Effects of heterologous platelet-rich plasma gel on standardized dermal wound healing in rabbits

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ABSTRACT

PURPOSE: To evaluate the potential of heterologous platelet-rich plasma (PRP) gel for surgical skin wound healing in rabbits

METHODS: Blood from a single healthy dog was used for PRP production, with calcium gluconate added to the PRP to form the gel. Two surgical excisions, one to the right and the other to the left of the dorsal midline, were made in six rabbits. One side was randomly allocated to topical application of a physiological solution, and the other was allocated to treatment with heterologous PRP gel. Clinical assessments (weight, pain sensitivity, coloring, edema, hyperemia, exudation, crust, and granulation) and morphometric evaluations were performed 0, 3, 7, 10, 14, and 17 days postoperatively. Histological analysis was performed on the 17th day.

RESULTS: With the exception of the presence of a crust at day 10, clinical variables did not differ significantly between the experimental groups. In both the control and PRP-treated groups, differences were identified when comparing time-points in terms of wound area reduction. Histological results indicated no significant differences between the control group and the PRP-treated group.

CONCLUSION: Heterologous platelet-rich plasma gel promoted dermal wound healing in rabbits with no adverse effects.

Key words: Platelet-Rich Plasma. Wound Healing. Rabbits.
Introduction

Due to clinical, scientific, and economic interest, skin healing has been the target of several studies and research\(^1\). Skin healing is a dynamic event that involves reactions and cellular interactions\(^2\) coordinated by cytokines and growth factors (GF) that attempt to repair the wounded area immediately after tissue injury\(^3\).

Platelets are a known source of several GF with beneficial effects in tissue healing\(^4\), including transforming growth factor-beta (TGF-β), insulin-like GF I (IGF-I), platelet-derived GF (PDGF), vascular endothelial GF (VEGF), fibroblast GF (FGF), and epidermal GF (EGF). This knowledge stimulated the development of a platelet concentrate with the purpose of increasing levels of local GF delivery at the injury site and improving the tissue repair process\(^5\).

Some researchers have found that autologous platelet-rich plasma (PRP) promote wound healing\(^1,6\). PRP has also been favorably incorporated in bone\(^7\) and cutaneous\(^8\) grafts for the treatment of tendinitis\(^9\) and is used as an adjuvant in plastic surgery\(^10\).

Despite the proven efficacy of autologous PRP in the aforementioned studies, another type of safe treatment can be useful when the patient’s general condition prevents the use of his/her own blood for the production of this concentrate\(^11\). In a recent study, Suzuki \textit{et al.}\(^2\) used heterologous blood for the production of PRP because they found it difficult to collect blood from small animals. Beneficial effects have been demonstrated with heterologous PRP in joint cartilage lesions\(^12\), and corneal ulcer healing was achieved in rabbits using a heterologous blood component associated with PRP\(^13\). On this basis, it is hypothesized that heterologous PRP in a gel form, when applied topically and repeatedly, can repair tissue injuries. The present study examined the morphological, morphometric, and histological aspects of healing of dermal wounds induced experimentally in the backs of rabbits that were treated with or without heterologous PRP gel.

Methods

Six white New Zealand rabbits (three male and three female) in good general condition aged 150 days and with an average weight of 3.0 kg were included. Animals were housed in individual cages with food and water \textit{ad libitum}, a controlled room temperature (22° ± 2°C), and a controlled photoperiod (12 hours light/dark). An acclimatization period of 7 days was observed before starting the experiment to allow for adaptation to the experimental conditions.

The sample size used in this study was set because of bioethical issues and to ensure that the number of animals included would provide reliable and valid scientific results in terms of statistical significance. In addition, previous work performed and published by the researchers corroborates the use of smaller sample sizes, as planned in this study\(^1,8,12,14,15\).

Surgical technique

Before surgery, rabbits were anesthetized with an intramuscular injection of a combination of 2% xylazine hydrochloride (Xilazin® 2%) and zolazepam (Zoletil® 50) at a dose of 15 mg/kg. The dorsal region (near the insertion of the scapula) was shaved using an electric clipper (AGC®) with blade n. 40. After outlining the skin, the region was prepared antiseptically. After administering 2% lidocaine (1.0 mL, SC) as a local anesthetic, two cutaneous lesions were induced on the right and left of the dorsal midline using a sterile punch 8 mm in diameter while preserving the muscle. One side was randomly allocated to receive treatment, while the other served as a control. After surgery, tramadol hydrochloride (0.5 mg/kg, IM) was administered twice a day, for three consecutive days.

