Histologic Evaluation of Leucocyte- and Platelet-Rich Fibrin in the Inflammatory Process and Repair of Noncritical Bone Defects in the Calvaria of Rats

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Purpose: This study aimed to evaluate the effects of leucocyte- and platelet-rich fibrin (L-PRF) on the inflammatory process, tissue repair, and expression of vascular endothelial growth factor (VEGF) on bone defects in the calvaria of rats. Materials and Methods: L-PRF was obtained from three animals submitted to cardiac puncture to prepare the membranes. Two noncritical defects with a diameter of 2 mm were created in the calvaria of 15 Wistar rats. The defects on the right side were filled with a blood clot (CTRL) and the left side with L-PRF. After 5, 15, and 30 days, the animals were euthanized and the specimens processed for histologic, histomorphometric, and immunohistochemical analyses. In order to measure the intensity of the inflammatory infiltrate and VEGF expression, scores were assigned from 0 to 3, with 0 being no expression, 1 discrete (up to 25%), 2 moderate (between 25% and 50%), and 3 intense (> 50%) expression. The area of bone neoformation at the edges of the defects was also quantified. Results: A less intense inflammatory infiltrate was observed in the defects filled with L-PRF compared with CTRL at all times analyzed (P < .05). At 5 days, no bone neoformation was observed in any of the groups evaluated. After 15 and 30 days, greater bone neoformation was observed in the group treated with L-PRF compared with the CTRL group (P < .05). At 15 days, 3,871.8 (1,070.15) μm² were recorded for the CTRL and 49,978.5 (14,360.7) μm² in the L-PRF. At 30 days, 62,284.5 (3,579.5) μm² were observed in the CTRL and 154,076.6 (31,464.9) μm² in the L-PRF. At all evaluated times, a lower inflammatory infiltrate was observed in the group treated with L-PRF compared with the CTRL. VEGF expression was observed in the initial phase and throughout the tissue repair process in both groups. At 5 days, there was no difference in VEGF expression between the groups. VEGF was present at the initial phase and throughout the tissue repair process in both groups. In the L-PRF group, a decrease in VEGF expression was observed at 15 and 30 days compared with the CTRL group. Conclusion: L-PRF had a positive effect on the regenerative process of bony defects, with a reduced inflammatory response and greater bone neoformation. INT J ORAL MAXILLOFAC IMPLANTS 2018;33:1206–1212. doi: 10.11607/jomi.6604

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The development of techniques that control the inflammatory process and promote bone healing after surgical procedures is among the major challenges faced by clinical research. It is well established that platelets play an important role in the healing process aside from their role in hemostasis. Many studies have demonstrated that granules secreted by platelets, such as α-granules, contain growth factors, including AB-platelet-derived growth factor (PDGF-AB), transforming growth factor β (TGF-β), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF).

VEGF plays an important role in tissue repair and bone formation, as it promotes angiogenesis, aggregation, and proliferation of endothelial cells and mesenchymal stem cells, not to mention its potent stimulant effect on osteoblast proliferation and differentiation.

In order to obtain high concentrations of growth factors, several techniques have been developed to
concentrate platelets. Initially, the most common approach was platelet-rich plasma (PRP), which has been exhaustively researched as a result of its potential bone regeneration capacity.9–11

The fact that different techniques are used in platelet collection and PRP preparation may in some cases contribute to premature platelet degranulation, besides providing different platelet concentrations, which could influence the outcome of such an approach.5,12 Some studies have questioned the effectiveness of PRP due to its short-lived growth factor release, peaking on the first day of treatment and decreasing to negligible values in subsequent days. Additionally, the use of thrombin during PRP manipulation results in abrupt polymerization, making it difficult to incorporate cytokines into the fibrin mesh, which causes premature release of growth factors, even before the arrival of osteoblasts.6,13,14

Regarded as the second generation of platelet concentrate, leukocyte- and platelet-rich fibrin (L-PRF) is defined as an autologous scar matrix.13,15–17 The process for obtaining L-PRF is relatively simple, without the need for anticoagulants or bovine thrombin.15 After centrifugation, blood is separated into three phases: the upper phase is acellular plasma, the lower phase is red cells, and the intermediate phase is L-PRF that is removed and shaped into a membrane using the shaping kit supplied by Intralock. L-PRF membrane near the red blood cells, which is considered to have the highest growth factor concentration, was used to fill the defects.

