Research article

Immunohistochemical Measurement of TGF-β1 and Factor VIII in the Skin of Horses Treated with Leukocyte-poor Platelet-rich Plasma: A Randomized Controlled Trial

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Abstract: Platelet-rich plasma (PRP) is a treatment used for several diseases of various species and different body regions. However, there is controversy about its therapeutic efficacy. The objective of this experiment was to immunohistochemically evaluate the expression of transforming growth factor beta 1 (TGF-β1) and factor VIII during the healing period of skin treated with leukocyte-poor platelet-rich plasma (LP-PRP) in seven healthy gelding crossbred horses. This study was a randomized controlled clinical trial. Wounds were surgically induced in gluteal region (treated group), and LP-PRP (0.5 mL) was injected into all borders of the wounds 12 h later. The contralateral region (untreated) was adopted as the control group. Five skin samples were obtained from the treated and untreated groups for immunohistochemical analysis. The last one was collected upon wound closure. We hypothesized that the treated wounds would exhibit higher expression of both TGF-β1 and factor VIII than the untreated wounds. There was no difference between groups in

TGF-β1 immunostaining. However, a difference was observed between groups in immunostaining of blood vessels for factor VIII. This difference ($P < 0.05$) occurred after macroscopic wound closure, with larger numbers of vessels stained in the treated wounds. The administration of a single dose of LP-PRP 12 h after wound induction in horses is not effective compared with the physiological healing of horse skin. Further research should not only take into consideration the association of the platelet-rich component with stem cells, which, in general, have provided adequate responses, but also evaluate other proteins present in PRP besides those typically studied.

Keywords: equine; growth factors; platelet-rich concentrate; wound; transforming growth factor beta 1; von Willebrand

1. Introduction

In the routine activities of medical and surgical equine practice, skin problems are a frequent occurrence. Sanchez-Casanova et al. [1] observed a prevalence of 6.8% for traumatic injuries in southern Mexico, while Pessoa et al. [2] reported that these injuries account for 23.04% of skin injuries in horses in the Northeast region of Brazil. Second-intention skin healing in the equine species is a slow process, although the time required for wound closure depends on the wound site and is longer in the limbs. There are four stages of skin healing (hemostasis, inflammation, proliferation, and remodeling) [3,4], although some of them occur simultaneously. These stages can be subdivided in a more detailed manner into proliferation and migration as well as remodeling and contraction [5]. Interactions among cells, cytokines and growth factors are critical to re-epithelialization and the structural and functional reestablishment of the new epithelium, which should appear as similar as possible to what occurs during the physiological healing process.

Platelet-rich plasma (PRP) is a kind of therapy used in a diverse range of diseases, but controlled studies on its use for the treatment of wounds in horses are still rare. Considering the concentration of platelets, leukocytes and erythrocytes, PRP may be obtained by automatic "selective blood filtration using commercially available technology" [6,7] or by semi-automatic (automated machine with commercial kits) [7–9] or manual (double centrifuging) methods [10–14]. The last technique is considered low cost and easy to implement [6,15]. Although PRP is rich in growth factors, it is also a source of other bioactive proteins. However, growth factors are still the most commonly studied proteins, particularly transforming growth factor β1 (TGF-β1) and vascular endothelial (VEGF), platelet-derived (PDGF), and basic fibroblast (bFGF) growth factors; those proteins are studied to evaluate their effectiveness for the treatment of various disorders in humans and animals [1,16–18].

PRP can be divided into categories according to the quantities of platelets and leukocytes. When pure, i.e., with the leukocytes totally removed, it is known as P-PRP [19]. However, when the PRP

contains leukocytes, it is considered L-PRP. In 2012, Draggo et al. [20] developed a new classification, in which PRP rich or poor in leukocytes was called LR-PRP or LP-PRP, respectively. LP-PRP should result in an acute inflammatory response of lesser intensity.

Recently, Fitzpatrick et al. [9] compared the efficacy of various kits for obtaining PRP in humans. Some authors have reported a satisfactory effect of LR-PRP on tendinopathy [21]. Additionally, LP-PRP appears to be effective in the treatment of osteoarthritis [22]. In human patients with symptomatic knee osteoarthritis, a single dose was sufficient to relieve pain and improve quality of life, but in a certain age range (over 67 years), there was no difference in outcome between LP-PRP and corticosteroid administration. In a review conducted by Bennell et al. [23] on the application of PRP in joints with osteoarthritis, the authors noted that it is not possible to determine whether the platelet-rich component is effective in treating this condition, since various methods are used to obtain PRP.

The effectiveness of LP-PRP in horse skin is still questionable [18,24]. Kraeutler et al. [25] mentioned in their review that LP-PRP has the advantages of reducing the inflammatory process and, particularly, anabolic changes. The authors suggest the need for future high-quality scientific studies comparing LP-PRP and LR-PRP.

Growth factors such as TGF-β1 and VEGF are released under hypoxic conditions [26] and help in neovascularization (angiogenesis), which is considered essential in the early tissue regeneration process. These proteins increase in concentration during the course of healing, subsequently reducing [27] during the wound remodeling phase. Pufe et al. [28] reported that, in the tendon, persistent angiogenesis can change the mechanical properties of the extracellular matrix and slow tissue repair. Furthermore, overproduction of TGF-β1 may result in exuberant granulation tissue [27,29,30], an undesirable consequence.

