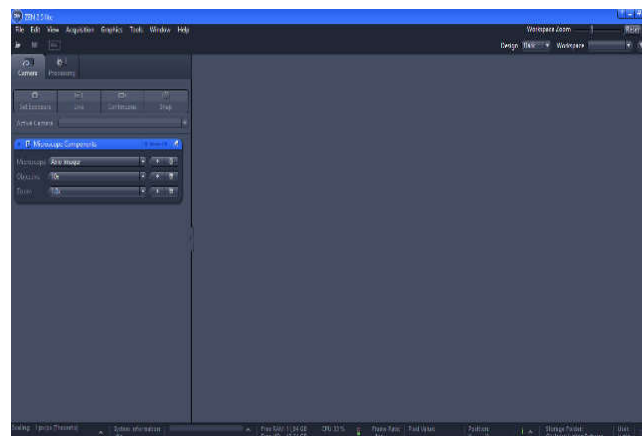

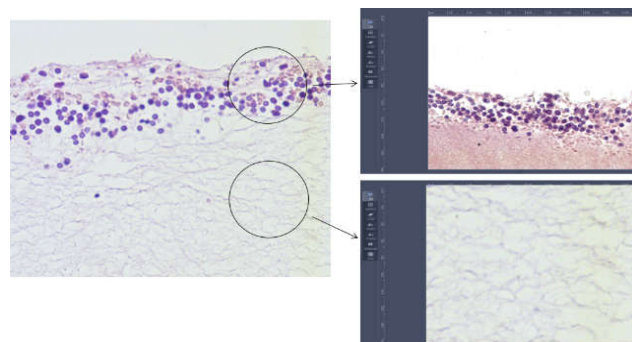**Figure 1. Screenshot of the semi-automatic capture and analysis software**

Figure 2. Counting software. Number of leukocytes counted using the semiautomatic Image J software. Points in yellow represent cells marked during counting
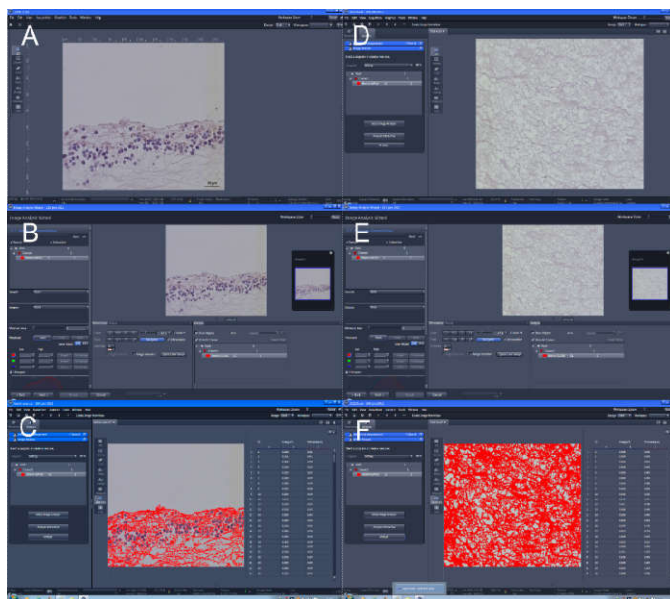