The animals were divided into three groups containing six animals. One animal from each group was sacrificed for blood collection through a cardiac puncture (Fig 1a). The blood from each animal was processed to produce the L-PRF membranes (Fig 1b), according to the Choukroun protocol.21 The blood was collected in a plastic tube with silica (9 mL), centrifuged at 3,000 rpm for 12 minutes, to obtain three phases, where the supernatant corresponded to the acellular plasma, the middle phase to the L-PRF, and the lowermost phase to the red cells. The L-PRF was removed with the aid of forceps and shaped into a membrane using the shaping kit supplied by Intralock. L-PRF membrane near the red blood cells, which is considered to have the highest growth factor concentration, was used to fill the defects.

The animals were submitted to trichotomy in the region of calvaria and subsequent antisepsis of the area with PVPI with 1% active iodine. To access the cranial cap of the animal, a 2-cm incision was made in the skin using a 15-inch scalpel blade in the median region of the skull, extending from the nasofrontal region to the occipital protuberance. The skin, the subcutaneous layers, the temporal muscles, and the periosteum were detached and laterally flipped to gain access with extensive exposure to the calvaria.

Two noncritical bony defects were made on either side of the median sagittal suture of the calvaria using a 2.0-mm trephine drill, exposing the dura mater. The defects were then irrigated with saline solution to remove any bone fragment from the defect22 (Fig 2a). The defects were then filled with L-PRF on the left side and with blood clot alone (CTRL) on the right side. After filling the defects, the surgical wound was sutured using single stitches and 4-0 needle-threaded mononylon (Fig 2b).

After surgery, the animals were placed in cages on a ventilated shelf, without restriction of movements, with feed ad libitum.

They received 0.5 mg/mL of Dipirone in the water immediately after the surgical procedures and also after 24 hours to ensure postsurgical analgesia.

Preparation of L-PRF and Bony Defects

The aim of this study was to evaluate the performance of L-PRF in bone repair in noncritical defects in the calvaria of rats, as well as to evaluate the extent of the inflammatory process and the expression of VEGF.

MATERIALS AND METHODS

Eighteen Rattus Norvegicus Albinus of the Wistar lineage composed the total sample of the study herein reported. The experiments were performed in the animal facilities of the São Leopoldo Mandic Research Center, with prior approval of the Research Ethics Committee for Animal Experimentation (Protocol No. 20150396). The animals were kept under controlled lighting conditions, with a light-dark cycle of 12 hours at 21°C, with balanced feed and water ad libitum.

The experiment was performed on 15-week-old animals weighing between 300 g and 400 g. The surgical procedure was performed under general anesthesia, using ketamine hydrochloride 50 mg/mL (Dopalen) and xylazine hydrochloride 20 mg/mL (Anasedan) for sedative, muscle relaxation, and mainly analgesia. The drugs ketamine and xylazine were administered to the animals intraperitoneally. The surgical procedures were performed respecting the principles of biosafety to avoid infection of the surgical wound.
The animals were euthanized on specific postoperative days, namely, 5 days (n = 5), 15 days (n = 5), and 30 days (n = 5). Euthanasia was performed by deepening anesthesia according to the following protocol: 90 to 150 mg/kg of sodium thiopental (71-73-8) combined with 10 mg/mL of Lidocaine (137-58-6), intraperitoneally. The areas of interest were subsequently accessed by dissection and removed using an autopsy oscillating saw.

**Histologic Processing**

The samples removed were prepared for standard light microscopy. They were immersed in 10% buffered formalin and subsequently demineralized in 20% formic acid.

The pieces were then included in histologic paraffin, and 4-μm sections were taken cross-sectionally from the central region of the defects, so that two cuts were made per sample at intervals of 10 μm.