According to Eppley et al. [31], PRP has six to nine times more VEGF than blood plasma. One way to evaluate angiogenesis is via immunostaining for factor VIII, also known as von Willebrand factor [12,32], which is present in and released by functionally active endothelial cells [33] and is a pro-cofactor for coagulation. However, factor VIII may originate from other sources, not only from the platelets. These last authors do not consider factor VIII to be a synonym for von Willebrand, as they mentioned that factor VIII can travel along with von Willebrand factor.

Despite its use in humans and animals, as mentioned earlier, the effectiveness of PRP is questionable, particularly because of the limited number of high-quality experimental studies, which are also rarely conducted with horse skin. The research carried out by DeRossi et al. [34] indicated that this therapy has benefits for wound healing, contrasting the findings of [35] on cutaneous wounds induced in the metacarpus of horses. According to Souza et al. [36], a single application of LP-PRP 12 h after injury induction in the gluteal region does not accelerate the wound closure process, which corroborates the observations of Monteiro et al. [35]. The aim of the present study was to evaluate the response of wounds treated (T) and untreated (UT) with LP-PRP by determining the immunohistochemical expression of TGF-β1 and factor VIII at different stages of the healing

process of surgically induced wounds in horses. The hypothesis tested in the study was that the treated wound exhibits higher expression of both factors.

2. Materials and Methods

This research was approved by the Ethics Committee of Animal Use of the Federal University of Viçosa (No. 935/2013). Seven healthy gelding crossbred horses in the age range of 16 to 17 (16.37 ± 0.52) years were used in the experiment. Only seemingly healthy animals with no dermatological disorders were included in the study. All horses were housed in stalls for fifteen days prior to the onset of the experiment. The animals received chopped grass and a mash diet for horses. Mineral salt and water were provided *ad libitum*. This management regimen was continued throughout the experimental trial.

All procedures—from skin biopsy to obtaining the samples for immunohistochemical analysis—were performed as described by Souza et al. [24]. Briefly, three rectangle-shaped (6.25 cm^2) skin injuries were created in the left and right gluteal regions of each horse. The epidermis, dermis and subcutaneous tissue were removed. The wounds were identified as A, B and C, from cranial to caudal, in both gluteal regions. The first biopsy collection was considered time zero (T0). The wounds healed by second intention and were monitored until complete closure, when the last biopsy (wound C) was performed. The surgical wound was dressed daily during the postoperative period. The animals received anti-tetanus serum (Vencofarma, Londrina, PR, Brazil). Pain was lessened using a single intravenous dose of butorphanol tartrate (0.08 mg/kg) (Butormin®, Holliday Scott S.A., San Isidro, Argentina). No anti-inflammatory or antibiotic was used.

Leukocyte-poor platelet-rich plasma was obtained using the procedures described by Souza et al. [18]. Blood was obtained from the external jugular vein and collected in tubes with 3.2% sodium citrate (0.199 mol/L). Blood samples were also added to EDTA-containing tubes for determining the quantities of platelets and leukocytes in whole blood. The adopted LP-PRP detection method was manual—double centrifugation ($120\times g$ for 10 min and $240\times g$ for 10 min) (CR3i multifunction, Thermo Electron Corporation, Thermo Electron Industries SAS, France). The procedures were performed in a flow hood, and the quantities of leukocytes and platelets were determined in the LP-PRP and in whole blood. Türk's solution (Haloquímica Indústria e Comércio Ltda, São Paulo, SP, Brazil) and Brecher's method were used to count leukocytes and platelets, respectively.

The LP-PRP was injected 12 h after the wounds were created. The plasma was prepared immediately and injected into each edge of the three wounds (A, B and C) at a dose of 0.5 mL/site, totaling 2 mL per wound and 6 mL per side (left or right) of the gluteal region (treated group = T). The wounds in the contralateral gluteal region (untreated group = UT) did not receive any injections, but only local cleaning, as was done for the treated wounds. Subsequently, the horses were kept in stalls, let out for a daily period of 2 h, and monitored throughout the experiment.

Skin samples were obtained as described by Deschene et al. [37], and the expression levels of TGF-β1 and factor VIII were analyzed immunohistochemically. Skin samples were obtained by biopsy (full thickness) using a 6-mm-diameter punch with the aid of a scalpel before (T0) and at 2 (T1), 7 (T2) and 14 (T3) days after induction of wounds A and B. A new collection was collected from the center of the healed area of wound C (which was still unpigmented) when the wound was completely closed (T4). The other biopsies were obtained from the peripheral region of the wounds.

Sections were evaluated by immunohistochemistry by the indirect immunoperoxidase technique [38]. The primary antibodies used are listed in Table 1. Citrate buffer (pH 6.0; 95 ℃; 30 min) was used in a water bath during the pretreatment for antigen retrieval. Afterwards, PBS was used for the wash. A 16% milk powder solution was used to block non-specific binding, and 3% H_2O_2 was used for a period of 30 min to block endogenous peroxidase. Sections were incubated with primary antibodies overnight at 4 ℃. Thereafter, the histological samples were incubated with anti-mouse and anti-rabbit universal immunoperoxidase polymer. The reaction was revealed using DAB (3,3'-diaminobenzidine) (Sigma, SP, Brazil). The positive controls for factor VIII were human breast carcinoma and exuberant granulation tissue, while canine placentae were used for TGF-β1. The negative control consisted of slides that did not have the primary antibody. Sections were counterstained with Harris hematoxylin (Labscience de Minas Instrumentos Cient ficos Ltd, Belo Horizonte, MG, Brazil).