Treating wounds

Dressing changes and treatments were performed twice a week at the day the wound was induced (M0) and at three days (M3), seven days (M7), 10 days (M10), and 14 days (M14) after wound induction. Initially, the lesions were cleaned with sterile gauze and sodium chloride solution at 0.9%\(^8\), after which topical application of this same solution to control wounds and that of heterologous PRP gel to the treated wounds (Figure 1). All wounds were protected with sterile rayon (1.0 cm\(^2\)) and hypoallergenic adhesive tape (Micropore 3M\(^8\)) at M0, which was replaced by a round adhesive bandage (Band-Aid\(^8\)) at the other time-points.
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Collection and preparation of the heterologous PRP gel

The blood samples for the preparation of PRP were collected from a single healthy adult dog (average weight, 25 kg) from the home University, which did not have a defined breed. The animal was kept in an individual kennel with water and food ad libitum.

To certify the animal’s health and obtain an initial platelet value, blood was collected from the external jugular vein of the donor dog prior to the induction of cutaneous wounds by using a vacuum system to obtain complete blood cell counts and an automatic platelet count (Sysmex Poch Diff 100iV-Roche®).

The PRP gel form was prepared shortly before its application to the treated group following the protocol proposed by Vendramin et al. 16

After repeating the puncture using the vacuum system, 4 mL of venous blood was collected from the donor animal, in a vial containing 10% sodium citrate anticoagulant. The sample was centrifuged (centrifuge Excelsa II Modelo 206-BL Fanem®) at 200 g for 10 minutes, allowing formation of an upper yellowish layer (plasma and platelets), a bottom reddish layer (erythrocytes), and an intermediate whitish layer referred to as the buffy coat (leukocytes and larger platelets). The entire upper layer and 200 µL of the buffy coat were pipetted into a dry, sterile vial and were centrifuged again at 400 g for 10 minutes. Another 200 µL of the buffy coat were transferred into a second dry and sterile vial.

After the second centrifugation, the platelet-poor plasma (supernatant) was discarded, and 200 µL of the reddish portion at the bottom of the vial (erythrocytic-platelet) was transferred into the vial containing the previously separated buffy coat. The vial was then homogenized, inducing platelet dispersion and PRP formation, and a total volume of 400 µL was obtained. For gel formation, 10% calcium gluconate was added at a ratio of 4:1 (400 µL of PRP to 100 µL of calcium gluconate), and the gel was obtained after five to 10 minutes in a water bath at 37°C. The final volume of the PRP in gel form was approximately 0.5 mL.

Morphological and morphometric assessment

Morphological and morphometric analyses of the lesions were performed at M0, M3, M7, M10, M14, and M17 (17 days).

Rabbits were weighed (kg) using a digital electronic scale (ELC-10®), and their lesions were photographed with a digital camera Coolpix P510 model (Nikon®).

Clinical parameters related to the evolution of the healing process were evaluated using a matrix analysis17. The incidences of the following events were observed and noted: pain sensitivity (0 – absent, 1 – present: considered present when one or more changes in animal behavior occurred, such as restlessness, agitation, vocalization, or head turning with the intent to bite after contact with the lesion); wound coloring (1 – pinkish, 2- yellowish, 3 – pale, 4 – cyanotic); edema; hyperemia; exudate; crust and granulation tissue (0 – absent, 1- present); and exudate characteristics when applicable (1 – serous, 2 – bloody, 3 – purulent).

To perform morphometric analysis, the animals underwent anesthetic re-intervention with the same protocol as that used for wound induction. Using a digital pachymeter graduated in millimeters (DC-60 Western®), the largest and smallest diameters of the wounds were measured, and the area was calculated using the mathematical equation described by Prata et al. 18: A = π × R × r, where A = area of the wound, R = larger radius, r = smaller radius. The calculation of the percentage of contraction (Pc) of the wound was expressed by the formula used by Ribeiro et al. 19. Pc = (Af – Ai)/Ai × 100, where Ai = initial area of the wound (M0) and Af = final area of the wound (M17).

Histological assessment

On the final day of the experiment (M17), after sedation of the rabbits using the same anesthetic protocol mentioned previously, a biopsy covering the central area and wound edges was performed using a punch 8 mm in diameter. The skin fragments were fixed in 10% formalin solution for 24 hours and paraffinized. Section of 5 µm were cut and stained using the hematoxylin-eosin (HE) and Masson’s trichrome protocols. The analysis was performed by the same pathologist who did not have prior knowledge of sample identification. The obtained data were classified according to intensity and transformed into quantitative variables according to an index for histological findings as follows: fibrin-leukocyte crust (0 – absent, 1 – present); amount of fibroblasts and collagen fibers (0 – absent, 1 – small, 2 – moderate, 3 - large); neovascularization (0 – absent, 1 – discrete, 2 – moderate, 3 – intense); inflammatory process.
(0 – no inflammation, 1 – mild intensity, 2 – moderate intensity, 3 – severe intensity); type of inflammatory cell (1 – neutrophil, 2 – lymphocyte, 3 – mixed); macrophage concentration (0 – absent, 1 - discrete, 2 – moderate, 3 – intense); and re-epithelialization (0 – absent, 1 – partial, 2 – total).