Samples were stained with hematoxylin-eosin and then mounted on glass slides with biologic resin (Permopar, Fisher Scientific). Photomicrographs were taken under a light microscope for the analyses described below.

**Histologic Analysis and Histomorphometry**

For histologic analysis, the presence of neutrophil, lymphocyte, and plasmocyte cells; granulation tissue represented by the proliferation of blood vessels and fibroblasts; and macrophages were considered. A classification score was adopted, considering the extent of the inflammatory process in the defect area from 0 to 3, with 0 being absent, 1 discrete (up to 25%), 2 moderate (25% to 50%), and 3 intense (> 50%). In addition, the presence of new bone formation was described at the edges of the defects.

The histomorphometry analysis of the histologic sections was performed by determining the area occupied by neoformed bone tissue at the edges of the defects created in the calvaria of the samples at different postoperative days.

The sections were analyzed using histomorphometry on a computerized image analysis system consisting of an Axioskop 2 plus light microscope (Carl Zeiss), connected to a microcomputer using the AxioVision rel. 4.8 image analysis software (Carl Zeiss), thus determining the measurements of the areas of neoformed bone (μm²). Sections were individually analyzed using a 4× objective, and the histomorphometry per se was performed under 40× objective.

**Immunohistochemistry**

Four-micrometer sections were obtained from the formalin-fixed and paraffin-embedded samples, and the material was placed on glass slides pretreated with 4% organosilane solution in acetone.

The sections were deparaffinized and hydrated. Endogenous peroxidases were blocked by immersion in 3% hydrogen peroxide solution (H₂O₂). Antigenic recovery was performed in a water bath with citrate buffer (pH 6.0). After antigen recovery, nonspecific protein block was performed (Dako). The slides were then incubated with the VEGF antibody (1:100, clone Sc-7269, St. Cruz Biotech., Inc.) for 18 hours, and amplification was performed using the Advance (Dako) system. The slides were subsequently stained with 3,3′-Diaminobenzidine (DAB) and counterstained with hematoxylin.

Endothelial cells were used as a positive internal control. The negative control of the reactions was obtained by omission of the respective primary antibody.

**Evaluation of the Immunohistochemistry Reactions**

Expression of the growth factor was analyzed semiquantitatively by percentage of cell positivity to VEGF, which was graded 0 to 3, where 0 corresponded to less than 10% positive cells, grade 1 from 10% to 25% positive cells, grade 2 from 25% to 50% positive cells, and grade 3 greater than 50% positive cells. Only cytoplasmic labeling was considered positive. When nuclear marking was observed, only those cases that accompanied concomitant cytoplasmic expression were considered positive.
Statistical Analysis
Data were statistically analyzed on SPSS 20 (SPSS), adopting a significance level of 5%. The Student t test and Tukey post hoc test were used to compare the groups with each other in the histomorphometry analysis, and the G test was used to evaluate inflammation scores.

The absolute and relative frequencies with which VEGF was expressed in the presence or absence of the L-PRF membrane were analyzed descriptively to compare the proportion of positive cells, classified according to the scale of scores from 0 to 3.

To investigate whether the intensity of VEGF expression was influenced by the presence of the L-PRF membrane, number of days postoperatively, or by the interaction of both variables, in view of the ordinal qualitative nature of the data, the Scheirer-Ray-Hare test was applied, a two-way analysis of variance (ANOVA)-like test for nonparametric data.

RESULTS

Histologic and Histomorphometric Findings
Representative images from inflammatory infiltrate and bone neoformation are shown in Fig 3.

After 5 days of evaluation, the presence of the intense nonspecific chronic inflammatory process was observed filling the whole defect, with the presence of lymphocytes, plasma cells, and macrophages in the CTRL and L-PRF groups evident in regions where the membrane was not used.

After 15 days, the presence of an inflammatory process and discrete bone neoformation was observed in the CTRL group at the edges of the defects. The L-PRF group showed neoformed bone on both edges of the defects, granulation tissue containing fibroblasts, and a discrete inflammatory process.

