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ORIGINAL PAPER

Effect of platelet-rich fibrin (PRF) membranes on the healing of infected skin wounds

Bruno Botto Barros Silveira[1] · Lucas Novaes Teixeira[2] · Richard J. Miron[3] · Elizabeth Ferreira Martinez[2]

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Abstract

Tissue engineering focuses on wound healing and tissue regeneration. Platelet-rich fibrin (PRF) is a fibrin matrix containing cytokines, growth factors and cells that are gradually released into the wound over time. This study aimed to evaluate the effect of PRF membranes on wound repair and microbial control in infected wounds. Skin wounds were performed on the dorsum of rats using a 6 mm diameter metal punch. The defects were randomly assigned into four groups ($n = 12$/each) accordingly to the treatment: G1, noninfected wound filled only with clot; G2, noninfected wound with PRF; G3, infected wound *(S. aureus*) without PRF; G4, infected wound (*S. aureus*) with PRF. After 7 and 14 days, macroscopic and histological analyses of the wounds were performed. Furthermore, the quantification of β-defensin in PRF was measured by ELISA. At 14 days, the groups with PRF (G2 and G4) had wound sizes significantly smaller than the original defects (6 mm) ($p < 0.05$) and significantly smaller than those not treated with PRF, in both the infected and noninfected groups ($p < 0.05$). Furthermore, the groups with infected wounds (G3 and G4) demonstrated a significantly lower inflammation score in the PRF group than in the noninfected groups ($p < 0.05$). In vitro analysis of β-defensin was performed in all PRF membrane groups, and the median value was 1.444 pg/mL. PRF in the wounds of both control and infected rats played an important role in the modulation of tissue healing, most notably in infected sites.

Keywords Leukocyte- and platelet-rich fibrin · Biomaterial · Tissue regeneration · Inflammatory response

Introduction

The development of techniques that control inflammatory responses and stimulate tissue healing after surgery is one of the main challenges in daily clinical practice given the complexity of the process itself and the cells and byproducts involved [1]. In tissue engineering, vascularization plays an important role by ensuring not only proper nutrient supply but also product and residue removal from the wound or the transplanted tissue [2]. Therefore, in addition to their main function related to hemostasis, platelets are very important for tissue healing [3].

During platelet degranulation, the release of cytokines and growth factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF), and insulin-like growth factor (IGF) is observed. Each has been shown to modulate cell proliferation, matrix remodeling, and angiogenesis [4, 5].

Platelet-rich fibrin (PRF) has been described as a second-generation platelet concentrate, resembling an actual autologous healing matrix [6]. General characteristics of PRF include the modification of centrifugation speed and time as well as the lack of anticoagulants/polymerization agents, differentiating it significantly from first-generation platelet concentrates or platelet-rich plasma (PRP). Unlike PRP, PRF delivers the biochemical structure of a fibrin clot, with a high affinity for platelets, cells and circulating cytokines, and growth factors [2, 4, 5, 7]. In addition, the release of growth factors is controlled and sustained owing to the fibrin scaffold, which can benefit the regeneration process [3, 8–10].

✉ Elizabeth Ferreira Martinez
dr.efmartinez@gmail.com;
elizabeth.martinez@slmandic.edu.br

[1] Division of Oral Implantology, Faculdade São Leopoldo Mandic, Campinas, São Paulo, Brazil

[2] Division of Cell Biology, Faculdade São Leopoldo Mandic, Instituto de Pesquisa São Leopoldo Mandic, Rua José Rocha Junqueira, 13, CEP, Campinas, São Paulo 13045-610, Brazil

[3] Department of Periodontology, University of Bern, Bern, Switzerland

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Angiogenesis, the immune/inflammatory response, circulating stem cells, and epithelial tissue over the wound are all key factors during tissue healing. The process is highly influenced by PRF, enabling earlier wound healing with faster scar tissue remodeling and significant decreases in postoperative infections [7].

Similarly, in epithelial tissue, microorganisms can stimulate epithelial cells to secrete antimicrobial peptides, which are components of the innate immune response. They protect the body by modulating the immune and inflammatory response or directly inactivating pathogens. Therefore, an increase in such peptides by epithelial cells has been shown to enhance host resistance to microbial infections [11]. Defensins stand out among the great diversity of antimicrobial peptides involved during the inflammatory response [12, 13].