Table 1. Information on manufacturers, codes and dilutions of the antibodies used in the study.

Primary antibody	Manufacturer	Code	Dilution
Anti-factor VIII (Rabbit polyclonal)	Dako	A0082	1:2000
Anti-TGF-β1 (Rabbit polyclonal)	Santa Cruz Biotechnology	SC-146	1:300

Quantitation of immunostaining for factor VIII and TGF-β1 was performed by three blinded observers upon review of four images obtained at each time in the form of a quadrant, i.e., from the region undergoing the healing process toward the dermis, with a subsequent return to the epithelium. The number of vessels immunostained for factor VIII was observed under $200\times$ magnification. Images of the slides were acquired using an optical microscope and AxioVision software v. 4.8.

For the quantification of cells immunostained for TGF-β1, only the region affected by the healing process was included. Cells constituting the epidermis (keratinocytes, melanocytes, Langerhans cells and Merckel cells) and attachments were not quantified, since immunostaining occurs at these locations regardless of the presence of injury. Moreover, the count was aimed at evaluating only the variations resulting from the healing process over time. Images were obtained using QCapture Pro 6.0 software (www.qimaging.com) (2010), and staining was assessed by careful identification of each cell immunostained for anti-TGF-β1 antibody under $400\times$ magnification.

Statistical analysis was carried out with GraphPad InStat 3.05 software (GraphPad Software, Inc, USA). The Mann-Whitney test was used for independent samples to compare the control and treated groups at each time point (T0, T1, T2, T3 and T4). Additionally, the different time points were compared with the initial situation (time 0, T0) in both groups using a t-test (unpaired data). For independent samples, Student's t-test was adopted to compare the mean values of platelet and leukocyte counts in the blood and LP-PRP as well as the mean wound-healing time in the groups. To compare the different times in both groups, we carried out repeated measures analysis of variance (ANOVA). All analyses were performed at 5% significance. Data were expressed as the mean $\pm$SEM.

3. Results

In the whole blood and LP-PRP, the platelet counts ranged from 100,000 to 150,000 platelets/μL and from 320,000 to 390,000 platelets/μL, respectively. Leukocyte values ranged from 5,600 to 10,900 cells/μL and 50 to 900 cells/μL in the whole blood and LP-PRP, respectively. The average number of platelets found in the blood was lower ($P = 0.000$) than that in LP-PRP.

Wound closure occurred in a maximum of 47 days, taking 36.85 ± 7.45 days for the untreated wounds and 38.85 ± 6.46 days for the wounds treated with LP-PRP. However, there was no significant difference ($P = 0.59$) between groups.

The immunohistochemical results regarding the expression of TGF-β1 are shown in Figures 1 and 2. There was no difference in immunostaining for this growth factor between wounds treated with LP-PRP and untreated wounds. Note that, in the treated wounds, there was a sharp rise in staining after one week of administration of the therapy, whereas in the control group this increase was gradual. A difference ($P < 0.05$) was observed over time with respect to immunostaining of the intact skin (T0), with an increase in immunostaining for the growth factor after one and two weeks in the treated and untreated wounds, respectively.

In healthy skin (T0), dark brown cytoplasmic immunostaining was observed in the skin, appearing in the epithelial tissue, sebaceous glands, hair follicles, vascular endothelium (see Figure 2A) and erector pili muscles. Staining was especially apparent in these last two structures. At 48 h (T1) after wound induction, wounds showed a discreet subtle inflammatory response that was characterized by lower immunoreactivity for TGF-β1, since polymorphonuclear cells and macrophages are immunoreactive, as described by Theoret et al. [38]. However, in this period of the healing process, immunostaining occurred haphazardly, staining the fibrin located in the papillary and reticular dermal regions. We also observed granulation tissue formation (see Figures 2B and 2C), characterized by neovascularization and fibroblast proliferation.
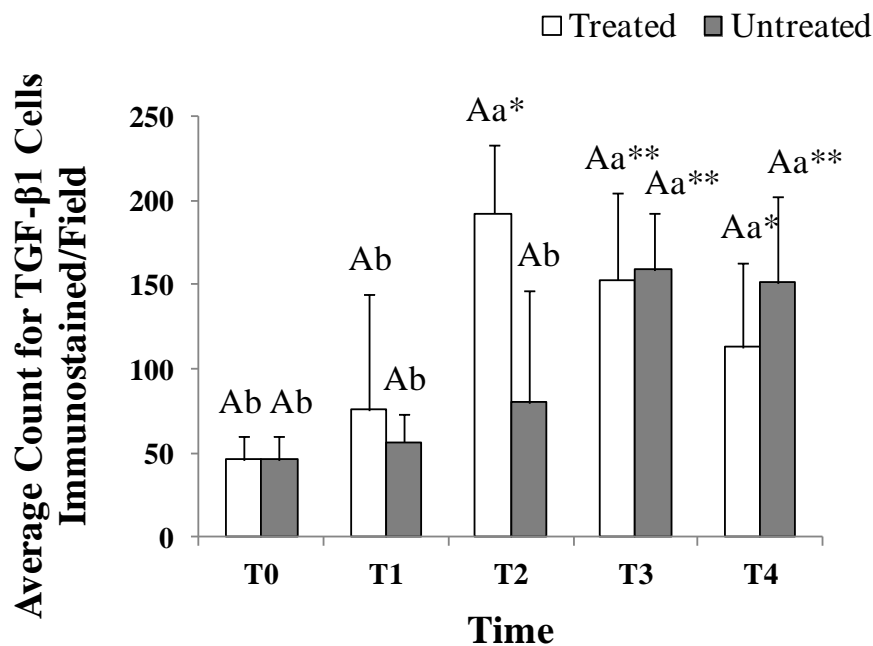
Figure 1. Average count of cells immunostained for TGF-β1 in surgically induced wounds treated and untreated with LP-PRP. The evaluation times were before (T0) and 2 (T1), 7 (T2) and 14 (T3) days following wound induction. The last evaluation was conducted upon macroscopic wound closure ($\pm$ 37 days) (T4). Differences were observed over time only in relation to the intact skin (T0), and such differences occurred in both groups. Mean values followed by the same uppercase letters do not differ between groups, and means followed by the same lowercase letters do not differ between time points in the same group ($P < 0.05$). * $P < 0.05$; ** $P < 0.01$.