Figure 3. Sequence of morphometry. Measurement of the density of fibers. A, B and C peripheral portion (edge of the blood clot). D, E and F internal region
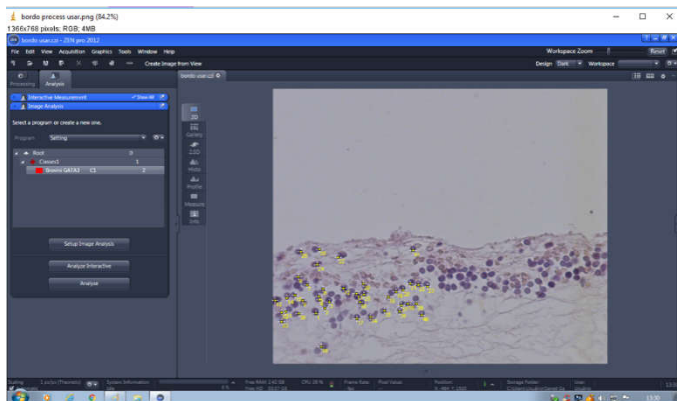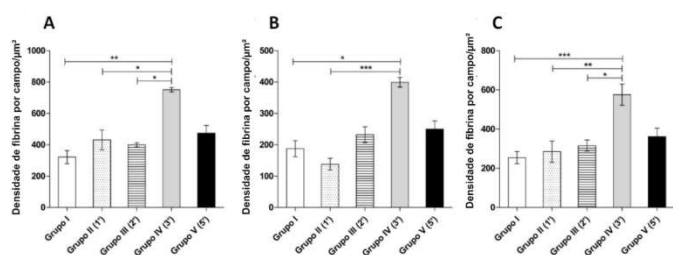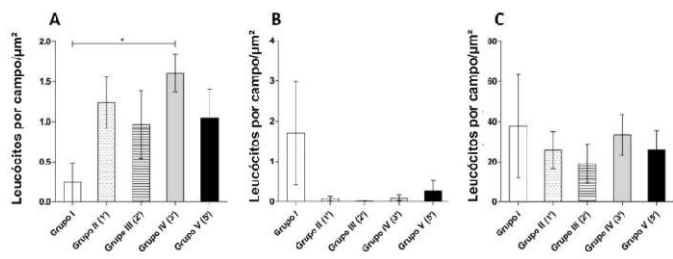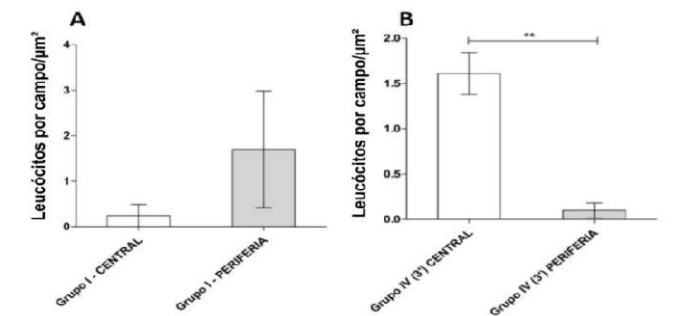
Figure 4. Counting software. Number of leukocytes counted using the semiautomatic Image J software. Points in yellow represent cells marked during counting
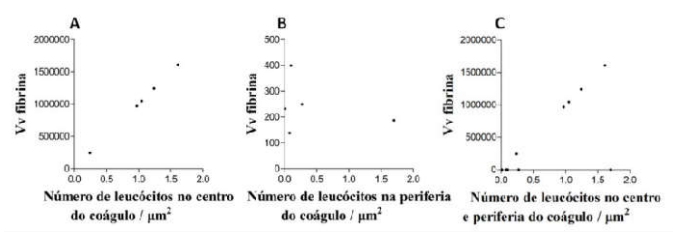

Graphic 1. Density of the fibrin network by groups. A inner portion. B peripheral portion. C blood clot as a whole

Graphic 2. Leukocyte count in fibrin clot by groups. A inner portion. B peripheral portion. C blood clot as a whole

Graphic 3. Leukocyte count in fibrin clot by region and group. A internal and peripheral region in the control group. B internal and peripheral region of group IV (3')

Graphic 4. Pearson correlation of fibrin volume among all the groups. A leukocytes only from the internal region Vs mesh volume. B Leukocytes only on the periphery of the groups in relation to the volume of the mesh. C Leukocytes in the whole clot among the groups in relation to the volume of the fibrin mesh

DISCUSSION

Harvesting and applications of platelet rich fibrin comprise a broad spectrum. Different protocols, forms of processing, and instruments to manipulate PRF are used, with diverse forms, trademarks and models, which can be analyzed under two viewpoints; the first, favoring access and the constant emergence of new protocol proposals within the universe of autologous biomaterials, in this case, PRF.(Almeida *et al.* 2016) The second is that such heterogeneity may provide difficulties to the researcher or clinician as to which harvesting and processing method or protocol to use which are more favorable to the objectives, as well as how to interpret results especially when the biomaterial is applied in combination with others of diverse natures, such as freeze-dried bones, hyaluronic acid, alloying materials, among others, providing the following questions:

"*To what extent can the results, in fact, be related to the use of PRF or the material associated with it? Can the benefits obtained with the application, combined or alone, be associated to the use of platelet-rich fibrin?*"

In a study with an animal model, (Yamashit *et al.* 2016) argued that PRF alone cannot act as a scaffold or membrane barrier when it comes to maintaining space in wounds, in order to favor healing. Several studies, especially in humans, are being continuously published, and despite various consensuses, different protocols and applications are still used. When comparing clinical trials in animals, the scarcity is even greater with regard to defined protocols, number of published studies and uniformity of results. (Oliveira *et al.* 2014) stated in their study with a Wistar animal model, that PRF had positive effects on bone regeneration, only when combined with demineralized bone (Bio-oss). However, (Sindel *et al.* 2017) argued in their study that PRF and demineralized bone did not differ, being superior only to the use of hyaluronic acid in the healing of bone defects, which contrasts the findings of (Dülgeroglu, 2011) in the same year, who evaluated bone healing in long bones and concluded that PRF is an excellent material in the healing of fractures. In skin wounds, (Camargo, 2013) found that their healing was not superior with the application of PRF compared to physiological solution, but superior when compared to the group where PRP was applied.