In addition to keratinocytes, circulating blood cells such as polymorphonuclear leukocytes (PMNs) may contain many defensin-rich granules fighting against pathogen invasion [14]. Among the several types of defensins, β-defensins (BDs) are secreted by epithelial cells and leukocytes in non-physiological situations, such as bacterial infection. Moreover, BD2 can exhibit its usual behavior, acting as a low-molecular-weight peptide to resist bacterial infections, and may promote cell proliferation and differentiation in bone tissue [15].

Given the lack of knowledge regarding biological events related to the use of PRF membranes during skin wound repair, this study focused on evaluating the effect of PRF on infected wounds in the rat dorsum in terms of healing and modulation of the inflammatory response. β-defensins in PRF were also investigated, given their role in microbial control.

Materials and methods

For this study, 54 male Wistar rats of the designation *Rattus norvegicus Albinus* were used (age: 90 days, mean weight: 320 g), with prior approval from the Research Ethics Committee for Animal Experimentation from Faculdade São Leopoldo Mandic (ethical protocol approval n. 2018/023). The study was carried out in compliance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiment). The animals were kept under controlled conditions of temperature and lighting, with a 12-h light–dark cycle, with balanced food and water ad libitum.

Sample groups and surgical procedures

The animals were divided into 4 groups according to the treatment used. Surgical procedures were performed using 5% ketamine hydrochloride (DopalenVetbrands, Jacareí – SP, Brazil) and 2% xylazine hydrochloride (Rompun Bayer, São Paulo – SP, Brazil), respecting the principles of biosafety to prevent infection in surgical areas.

The animals were placed in a prone position, submitted to trichotomy in the region of the dorsum, on a 24 mm^2 area (6 mm long × 4 mm wide) situated caudally to an imaginary line along the forelimbs. Povidone-iodine (PVP-I) with 1% active iodine was used for antisepsis. Skin demarcation was performed in the center of the previously shaved area of each rat by rotation of the cutting edge of a 6 mm diameter metal punch. The next step was to perform resection of a circular skin segment alongside the punch demarcation, deepening the incision until the muscle fascia was exposed (Fig. 1a).

After homeostasis was achieved, the defects were randomly assigned to the four groups ($n = 12$/each) as follows: G1, noninfected wound filled only with clot; G2, noninfected

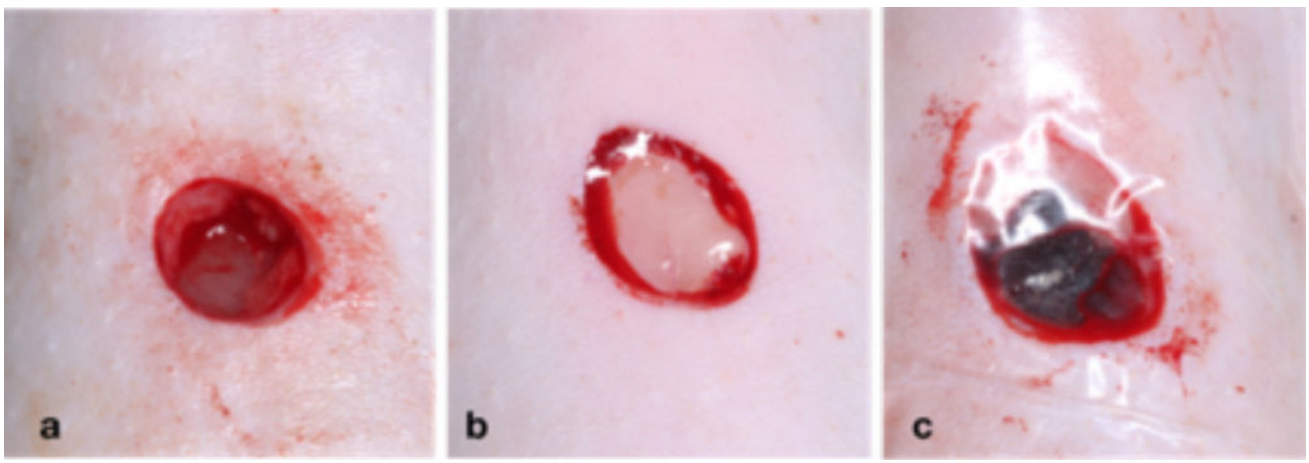

Fig. 1 Surgical procedures. **a** Wound created using a 6 mm punch and muscle fascia exposure; **b** PRF membrane on wound; **c** Tegaderm™ Film on the defect

wound with PRF; G3, infected wound *(S. aureus)* without PRF; G4, infected wound (*S. aureus*) with PRF.