Immunohistochemistry of tissue biopsied one week (T2) after application of LP-PRP showed that the inflammatory infiltrate in the dermal region reached a more extensive area than at earlier times (see Figure 2D). After one week, the color intensity, an indicator of TGF-β1 expression in inflammatory infiltrates, increased from light to dark. In the treated group, acellular gaps in the dermal region were observed where there were a large number of inflammatory cells in the evaluation performed after 48 h.

Two weeks after the wound was created, the inflammatory cells were concentrated in the papillary dermis in both groups (see Figure 2E). The amount and intensity of immunostaining were similar between the groups and higher than at T0 in both groups ($P < 0.05$). The inflammatory infiltrate was no longer present in the evaluation performed after the surgical wound had healed (T4). At that time, immunostaining was concentrated in connective tissue cells and fibroblasts (see Figure 2F). However, there was no restructuring of the epidermal appendages, indicating that the epidermal repair process was still in progress.
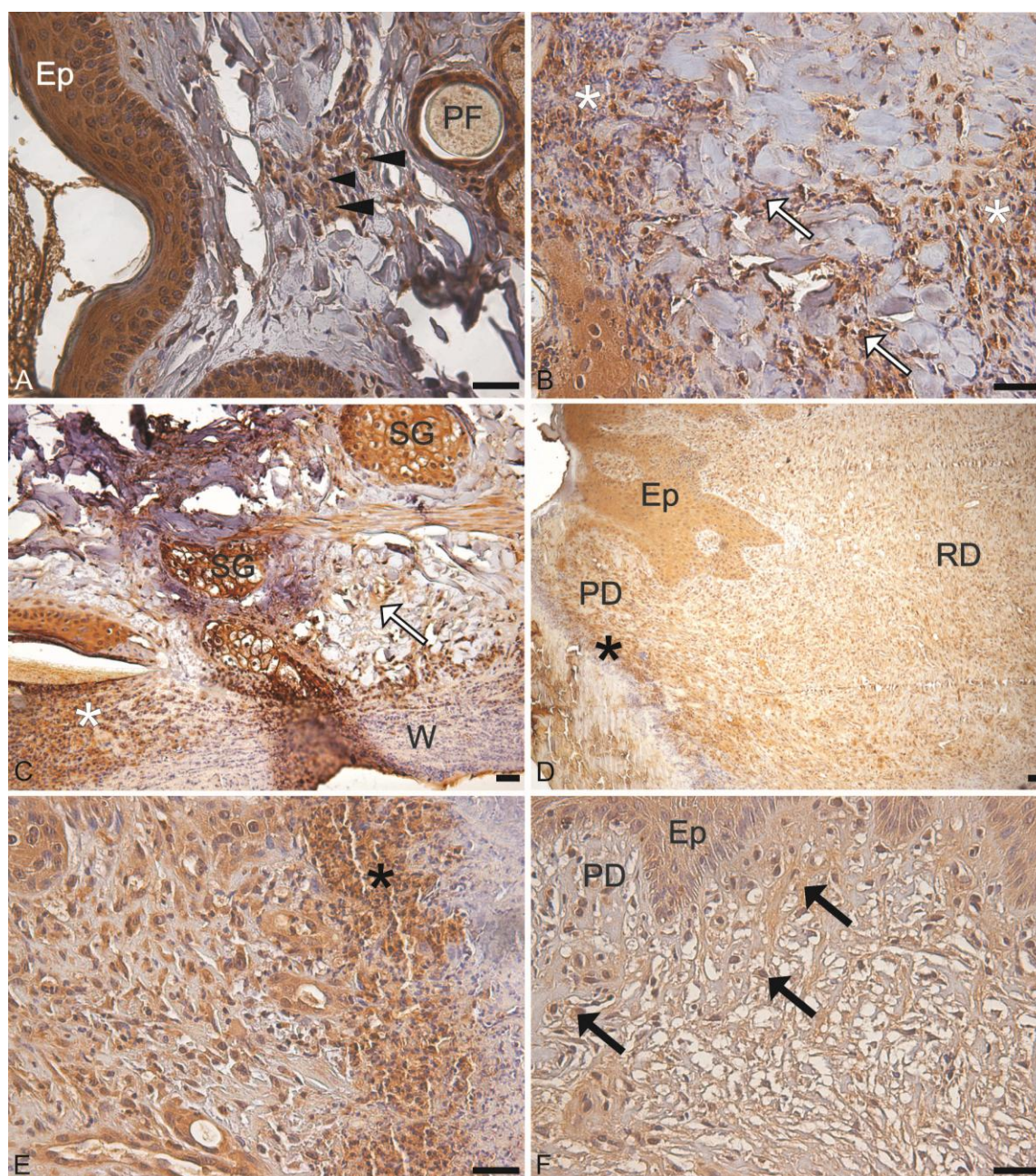
Figure 2. Immunohistochemistry for TGF-β1 in horse skin treated or untreated with LP-PRP.
(A) Healthy skin with cytoplasmic immunostaining for TGF-β1 in the epidermis (Ep), hair follicle (HF) and blood vessels (head arrow). (B) Dark brown immunostaining for TGF-β1 in inflammatory cells (*) and granulation tissue (white arrow) in the dermis 48 h after LP-PRP administration. (C) TGF-β1 labeling in areas with inflammatory infiltration (*) in the wound site (W) and absence of immunostaining in the granulation tissue (white arrow) at the same sampling time, i.e., T1, in the untreated group. (D) After two weeks, this wound treated with LP-PRP presented an extensive dermal area with dense inflammatory infiltrate (*), which was particularly present in (E) the papillary dermis (PD). Image obtained after closure of treated wound (T4). (F) Immunostaining for TGF-β1 was observed in connective tissue cells and fibroblasts (black arrow). RD, reticular dermis. Bars = 50 μm.