At 30 days, a moderate nonspecific chronic inflammatory process was observed in the CTRL group, with the presence of granulation tissue and a large number of fibroblasts and collagen fibers; bone neoformation was restricted to the edges of the bony defect; and none of the specimens presented complete closure of the defect. The L-PRF group presented bone neoformation with osteoblasts at the borders of the defects, no granulation tissue or inflammatory process (score 0), and a well-vascularized defect. In this group, none of the specimens presented complete closure of the defect.

Regarding the inflammatory infiltrate, the samples submitted to the treatment with L-PRF showed less inflammation compared with the CTRL at all the evaluated times, being statistically significant at 5 days ($P = .025$), 15 days ($P = .03$), and 30 days ($P = .03$). In the same way, according to the longer evaluation time,
there was a decrease in the intensity of inflammation, both in the CTRL group ($P = .046$) and in the PRF group ($P = .03$) (Table 1).

Table 2 shows the values of means and SD of bone neoformation at the edges of the defects. After 5 days of evaluation, no neoformed bone was present in any of the groups evaluated. After 15 days, neoformed bone area amounted to 3,871.8 (1,070.15) and 49,978.5 (14,360.7) μm² for the CTRL and L-PRF groups, respectively. After 30 days, the amount of neoformed bone was 62,284.5 (3,579.5) for the CTRL group and 154,076.6 (31,464.9) μm² for the group treated with PRF. At both evaluated times, bone neoformation in the L-PRF group was statistically higher than the CTRL group ($P < .05$).

**VEGF Immunoexpression**

VEGF expression was observed in the nucleus and cytoplasm of cells, mainly endothelial cells, fibroblasts, and osteocytes.

In the defects filled with the L-PRF membrane, VEGF expression at 5 days scored 2 (positivity in 25% to 50% of cells) in one case (33.3%), and 3 (positivity in more than 50% of the cells) in the remaining two cases (66.7%) (Fig 4 and Table 3). After 15 and 30 days, there was a decrease in VEGF expression, with two cases (66.7%) scoring 1 (positivity in 10% to 25% of cells) and one case (33.3%) scoring 2 (Fig 4 and Table 3).

After 5 and 15 days, all CTRL cases (100%) scored 3 (positivity in more than 50% of the cells). After 30 days, a slight decrease in VEGF expression was observed, with one case (33.3%) scoring 2 and two cases (66.7%) scoring 3 (Fig 4 and Table 3).

The Scheirer-Ray-Hare test revealed that VEGF expression scores were significantly lower in the presence of L-PRF compared with the CTRL at all time points ($P = .003$) (Table 4).

**DISCUSSION**

Bone healing is a complex process, involving different types of cells, tissues, and chemical mediators. This process is commonly divided into four steps: inflammation (granulation tissue), soft callus formation, hard callus formation, and bone remodeling.23 In this context, several biomaterials have emerged aiming to improve bone healing and reduce healing time. Platelet concentrate has been used to optimize oral tissue repair, with a main advantage of growth factor release, such as VEGF, which is extremely important in the regulation and control of inflammatory as well as healing processes.24 However, little is known on the performance of platelet concentrates in the cells and growth factors involved in the healing process, especially at the inflammation phase.
In this study, the calvaria of rats was chosen as the region for experimentation, as it is anatomically favorable for transoperative manipulation and postoperative control, minimizing the chances of surgical site infection. In addition, it is a region free from mechanical stress, with relative stability of the structures surrounding the defects, having the dura mater as the base of the bony defect, whereas the cranial periosteum and the temporal and frontal muscles formed the outer cover. This model allows the evaluation of interactions between the neoformed bone and the bone in situ.

According to Yoshimaki et al., calvarial defects in rats with less than 2.7 mm are considered noncritical defects. Thus, bone defects were created with a 2.0-mm-diameter trephine, aiming to devise an experimental model that could provide data on the healing and inflammatory processes from a regenerative potential perspective. Because it is a noncritical defect, it allows a comparative analysis of the CTRL group without the need for additional biomaterials. No macroscopic phlogistic signs or wound exposure were observed in any of the evaluated specimens. Therefore, the experimental model based on bilateral circular defects of 2.0 mm diameter was deemed to be the most suitable for this experiment, allowing a control group with high precision for comparison between the sides within the same animal.