After positioning the tissues, the wound was closed in all animals using the continuous technique of suturing with 4–0 absorbable polyglactin 910 thread (Vycril®, Ethicon, Johnson & Johnson, USA). All skin wounds were covered with a transparent and sterile polyurethane film (Tegaderm™ Film, 3 M Health Care, St Paul, MN, USA) to maintain the clot (G1) and the PRF membrane (G2 and G4) and to preserve the ongoing bacterial infection (G3 and G4) (Cross et al., 1995).

Wound contamination

Skin wounds (G3 and G4) were infected with a *Staphylococcus aureus* strain (ATCC 25,923, National Institute of Health, USA), as described by Vecchio et al. [16].

S. aureus was cultured in BHI (Brain Heart Infusion, Imedia, India) broth. For the experiments, a suspension equivalent to 0.5 McFarland (Nefolobac, Brazil), corresponding to 1×10^8 colony forming units (CFU)/mL, was used. The wounds were inoculated with the suspension using sterile swabs [17].

After the procedure, the animals were kept in individual cages under temperature control and with no restrictions in relation to movement or food.

PRF membrane preparation

Blood was collected via the cardiac puncture technique from 2 animals. The left ventricle was directly punctured with a 21 g vacuum blood collection set [18]. Due to rapid blood clotting in this species, a 10 mL plastic tube (Vacutube Seco, Biocon®, Brazil) without anticoagulant was used, as previously described by do Lago [19]. After drawing the blood, the membranes were produced using a protocol of 2700 rpm for 12 min and an RCF-clot (relative centrifugal force at PRF clot) of 637×g using a Spinplus Digital Centrifuge device (Spinlab®, China) (45° rotor angulation, 78 mm radius at the clot, 105 mm at the maximum, RCF-max = 857×g). PRF membranes near the red blood cells, which are considered to have the highest growth factor concentration, were used to protect the defects in the G2 and G4 groups.

The animals were euthanized on postoperative days 7 and 14. Euthanasia was performed by deepening anesthesia according to the following protocol: 90–150 mg/kg sodium thiopental (71-73-8) associated with 10 mg/ml lidocaine (137-58-6) via intraperitoneal injection.

The wounds were monitored daily until euthanasia was performed after 7 and 14 days.

Macroscopic analysis

The assessment considered the following parameters: wound size (in millimeters), bleeding, and secretion. The method chosen was visual assessment and the use of a digital caliper to measure wound closure.

Histologic processing

On days 7 and 14, the parts surrounding the demarcated dorsum were dissected, reduced, and subsequently fixed in 10% buffered formalin. The pieces were then embedded in histological paraffin, and 4 µm sections were taken cross-sectionally from the central region of the defects at intervals of 10 µm.

Samples were stained with hematoxylin–eosin and then mounted on glass slides with mounting resin (Permount, Fisher Scientific). Photomicrographs were taken under a light microscope using a computerized image analysis system consisting of an Axioskop 2 plus light microscope (Carl Zeiss) connected to a microcomputer using AxioVision rel. 4.8 image analysis software (Carl Zeiss),

For histologic analysis, the presence of vascular proliferation, polymorphonuclear and mononuclear cells, collagen fibers and re-epithelialization were considered. A classification score from 0 to 3 was adopted, considering the extent of the inflammatory process in the defect area as follows: 0 absent, 1 discrete (up to 25%), 2 moderate (25–50%), and 3 intense (> 50%) [18, 20, 21].

Enzyme immunoassay for quantification of beta-defensin

The quantification of β-defensin in PRF membranes ($n = 4$) was measured by enzyme-linked immunosorbent assay (ELISA). Immediately after attaining the membranes, they were immediately immersed in RIPA buffer (10 mM Tris–Cl, pH 8.0; 1 mM EDTA; 1% Triton X-100; 0.1% sodium dodecyl sulfate; 140 mM NaCl) with 1% protease inhibitor (Sigma). The membranes were sonicated in 1 mL of RIPA buffer, and the supernatant was aspirated and centrifuged at 5000×g for 15 min at 4 °C. Aliquots of each sample were measured by ELISA to determine the levels of β-defensin to be analyzed in accordance with the manufacturer's recommendations (R&D Systems, USA). Then, 100 µl of the detection antibody solution was added to each well for incubation for 1 h at room temperature. The plates were washed with buffer solution (0.05% Tween 20 in PBS), and 100 µl of streptavidin and peroxidase conjugate was added to the plates for incubation for 30 min at room temperature. The plates were washed once again, and 100 µl of substrate (tetramethylbenzidine) was added to each well and incubated for 15 min with

protection from light. The reaction was completed with 50 µl of 2 N sulfuric acid (H_2SO_4) added to the substrate solution in each well; the color was measured in a spectrophotometer (Epoch, Biotek, Winooski, VT, USA) at a wavelength of 450 nm. The amount of β-defensin was determined in picograms (pg/mL).