Results for the immunohistochemical expression of factor VIII are shown in Figures 3 and 4. All microvessels were immunostained. Differences between treated and untreated wounds occurred only at time T4, although lesions treated with LP-PRP had a higher number of immunostained vessels. In the treated wounds, this finding occurred after the first week, while in the untreated lesions significant staining was already observed two days after wound induction, with a gradual reduction in both groups.
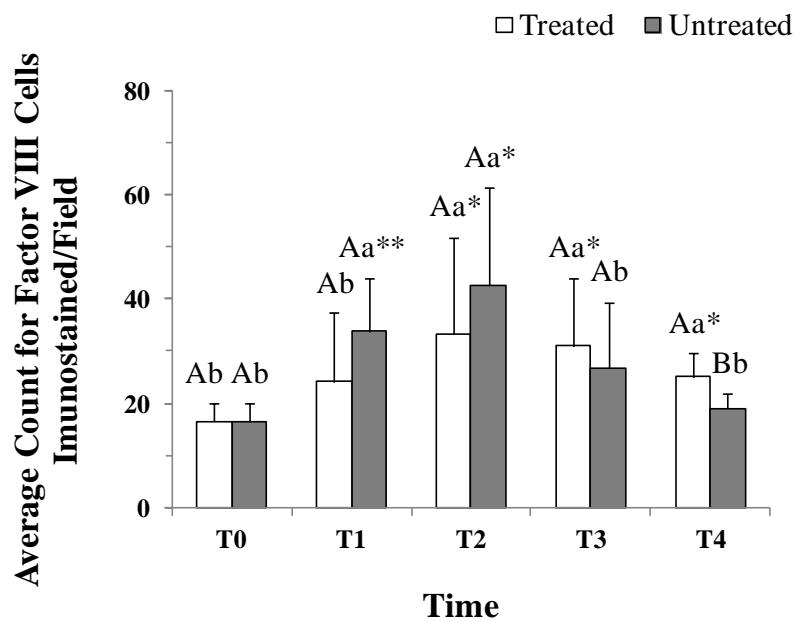