In order to enable rapid blood collection according to the time frame established by the protocol, sampling for the production of the membrane in this experiment was performed through cardiac puncture. Concerns regarding blood sampling from animals were raised by Dohan Ehrenfest et al. because some studies reported blood collection by dripping, which is time consuming, where time between collection and centrifugation is a critical factor in the PRF protocol. In a study by Oliveira et al., no significant differences were found between the group of rats in which autologous PRF was used to fill critical defects in the calvaria and the group that used homogenous PRF, although the highest percentage of bone neoformation was observed in the homogenous PRF group. This demonstrates that the use of a homogenous membrane of L-PRF would not be a limitation to the experiments reported herein.

Several studies have been performed trying to establish the true regenerative potential of PRF. Studies in vivo evaluating the performance of PRF in the healing process in defects made in the calvaria of rats have demonstrated the superiority of this model to assess tissue regeneration. Such findings corroborate the results of the present study, which demonstrated that PRF promoted greater bone neoformation as well as a shorter and less intense inflammatory response compared with the clot-filled group (CTRL).

The results obtained in this study confirm the ability of L-PRF to reduce inflammatory process time and to consequently improve tissue healing. During the histologic evaluation over time, it was possible to verify that after 5 days of treatment in the group that received the L-PRF membrane, the inflammatory process was much less intense than in the control group, which showed an intense inflammatory infiltrate throughout the extension of the defect \( P = .025 \). Also, no evidence of bone neoformation was observed in any of the animals at this experimental time. At 15 days, although both groups showed evidence of bone neoformation, the CTRL group still presented an intense inflammatory process and lower bone neoformation, whereas the L-PRF group showed greater bone neoformation, granulation tissue, and an inflammatory process in remission. After 30 days, the presence of an inflammatory infiltrate and granulation tissue as well as bone neoformation was restricted to the edges of the defects in the control group, while the group treated with L-PRF had a higher amount of neoformed bone with no granulation tissue or inflammatory infiltrate.

In view of the findings from the histologic analysis, the groups that received the L-PRF membrane behaved as if they were at an advanced repair stage compared with the CTRL, corroborating the findings by Tatullo et al., in which a reduction in healing time was observed, favoring bone regeneration. Other studies have also reported superior results of neoformed bone in groups treated with PRF compared with controls using quantitative histomorphometry.

The angiogenic potential of L-PRF is one of the factors that contributes to accelerate the regenerative process favoring predictability. The combination of growth factors, especially VEGF, has shown an acceleration in bone repair and stimulation of fibroblasts, osteoblasts, and undifferentiated and endothelial mesenchymal cells, resulting in increased vascularization and collagen synthesis, which play a key role in bone formation.

From the analysis of the data obtained in this study, high VEGF expression was observed after 5 days in both groups, ie, superexpression of VEGF at the beginning of the repair process, when granulation tissue with a large quantity of newly formed vessels is observed. After 15 and 30 days, high expression of VEGF in the CTRL group was still identified, whereas in the PRF group, decreased VEGF expression was observed, indicating that the latter approach decreases the initial phase of the bone repair process (granulation tissue), as observed by Can et al. In addition to growth factors, L-PRF is mainly composed of a fibrin network, which is a natural conductor of angiogenesis and provides protection to injured tissues, behaving like a biologic dressing. Many authors have demonstrated that such fibrin matrix
provides excellent support for undifferentiated mesenchymal cells, contributing to the regeneration of bone cells and many other tissues as well as assisting in the repair of bone defects.\textsuperscript{30–32}

Based on the results obtained and the limitations of the present study, L-PRF had a positive effect on the regenerative process of noncritical bony defects, with a reduced inflammatory response and greater bone neoformation compared with controls, due to less intense VEGF expression and inflammatory response, which optimizes the early stages of tissue repair and supports a potential role for L-PRF in sites that require faster repair.

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The authors declare that they have no competing interests.

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