All experiments were conducted in biological triplicate for each membrane.

Statistical analysis

Descriptive and exploratory analyses of the data were performed. An asymmetric distribution was observed, and the data were analyzed using generalized models. Subsequently, nonparametric analysis was applied (Mann–Whitney test). Analyses were performed using the R Core Team 2019 program with a significance level of 5%.

Results

Macroscopic evaluation

The macroscopic analysis of the wound size measurement is shown in Table 1. A significantly smaller skin wound was observed after 14 days than after 7 days in all evaluated groups ($p < 0.05$). Additionally, wound size was significantly smaller in the PRF group for the noninfected wounds at 14 days and for the infected wounds at 7 and 14 days ($p < 0.05$). Wound size was also significantly and noticeably decreased in the PRF groups with infection at 14 days ($p < 0.05$) (Fig. 2).

Histological assessment

H&E-stained histological images are shown in Figure 3. At 7 days, the group without infection and without PRF (control G1) showed little wound repair (Figure 3a). However, in the presence of a PRF membrane (G2), complete wound closure and an epithelial layer under the overlying PRF membrane were observed (Figure 3c).

In the presence of infection and without a PRF membrane (G3), no wound closure was observed. In addition, an inflammatory infiltrate, typically of mononuclear lymphocytes and blood vessels, was observed in the underlying dermis (Fig. 3e). In the presence of PRF (G4), epithelialization of the wounds and intense vascularization in the dermis was noticed, indicating a moderate to mild presence of lymphocyte inflammatory cells (Fig. 3g).

At 14 days, the wound edges in G1 and G3 were still not fully epithelialized, while wounds in G2 and G4 were fully closed, with re-epithelialized edges in continuity. In the groups with PRF (G2 and G4), remnant particles of the fibrin membranes were not observed. Moreover, regardless of infection, these groups showed an intense presence of blood vessels in comparison to the control groups.

Inflammatory infiltrate intensity

The inflammation score (Table 2) was significantly lower at 14 days than at 7 days in all groups ($p < 0.05$). On the one hand, the groups without PRF (G1 and G3) presented a score significantly higher with infection than without infection ($p < 0.05$). In contrast, the groups with PRF (G2 and G4) showed no significant difference with and without infection ($p > 0.05$).

Similarly, there was no significant difference between the groups with/without PRF (G1 and G2) in the absence

Table 1 Clinical dimensions of the wounds (mm) in relation to the presence of PRF, infection and time

Period (days)	G1		G2		G3		G4	
	Average (standard deviation)	Median (minimum and maximum value)	Average (standard deviation)	Median (minimum and maximum value)	Average (standard deviation)	Median (minimum and maximum value)	Average (standard deviation)	Median (minimum and maximum value)
7	2.33 (0.21)	2.40 (2.10–2.50)	1.85 (0.57)	2.10 (1.00–2.20)	2.68 (0.19)	2.75 (2.40–2.80)	1.95 (0.13)	[c]1.95 (1.80–2.10)
14	1.06 (0.11)	[b]1.10 (0.90–1.20)	0.00 (0.00)	[bc]0.00 (0.00–0.00)	1.30 (0.14)	[ab]1.25 (1.20–1.50)	0.00 (0.00)	[bc]0.00 (0.00–0.00)

G1 noninfected wound (*S. aureus*) without PRF, *G2* noninfected wound (*S. aureus*) with PRF, *G3* infected wound (*S. aureus*) without PRF, *G4* infected wound (*S. aureus*) with PRF

[a]Difference between G3 at 14 days and G1 at 14 days, $p < 0.05$

[b]Difference between: G1 at 14 days and G1 at 7 days; G2 at 14 days and G2 at 7 days; G3 at 14 days and G3 at 7 days; G4 at 14 days and G4 at 7 days, $p < 0.05$

[c]Difference between: G2 at 14 days and G1 at 14 days; G4 at 14 days and G3 at 14 days), $p < 0.05$