Figure 3. Average count of vessels immunostained for factor VIII in surgically induced wounds treated and untreated with LP-PRP. Evaluation times were before (T0) and 2 (T1), 7 (T2) and 14 (T3) days following wound induction. A final evaluation was performed upon macroscopic wound closure (approximately 37 days) (T4). Note that there was a significant difference between the groups only at T4. As observed over time for TGF-β1, both untreated and treated wounds showed differences in immunostaining for factor VIII in relation to the healthy skin. Mean values followed by the same uppercase letters do not differ between groups, and means followed by the same lowercase letters do not differ between times in the same group ($P < 0.05$). * $P < 0.05$; ** $P < 0.01$.
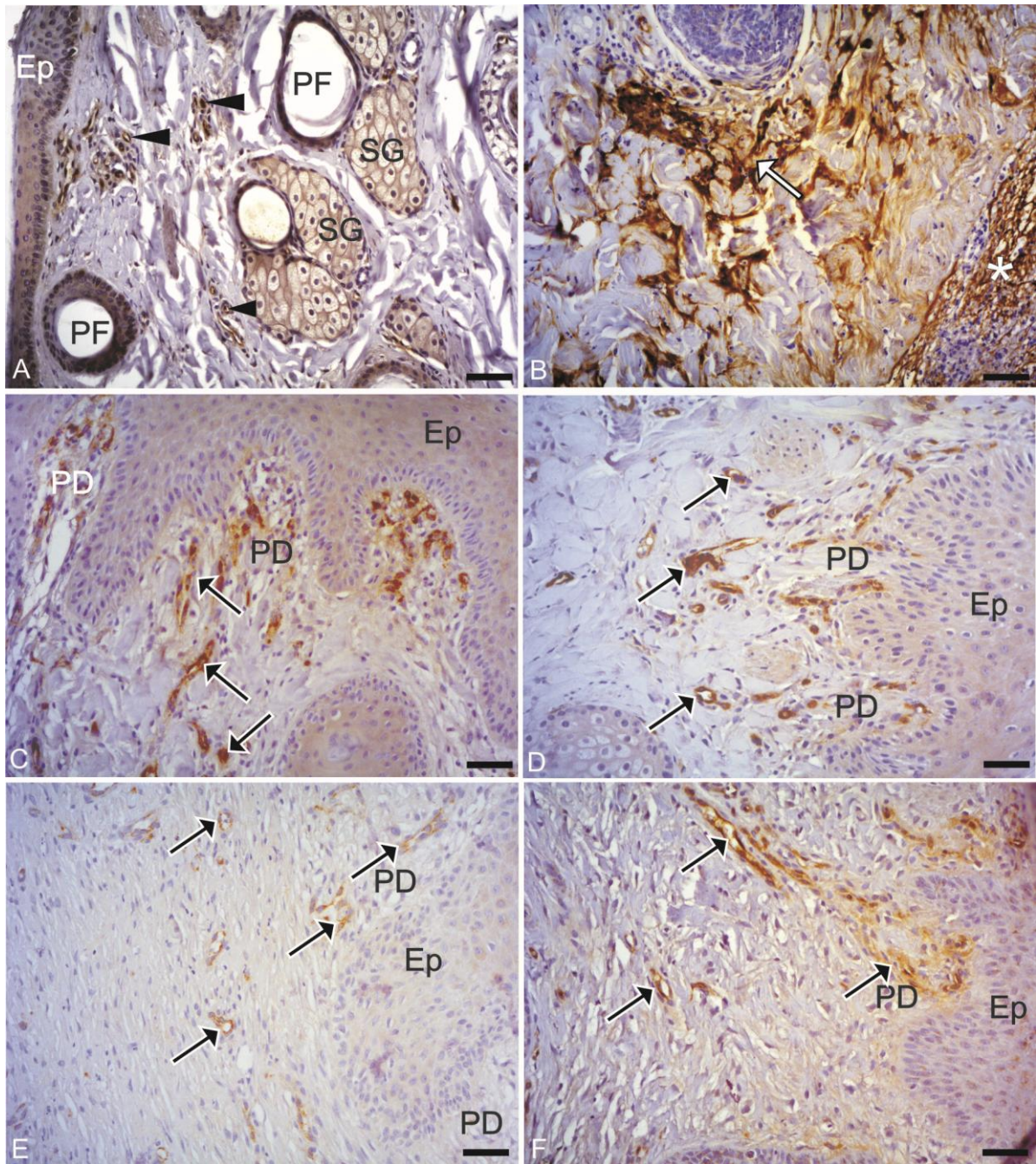
Figure 4. Immunohistochemistry for factor VIII in horse skin treated or untreated with LP-PRP. (A) Few blood vessels (black arrow head) were labeled in healthy skin, and hair follicles (HF), epidermal appendages and sebaceous glands (SG) were present. (B) T1, inflammatory infiltrate (*) and granulation tissue (white arrow) in the treated group. (C-D) T2, blood vessels more organized in the papillary dermis (PD) compared with T1 (arrows). Note that immunostaining was evident in vascular structures with squamous cells (arrows) in both groups. (E) T3, capillaries and venules with well-defined dark brown staining (arrows) in both groups. (F) Final time point, corresponding to the completion of treated wound healing; immunostaining of endothelial cells is more intense, and numerous vessels (arrows) can be observed. Ep = Epidermis. Bars = 50 μm.

Immunohistochemical staining for factor VIII is observed in the cytoplasm of endothelial cells, showing the formation of blood vessels. Healthy skin has few immunostained vessels (see Figure 4A). The granulation tissue was vascularized, with immunoblots distributed sparsely at T1. This staining occurred in regions where there was invasion of inflammatory infiltrate (see Figure 4B), characterized by the presence of polymorphonuclear cells.

In the evaluation performed at one week (T2), staining was evident, with circular structures consisting of elongated cells and flattened nuclei, which indicates the formation of small vessels in the dermal papillae (see Figures 4C and 4D). The blood vessels were immunostained light brown, as they were at the previous time, although the number of microvessels in the dermis under restructuring was large. The microvessels in the dermis were well delimited with dark brown in both groups at T3 (see Figures 4E and 4F). In the evaluation at the macroscopic closure of the wound (T4), as observed at the previous times, there were still no epidermal appendages present.

4.   Discussion

The similarity in the re-epithelialization process, contraction capacity and possibility of development of fibroproliferative lesions in skin wounds of horses and humans indicates the similarity of the healing process between the two species [27]. However, this does not occur in mice, which, unlike humans and other thick-skinned animals, have the *musculus panniculus carnosus*, responsible for starting wound contraction in these animals [39].

Contradictory results of studies on the efficacy of PRP might be related to a number of factors. Some of these factors are lack of standardization in the production method (quantitative, semi-quantitative or manual), the amount injected into the lesion, the presentation of the final product, the number of applications, and the interval between applications [36]. This problem occurs in several species, including humans. Recently, Fitzpatrick et al. [9] mentioned that "There is a need for standardization of PRP for clinical use." According to Pruna [40], the conflicting results may also be related to "interindividual differences in the presence of single nucleotide polymorphisms (SNPs) in genes related to PRPs and/or their receptors." These SNPs may control the response to therapy and the recovery time of the involved tissues.