Statistical analysis

The statistical analysis was performed using the computer software SPSS, v. 13.0. For the body weight variable, the ANOVA test for repeated measures was used with validation of the assumption of sphericity by the Mauchly test and contrasts by the Sidak method. The same statistical test was used for the morphometric assessment of the control and treated wounds, while the comparison between the control group (CG) and treatment group (TG) for each observation time-point was conducted using the unpaired t-test. The scored macroscopic results were analyzed with the non-parametric Friedman test, with contrasts analyzed by the Dunn test, and the between-group comparison was performed using the non-parametric Mann-Whitney test. The non-parametric Wilcoxon test was used to verify possible differences in histological data between the experimental groups. p<0.05 indicated statistical significance.

Results

Platelet count and clinical analysis

The automatic initial platelet count of the donor animal was 266 × 10³ platelets/µL, indicating that samples were viable for the preparation of PRP.

The rabbits tolerated both the anesthetic and the surgical procedures quite well and without exhibiting significant weight loss, apparent stress, or any other type of behavior suggestive of pain.

There were no statistically significant differences in coloring, edema, hyperemia, or exudate and granulation between the CG and TG at the five observation time-points. Both control and treated lesions appeared pinkish in color, and had no macroscopic characteristics of contamination or the presence of necrotic tissue. Discrete edema was observed around the wounds treated with PRP gel (2/6) at M3, one of which was maintained at M10. These completely disappeared at subsequent time-points. Mild hyperemia was observed in one of these wounds at M3. Similar clinical features were not observed in the control group. Exudate was not present in any of the lesions in this study.

A dry crust was formed on M7 in 80% of the total wounds studied. The number of samples showing crust formation was found at M10 was significantly different (p=0.03) between the control group (5/6) and the treated group (1/6). This difference was not observed at M14 and M17 (p>0.05), consistent with the results of the histological analysis carried out on M17. A few crusts over the wounds appeared spontaneously at M10.

In our study, the presence of granulation tissue was imperceptible in both groups at the various time-points.

The evolution of the healing process was similar between the control and treated wounds (Figure 2). Complete repair was observed earlier in the control group (1/6) at M14, whereas none of the wounds treated with PRP healed fully in the same period. At the subsequent time-point M17, the number of lesions with complete closure was identical in both experimental groups (3/6).

Morphometric analysis

A statistically significant decrease (p<0.05) in wound area was observed over time in both groups. However, a comparison of the CG and TG shows no statistically significant differences in the reduction of the surgical area (Figure 3) and the percentage of contraction on observation days (Figure 4 e Table 1).
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**Histological analysis**

On day 17 post-surgery (M17), the prevalence of a moderate amount of fibroblasts (Figure 5 c and d) and collagen fibers (Figure 5 e and f) as well as the discrete presence of newly formed blood vessels (Figure 5 c and d) were observed in both sample groups.

Although not statistically significant (p>0.05), a mild-to-moderate inflammatory infiltrate of the mixed type was observed in 90% (5/6) of the wounds treated with PRP gel (Figure 5 d), while the inflammatory response was absent in 90% of the control wounds (5/6) (Figure 5 c). A discrete concentration of macrophages was found in a smaller number of the CG (4/6) than the TG (5/6) (p>0.05).

Microscopic analysis showed that 100% (6/6) of the wounds treated with PRP were completely covered with keratinized stratified squamous epithelial tissue, which was not significantly different from the 90% result in controls 90% (5/6) (Figure 5 a and b).

![Graph](image.png)

**FIGURE 4** - Percentage of contraction in control and treated wounds. Note the similarity in tissue regression between the control group (CG) and heterologous PRP gel-treated group (TG) over the observation days. ANOVA p>0.05.

**TABLE 1** - Mean values and standard deviations of wound contraction (%) in the control group (CG) and heterologous PRP gel-treated group (TG).