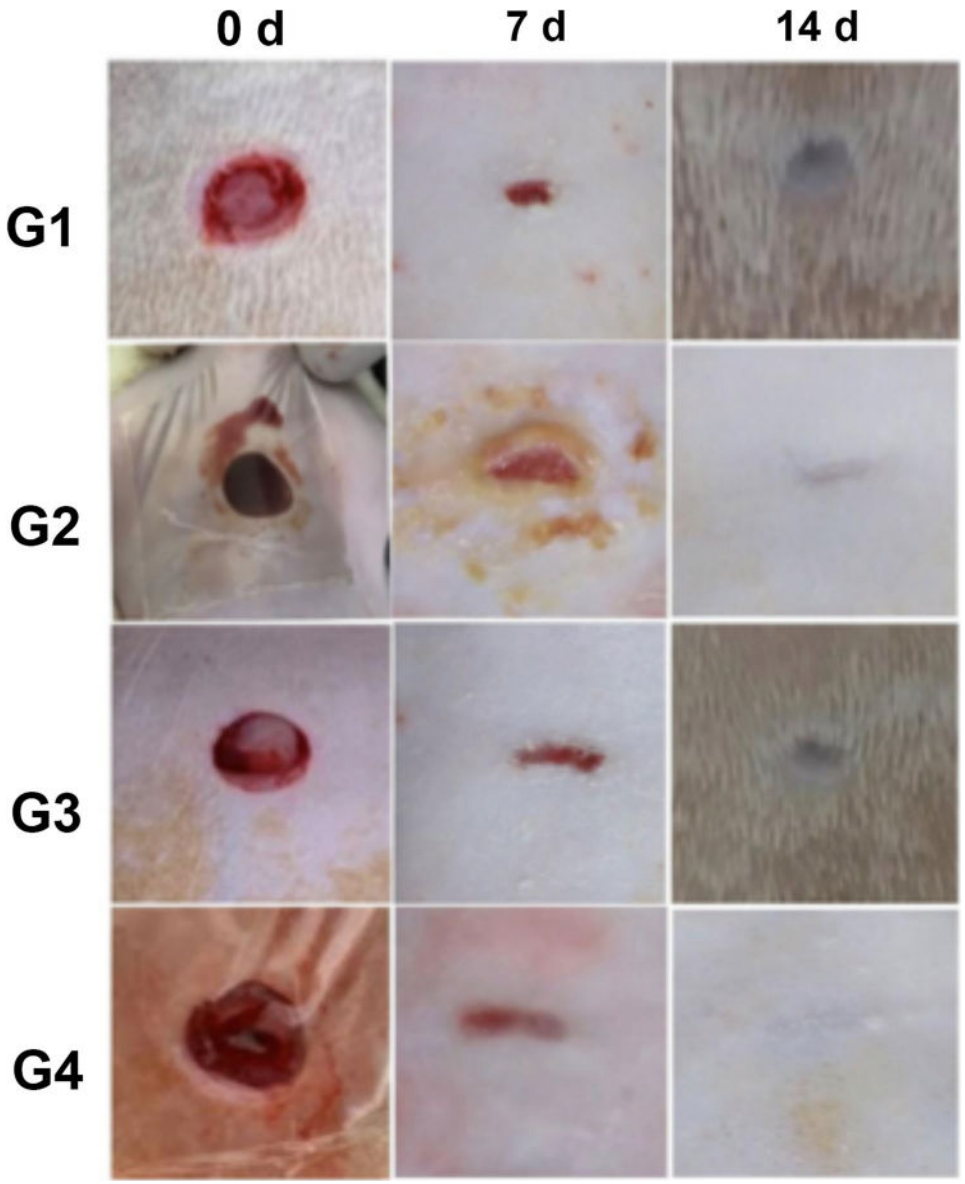

Fig. 2 Image representing macroscopic evaluations of the different groups at 0, 7 and 14 days. *G1* without infection and without PRF (control), *G2* without infection and with PRF, *G3* with infection and without PRF, *G4* with infection and with PRF

of infection ($p > 0.05$). In the infection groups, the p value was very close to the threshold ($p = 0.0583$) at Day 7, with a median score of 3 in the group without PRF and 1 in the group with PRF. On the 14th day and in the groups with infection (G3 and G4), the inflammation score was significantly lower in the group with PRF (G4) than in the group without PRF (G3) ($p < 0.05$).