The manual technique is considered less suitable for obtaining PRP because of the possibility of contamination [41]. However, research carried out by members of the present study's team on both horse tendons [12,42] and skin [18,35] did not result in infection of the platelet-rich component or the treated area. This is probably because all necessary care was taken to prevent contamination. In addition, although one school of thought argues for the necessity of a large quantity of platelets, which can be obtained using automated or semi-automated methods, some researchers refer to any amount greater than 300 platelets/µL [43] or 2.5 times that present in total blood [44] as sufficient to achieve the desired effect. The latter authors even mentioned that high amounts, i.e., 5.5 times that

found in whole blood, may be detrimental to the tissue. In this regard, the number of platelets obtained in this study was considered adequate to demonstrate differences between groups.

Wound closure occurred in a maximum of 47 days, faster in untreated wounds. However, there was no significant difference between groups. Slower closure of skin lesions receiving PRP with no significant difference between groups was observed by Monteiro et al. [35] in wounds induced in the metacarpal region that were treated with platelet-rich plasma gel. This faster closure may be related to individual and physiological aspects.

It is possible that the persistence of some proteins present in PRP, including TGF-β1, is related to the non-significant delay in the closure of the treated wounds. It is noteworthy that, as mentioned by Andia et al. [45], platelets have over 300 proteins. Other proteins besides the traditionally studied growth factors should be considered in other studies.

A recently published result from Farghali et al. [46] on the use of PRP activated with calcium chloride and administered weekly (four applications) in five male mongrel dogs after surgical induction of skin wounds in the thoracic region revealed better re-epithelization of the treated wounds . In contrast, Karayannopoulou et al. [47] reported that intralesional administration of PRP in wounds induced on the trunk of six Beagle dogs did not result in faster healing of the treated wounds, as observed in the present study in horse skin. Again, the effectiveness of PRP was not demonstrated in terms of wound closure time. In an experiment with rabbits [48], the wound contraction speed and the amount of collagen did not differ between the group receiving PRP gel and that receiving 0.9% sodium chloride. In all cases, there were differences in the methodology adopted, such as the technique used to obtain PRP, its activation with pharmacological agents, its consistency, and the number of applications. In the last two studies, in which no difference was detected for wound closure time, the authors did not address the reason for this occurrence. Fitzpatrick et al. [9] compared the effectiveness, in terms of concentration of platelets, of various kits used to obtain PRP semi-automatically; they found great variation, reinforcing the need for a standardized technique to obtain this biomaterial. Such results may compromise responses to the therapy.

As with the regenerative process of the skin, the use of PRP has been regarded as a common therapy in the process of treating lesions in equine tendons [49]. However, as described for skin, the results for tendons may be conflicting because of the various PRP preparation methods, and, as stated by Fisher & Mauck [50], there is no scientific evidence for consistent effects of the treatment when it is administered via local injection.

Immunohistochemical techniques are considered effective to determine the expression of several proteins, but controlled studies using this methodology to evaluate skin healing are rare. Some examples are studies conducted by Souza et al. [24], Theoret et al. [38], Morgan et al. [51] and Schwartz et al. [52]. Dark brown cytoplasmic immunostaining by TGF-β1 on the skin was observed, appearing in the epithelial tissue, sebaceous glands, hair follicles, vascular endothelium and erector pili muscles. Polymorphonuclear cells and macrophages are also immunoreactive, as described by Theoret et al. [38].

In the present study, the staining of inflammatory cells was variable, with dark to light brown shades, irrespective of the evaluated group. There was no staining in the sites where there was evidence of granulation tissue (see Figure 4B). Similar findings were mentioned by Theoret et al. [38] in the evaluation of TGF-β1 in "full-thickness dermal wounds" of horses.

As previously stated, our results showed that cytoplasmic immunoreactivity of the epidermis for TGF-β1 occurred at all studied time points. Epithelial tissue, hair follicles and sebaceous glands as well as the vascular endothelium and the erector pili muscles were also appropriately stained. However, the staining was more subtle. At all evaluated times, the cells participating in the inflammatory process showed immunoreactivity, since the epithelial layer that covered the site of the wound edge thickened two days after wound induction. This finding was mentioned by Beldon [53] in human wounds after trauma.

According to Harper et al. [4], in the first 48 h after a skin injury, TGF-β1 acts as a chemotactic factor for neutrophils, which are widely known to be the first inflammatory cells to arrive at the injured region. The peak concentration of macrophages occurs between 48 and 72 h. According to Schultz et al. [54], the presence of macrophages indicates a transition stage between the inflammatory and proliferative phases. As mentioned previously, the dynamics of TGF-β1 expression during the progression of wound healing is described by an increase in the early stages with a subsequent reduction during remodeling. These dynamics were observed in this study in treated and untreated animals. It is noteworthy that LP-PRP induced an increase in TGF-β1 in the evaluation performed one week after treatment, while the untreated wounds reached high levels of this growth factor in two weeks. However, this difference did not lead to different outcomes. The gradual reduction of TGF-β1 in both groups is considered suitable for wound healing, since permanent presence of this growth factor can stimulate the formation of exuberant granulation tissue.

In the present study, all microvessels were immunostained for factor VIII. It is important to note that the endothelial cells must be intact [33] for this factor to be stained. All microvessels stained positive. The capillaries at the wound site originate from the adjacent venules, and endothelial cells are responsible for producing enzymes that degrade the extracellular matrix, resulting in changes that will lead to capillary infiltration, with subsequent formation of the capillary network, arterioles and venules, as reported by Beldon [53].