<table>
<thead>
<tr>
<th>Moments (days)</th>
<th>Wounds contraction (%) (CG)</th>
<th>Wounds contraction (%) (TG)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M3</td>
<td>18.42±14.21</td>
<td>23.28±12.24</td>
<td>0.540</td>
</tr>
<tr>
<td>M7</td>
<td>27.68±18.08</td>
<td>38.08±13.34</td>
<td>0.283</td>
</tr>
<tr>
<td>M10</td>
<td>43.46±25.33</td>
<td>50.54±10.86</td>
<td>0.543</td>
</tr>
<tr>
<td>M14</td>
<td>80.64±28.60</td>
<td>84.14±12.40</td>
<td>0.789</td>
</tr>
<tr>
<td>M17</td>
<td>96.44±7.93</td>
<td>96.74±4.69</td>
<td>0.938</td>
</tr>
</tbody>
</table>

![Images](image.png)

**FIGURE 5** - Photomicroscopy of the control wounds (left), represented by the letters (a), (c), and (e), and the PRP-treated wounds (right), represented by the letters (b), (d), (f). HE. (a) and (b) (x100 magnification) – total re-epithelialization, (c) (x400 magnification) – discrete neovascularization, no inflammatory infiltrate, and moderate number of fibroblasts (arrowhead), (d) (x400 magnification) – discrete neovascularization, moderate neutrophil-lymphocytic inflammatory infiltrate. Note the presence of lymphocytes (smaller arrow) and a moderate number of fibroblasts (arrowhead). Masson’s trichrome. (e) and (f) (x100 magnification) – detail of collagen fibers (large arrow).
Discussion

The PRP platelet count is directly related to the initial platelet count, which in the present study is in agreement with the values of basal blood of donor species. In order to obtain PRP, we performed double centrifugation with adequate rotation force to avoid the premature release of the GF in the platelets, and thus, promoted its action in the wound repair process. Platelet activation by the addition of 10% calcium gluconate immediately prior to the treatment of these lesions ensured, as indicated by Maia et al., a suitable concentration of these factors in the PRP, the most important of which are TGF-β, PDGF, and IGF-I.

Consistent with our observations in this study, some authors who studied wound healing reported that PRP directly reduces lesion site pain, as well as the demand for analgesics in postsurgical patients. In the current research, some lesions treated with PRP gel had a different reaction, from that reported in the study by DeRossi et al., who had identified a more intense inflammation process in control wounds than in the group treated with PRP. However, in our study, the fact that some lesions showed a different reaction did not impair the repair process of the treated wounds, since the inflammatory responses were mild and almost imperceptible, with a decrease starting at day 10 post-surgery.

The use of heterologous PRP was not associated with the presence of signs suggestive of infection, similar to that reported by Rezende et al., who used a heterologous PRP source. Similar results were also observed with autologous PRP treatments. This absence of infection may be explained by the variable amount of leukocytes with antimicrobial activity present in PRP. Meanwhile, it is important to note that in the present experiment, surgical wounds were cleaned, and the application of an aseptic technique during preparatory blood handling and PRP use also contributed to this finding.

The dried crusts were easily removed during cleaning so that the PRP gel and the physiological solution could be applied topically and directly over the bed of the wounds, and to facilitate morphological and morphometric assessments.

According to Ribeiro et al., factors responsible for promoting tissue hypergranulation include lesion location, mobility of the region, altered tissue perfusion, infections, and trauma. Corroborating the justification pointed out by the authors, since none of the above factors was present, it is possible to understand why hypergranulation was not observed in this study.

Histological results from the CG and TG samples suggest components of the remodeling process of tissue healing. Complete re-epithelialization was already present in all wounds treated with PRP, similar to the description in the study by Bauer et al. However, at M17, the microscopic analysis of samples from the treated group revealed the presence of inflammatory cells consistent with the initial phase (inflammatory) of healing. Similarly, on the basis of an analysis of synovial fluid, Yamada et al. concluded that treatment of joint lesions with PRP did not significantly minimize the degree of inflammation compared to saline solution. We agree with the authors that it is most likely that, despite the anti-inflammatory effects of PRP in some studies, de-granulated platelets in tissue lesions may activate chemical mediators such as cytokines, and therefore, may alter the inflammatory response during tissue repair.

The morphological, morphometric, and histological assessments showed no delay in the tissue healing process in any of the study groups, and indicated that all lesions had characteristics consistent with the normal repair process. In this study, where we focused on wounds not associated with compromised blood and located in regions with good blood circulation, heterologous PRP may not induce faster wound reduction or alterations in tissue contraction during the repair process, similar to the results by Bauer et al. In contrast, some studies that presented superior effects of PRP studied chronic wounds in peripheral regions or in patients with some type of circulatory compromise.

Conclusion

Treatment with heterologous platelet-rich plasma gel promoted the healing of dermal wounds in rabbits and showed no adverse effects.

References

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