Quantification of defensin

The results of defensin quantification in PRF are depicted in Table 3. The median amount was 1.444 pg/mL, ranging from 0.8670 to 1.664 pg/mL, which confirms the presence of β-defensin in the PRF membranes, which contributes to host defense.

Discussion

Cicatrisation is a process related to all wounds, and regardless of the cause, it is a systemic and dynamic event directly related to the general conditions of the body [22]. Wound healing is a perfect and coordinated ripple effect of cells and molecular and biochemical events collaborating to tissue repair, and it depends entirely on initial tissue homeostasis. At first, the blood interacts with the wound by means of a ripple effect of cell and molecular events that enable platelet aggregation. Platelets thus not only act as a hemostatic agent but also produce a large concentration of fibrinogen and fibrinogen enzymes in wounds and release several regeneration mediators [23]. Growth factors such as platelet-derived growth factor (PDGF), transforming growth factor (TGF-beta), and vascular

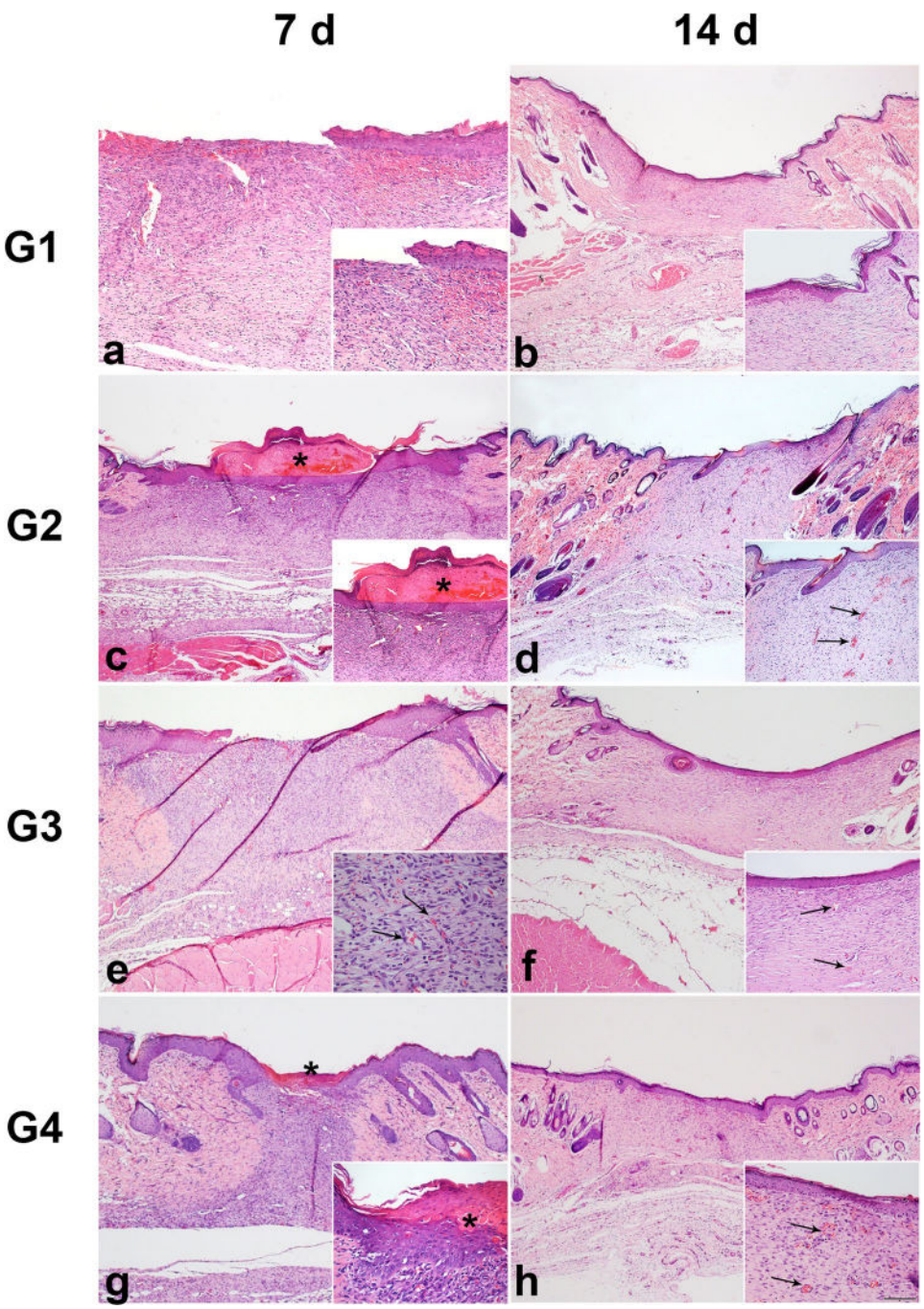

Fig. 3 Representative H&E-stained histological images of the groups at 7 and 14 days. *G1* without infection and without PRF (control), *G2* without infection and with PRF, *G3* with infection and without PRF, *G4* with infection and with PRF. *PRF membrane; arrows = blood vessels. Bars: **a**–**h** = 100 µm; inset: **a**–**d** = 50 µm, **e**, **g** = 10 µm, **f**, **h** = 25 µm

endothelial factor (VEGF), as well as fibrinogen, fibronectin, and vitronectin contained in platelet α-granules, enable platelets to perform key roles in the modulation of several phases of wound healing, such as hemostasis and neoangiogenesis [1].

Studies have now convincingly shown that platelet concentrates can be used in surgery as an effective adjuvant treatment to aid in tissue repair [24, 25]. These are autologous growth factor additives developed for surgical use, prepared from a simple blood draw from the patient [4].

The PRF protocol is favored over the original PRP protocols because it is simpler, less expensive and does not use anticoagulants and thus allows wound healing and tissue repair to be more physiologically normal. Clinical data reveal that this biomaterial may be a favorable matrix for healing without an exacerbated inflammatory response [2]. After all, it is derived from human blood and contains a variety of blood cells, including platelets, B and T lymphocytes, monocytes, stem cells and neutrophils (granulocytes), as well as their growth factors [26].