Differences in factor VIII immunostaining between treated and untreated wounds occurred only at T4. However, lesions treated with LP-PRP had a higher number of immunostained vessels. Changes over time in healthy skin were highly variable. In the treated wounds, this finding occurred after the first week, while in the untreated lesions, significant staining was already observed two days after wound induction, with peak cell immunostaining for factor VIII observed at one week. Finally, there was a gradual reduction. This effect is considered beneficial, since it restores blood supply and local oxygen. On the other hand, the difference between groups at the last time point of evaluation, i.e., in an advanced stage of healing, can be detrimental. According to Pufe et al. [28], persistent angiogenesis interferes with the mechanical properties of the extracellular matrix and retards the

wound healing process. It has also been reported to promote pain [54]. It is important to remember that, although the difference was not significant, the treated wounds took a slightly longer time to heal, as observed in the study of Monteiro et al. [35] in wounds in the metacarpal region that were treated with manually obtained PRP.

Neovascularization was evaluated by Bosch et al. [32] using immunostaining for factor VIII in the surgically injured superficial digital flexor tendon and by Zandim et al. [12] by collagenase application. These authors used another methodology to determine staining. Zandim et al. [12] found no differences between groups treated with PRP and with saline, but they evaluated angiogenesis only three and 16 days after administration of PRP. By contrast, Bosch et al. [32] evaluated immunostaining 23 weeks after treatment with PRP and found differences between treated and placebo groups, with increased angiogenesis in the tendon treated with PRP ($P = 0.026$). In the present study, despite a similar finding, which we consider important, there was no difference between groups in macroscopic wound closure, although it was faster in the wounds not treated with LP-PRP. These results demonstrate the need to perform biopsies at times later than those used in this study, since the wound remains subject to chemical and structural changes even after it is seemingly re-epithelized, as mentioned by Yamauchi & Mechanic [55]. In fact, chemical and structural changes may remain for up to a year after the occurrence of a skin injury [56], and in the opinion of Harper et al. [4], the real maturation may be observed up to two years after the injury is produced. Lastly, Bosch et al. [32] reported, after a study on equine tendons, that the effect of a single application of PRP is long lasting. Given this context, it is plausible that different results could be obtained in a sample taken after macroscopic wound closure. This possibility draws the attention to the need for future evaluations.

The blood vessels were immunostained light brown as they were at the previous time, although the number of microvessels in the dermis that were being restructured was large. It is during the proliferative phase, which begins 48 h after the appearance or induction of a wound, and which extends up to two weeks, that vascularized connective tissue is observed, followed by the formation of granulation tissue, as mentioned by Beldon [53] and Schultz et al. [5,54].

It is noteworthy that under physiological conditions, growth factors are present in the wound. Additionally, neovascularization is complex and depends not only on angiogenesis but also on the arteriogenesis and vasculogenesis stimulated by growth factors. Extracellular angiogenin and other cytokines that act in the healing process or physiological repair stimulate proliferation, differentiation and migration of endothelial cells. The hypothesis that TGF-β1 acts as a stimulant of angiogenesis [57] and VEGF synthesis can be discarded in this case, since immunostaining did not vary between groups.

The hair follicle, the epidermis, and the dermis are known to have stem cells essential for tissue regeneration, as reported by Broeckx et al. [58]. Roubelakis et al. [59] studied PRP in skin ulcers with different etiologies in patients. The platelet-rich component was obtained by a semi-automatic system, unlike the manual procedure performed in the present study. The authors reported that the

therapy can be of great value in several therapeutic procedures, as it can accelerate the healing of ulcers and promote faster neovascularization in the affected region. Additionally, the *in vitro* study revealed that PRP can attract mesenchymal stem/stromal cells (MSCs) and dermal fibroblasts (DFs) to the lesion site.

The main limiting factor of the present study is that no biopsies were performed after macroscopic wound closure, since remodeling continues afterward, and other findings could be obtained then. According to Souza et al. [24], the clinical appearance of wound closure does not necessarily correspond to microscopy results, as the authors observed tissue with better histological quality in treated wounds, although no significant differences were seen between groups in the evaluated histological features. In contrast, the obtained data are unprecedented and thus of great value in that they contribute to deciphering some of the conflicting results related to the therapy. According to some authors [60–63], the use of PRP does not yet have results that demonstrate its benefits in animals and/or human beings. In this sense, *in vitro* findings with human cells reported by Lynch & Bashir [64] did not provide sufficient evidence confirming the efficacy of PRP or whether it is appropriate for use in the practice of dermatology.

A comparison between results obtained from studies performed on skin and those from studies on tendons and ligaments is rather complicated. According to Frairia & Berta [65], there is a high degree of heterogeneity among fibroblasts present in different body tissues.

It is believed that the unsatisfactory results obtained in the present study are not related to the low leukocyte concentration, since, as mentioned by Wasterlain et al. [66], the use of PRP with a low concentration of white cells is considered adequate when it is intended to provide more of an anabolic than a catabolic effect. This is particularly important when working with uncontaminated wounds. Additionally, according to Boswell et al. [67], high amounts of leukocytes may be more relevant in cases of large and infected cutaneous wounds.

In the current experimental conditions, LP-PRP was not effective compared with the physiological healing of horse skin. The hypothesis that the treated wound would exhibits greater expression of both TGF-β1 was not supported by the data. However, these wounds presented larger numbers of stained vessels after macroscopic wound closure. Further research should not only take into consideration the association of the platelet-rich component with stem cells, which, in general, have provided adequate responses, but also evaluate other proteins present in PRP besides those typically studied.

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Conflicts of Interest

There is no conflict of interest regarding this paper.

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