When assessing skeletal muscle healing, (Gigante *et al.* 2012) reported that the application of PRF improves healing and vascularization in the long term, being a safe and useful product for the treatment of muscular injuries. Within this spectrum, the present work sought to combine a method of obtaining and processing the clot in a membrane that followed parameters more analogous to what is essentially applied to humans, such as: a single step method of harvesting and centrifugation; without chemical additives; tubes with internal surface treatment; suitable compression and collection kit (FibrinBox); calibrated microtome in the direction of the long axis of the membrane. The computer analysis was performed by areas (peripheral and internal) so named after observations in several studies, when histological images are presented where the leukocytes tend to concentrate in proximal portions and in the borders of the clot with aspect of aggregated cell chains in greater number in this region, being more dispersed throughout the remaining clot.(Dohan *et al*, 2006) In addition, evaluating the behavior of the fibers and the cellularity throughout the body of the clot in the longitudinal direction allowed an enlarged view of the behavior separated by region, and of the clot as a whole regarding its compression and leukocyte number. The results of the study suggested that the 3'drainage period favors a better accommodation of the fibers (pore compaction/increase in density) compared to the other times in the internal portion (Figure 1A); which only occurs between GI and GIV when the peripheral region is analyzed (Figure 1 B).

These results suggest that the clot conformation after drainage is less homogeneous than when fresh, which may favor the exit of fluid and cytokine content, and especially the periphery must be less compact to favor drainage without considerably compromising the structure

under pressure. When the clot is evaluated as a whole (Figure 1C), the 3'period is again emphasized compared to the others except for V suggesting that after the 3' drainage, there is no statistical significance for the body of the clot regarding compression with a 77g glass plate in an animal model. When taking into account the leukocyte number in the internal portion, GIV again stands out differing only from the control (Figure 2A). This data was analyzed as a response of the cells to the pressure exerted by the glass plate at this drainage period, where they tend to concentrate on the inner portion, along the clot, because, when one considers the periphery of this (Figure 2 B), in spite of the variability of the sample, no statistical differences were found. The leukocyte count in the blood clot as a whole, (Figure 2 C) also did not present statistically significant differences, suggesting that there is a compensation in the arrangement and general stability of the cell count along the clot. This analysis can be enhanced when GIV is evaluated individually by associating it with the internal and peripheral portions (Figure 3 A) where there is statistically significant difference and high numerical or quantitative difference compared to the periphery. Thus, it seems that the drain time of 3' increases the density of the fibers and the number of leukocytes in the inner portion along the body of the clot. When the same specific evaluation is performed with the control GI (Figure 3 B) where compression was no performed, being fixed immediately, there are more leukocytes in the periphery, as already stated, and uniform aspect of the fibers, visible in the photomicrographs in a general way as empirical finding, and the data of this study, suggested that this is probably due to the action of the g force and angle of rotation of the rotor from the centrifuge.

Multiple findings such as those presented in this work also suggested an evaluation of the possible dynamics between the variables considered in this study (volume/density of the fibrin mesh and leukocytes) among all groups in an attempt to understand whether to submit the PRF clot to a given stress, such as the provision of a plate with weight far superior to the clot, generating internal tensions in the whole body and consequently in each area assessed, observing any relationship of cause and effect, or simplyrandom dynamics. For such, a Pearson correlation was held with all groups for each area and the clot as a whole vs the volume/density of fibrin. When taking the internal region of the clot in relation to the volume of fibrin (Figure 4), the groups establish a perfect interrelationship (equal to 1) suggesting that a variable changes in relation to another, which does not occur when the peripheral region is evaluated (Figure 4 B) given the dispersion graph between the groups. When the clot is considered as a whole (Figure 4 C), the correlation between the variables tend to be more even, suggesting that despite the differences between the regions of the clot evaluated separately, when evaluated in general and submitted to stress, they tend to behave in a compensatory manner while maintaining a certain stability among physical structure (fibrin mesh) and their cellular content.

Conclusion

The 3' drainage time is an important and relevant factor when the density (compression) of the fibers as well as cellularity is compared to the other periods, even when higher such as 5'. Depending on the desired application in each case, clinicians should consider that the drainage time of the clots will considerably affect its structure and cellular composition even when there is a difference of 1' between the times of compression.

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