Table 2 Median (minimum and maximum value) inflammation score in relation to PRF, infection and period

Period (days)	G1	G2	G3	G4
7	1.0 (0.0; 1.0)	1.0 (1.0; 2.0)	[a]3.0 (2.0; 3.0)	1.0 (1.0; 2.0)
14	[b]0.0 (0.0; 0.0)	[b]0.0 (0.0; 0.0)	[ab]1.0 (1.0; 1.0)	[bc]0.0 (0.0; 0.0)

G1 noninfected wound (*S. aureus*) without PRF, *G2* noninfected wound (*S. aureus*) with PRF, *G3* infected wound (*S. aureus*) without PRF, *G4* infected wound (*S. aureus*) with PRF. Inflammation score: 0 = absent, 1 = up to 25% of inflammatory infiltrates (mild), 2 = 25% to 50% (moderate), and 3 = more than 50% (intense)

[a]Difference between: G3 at 7 days and G1 at 7 days; G3 at 14 days and G1 at 14 days), $p < 0.05$

[b]Difference between: G1 at 14 days and G1 at 7 days; G2 at 14 days and G2 at 7 days; G3 at 14 days and G3 at 7 days; G4 at 14 days and G4 at 7 days, $p < 0.05$

[c]Difference between G4 at 14 days and G3 at 14 days, $p < 0.05$

Table 3 Descriptive analysis of the amount of β-defensin in PRF membrane (pg/mL)

Statistics	Quantification of β-defensin
Minimum Value	0.8670
First quartile (25%)	1.2163
Median (50%)	1.4440
Third quartile (75%)	1.5768
Maximum value	1.6640

The blood physiology of rats differs from that of humans, particularly in terms of coagulation. Blood coagulation in Wistar rats is faster than that in humans since the rats have a higher number of platelets (837–1455/mm^3) than humans (100–150/mm^3) [27]. Therefore, this research adapted one of the faster protocols proposed initially to make PRF membrane production feasible (400 RCF-max for 10 min). Additionally, plastic tubes were used instead of glass tubes to further delay clotting, as previously described by Padilha et al. [18] and do Lago et al. [19]. Glass tubes are more hydrophilic and stimulate faster blood coagulation, preventing the dissociation of blood components and thus making the production of PRF membranes more feasible in humans. However, in this rat experiment with up to 2–3 times higher platelet counts, plastic tubes were used to generate PRF. This adaptation and standardization in the protocol used for the production of PRF in different clinics make it possible to increase the probability of the PRF presenting similar biological and clinical characteristics [18, 19, 28].

Experiments in animals have shown that wound contraction is 0.6–0.75 mm per day, regardless of its size, due to the contractile characteristics of myofibroblasts [29, 30]. In this context, wound healing should be completed in an average of up to 18 days in a 6 mm defect [31]. For all groups analyzed, the results demonstrated that the size of the wound at 14 days was significantly smaller than that at baseline ($p < 0.05$). The p value was close to the threshold in relation to the other periods, pointing to the necessity of further studies with larger samples. Nevertheless, the comparison between the groups with and without PRF revealed that the wound size was significantly smaller in the groups with PRF, regardless of infection. Therefore, the results suggest that treatment with PRF membrane may speed up wound healing in comparison to natural wound repair, and this may even be of additional benefit in infected sites.

It has previously been reported that the characteristics of PRF help open infected wounds since PRF can speed cell recruitment and facilitate the migration of the epithelium on its surface, as well as stimulate fibroblasts to produce collagen, promote angiogenesis, and regulate the immune system. Additionally, PRF membranes protect the second intention open wounds owing to their leukocyte incorporation.

Understanding the degradation timeline of biodegradable materials such as PRF membranes is extremely relevant to understanding how these biomaterials can benefit tissue regeneration. If degradation occurs before the formation of new tissue, insufficient tissue regeneration may follow. According to Kawase et al. [32], the degradation of PRF membranes occurs from one to two weeks as a result of intrinsic plasmin and other local cells or external agents. In this study, a total lack of PRF membranes was observed at 14 days. Membranes were observed in all groups at 7 days, including those with infection. It would be relevant to better understand the degradation properties of PRF both in infected and noninfected sites in various animal and human models. Future research could better address this topic.

Several potential pathogenic microorganisms are in constant contact with human skin. These threats may impair tissue repair. To overcome this risk, human skin has developed a defense system that involves not only mechanical barriers but also the release of antimicrobial agents. The literature shows that cells can use defense peptides (defensins) against invading agents [11, 12, 33]. Given that human beta-defensins (hBDs) are found in the epithelium, they become the first line of innate defense against invading pathogens [12, 13].

Studies have shown that beta-defensin-2 (hBD-2) is a human peptide resulting from the stimulation of epithelial

cells in contact with microorganisms, such as gram-negative bacteria [34]. In addition to acting as a bactericidal peptide, hBD-2 can enable adaptive immune responses by leading dendritic and T cells to the site of microbial invasion [35]. In addition to antimicrobial and immunomodulatory effects, such peptides act as angiogenesis modulators, which suggests an even more relevant role of hBD-2 in tissue healing [36]. Although the literature indicates that hBD-2 results from a tissue response to an inflammatory or infectious agent [14], this study demonstrates the existence of such peptides in the constitution of PRF membranes. Therefore, hemoderivatives contain not only growth factors, chemokines, and cellular components but also antimicrobial peptides that enhance the biological signature of hemoderivatives. This can be important during tissue healing.

In addition to its microbicidal effect, PRF is a concentrate that can regulate the inflammatory response and stimulate chemotaxis [35]. Platelets, leukocytes, growth factors and cytokines play a key role in the biology of the concentrate. Angiogenesis, immune response and epithelial covering are the main factors in tissue healing and maturation; PRF can simultaneously support the development of these three phenomena.

In relation to the groups with infected wounds (G2 and G4), the analysis of the inflammatory response showed that the inflammation score was significantly lower in the group with PRF (G4) in comparison with wounds treated without PRF (G2) ($p < 0.05$) at 14 days. At 7 days, the p value was close to the threshold ($p = 0.0583$) in the infected wounds, signaling a limitation of this study in terms of reduced sampling. For the groups without PRF (G1 and G3), the group with infection (G3) had a higher inflammation score than the group with no infection (G1) at both time points ($p < 0.05$).

Conclusion

Therefore, the findings from the present study suggest that wounds treated with PRF show a better response, speeding the healing process, especially in infected wounds. These findings reinforce that the use of PRF can be an interesting treatment strategy for difficult tissue repair due to infection.

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Author contributions The study design was conceived by BBBS and EFM. The study was executed by BBBS and EFM. Data analysis was performed by BBBS, LNT and EFM. Interpretation was performed by BBBS, LNT, RJM and EFM. The manuscript was written by BBBS, LNT, RJM and EFM.

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Declarations

Conflict of interest The authors report no declarations of interest.

Ethical approval The research protocol was approved by the Faculdade São Leopoldo Mandic's Committee of Ethics in the Use of Animals (number 2018/023).

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