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Original Article

Macroscopic and histomorphometric evaluation of different healing stages of skin wounds in horses treated with leukocyte-poor platelet-rich plasma

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ABSTRACT

Skin injuries are frequent in horses, and one of the treatments used for such injuries is the platelet-rich plasma (PRP). The objective of this experiment was to macroscopically and microscopically investigate the process of healing by second intention in skin wounds of eight healthy gelding crossbred horses treated (T) or untreated (UT) with a single dose of leukocyte-poor PRP (LP-PRP). Three square-shaped wounds were induced in both gluteal regions. After 12 h of wound induction, 0.5 mL LP-PRP was administered in each border of the lesion in one of gluteal region. Contralateral wounds were UT. Six skin biopsies were obtained with a 6-mm *Punch*. Macroscopic variables of one of the non-biopsied wounds were evaluated. Samples were processed for histomorphometric evaluation. No difference was observed between the time required for wound closure in the two groups. Histomorphometric analysis performed 14 days after wound induction revealed higher ($p = 0.034$) angiogenesis and number ($p = 0.0179$) of total leukocytes in T wounds. Fibrocytes numbers increased significantly ($p = 0.023$) on day 7 after injury in the UT group. General microscopic evaluation performed independently of scores and morphometric analysis revealed that the majority of the T wounds showed better healing variables in the sections analyzed after complete macroscopic closure of the wound. A single injected dose of LP-PRP 12 h after wound induction in horses does not interfere with the wound healing process but reveals that majority of the T wounds exhibit better healing histomorphometric characteristics.

INTRODUCTION

The skin is an essential organ for body protection. Skin thickness in horses ranges from 1 to 6.3 mm depending on the site, and it is thicker on the lumbosacral and gluteal regions. In addition to acting as a barrier against microorganisms, it represents a passive barrier against fluid losses, which favors the maintenance of homeostasis.

Skin wounds are common in the equine species. Depending on the site and extent of the injury, it can partly or completely compromise the use of horses in sport activities or employment practices. Therefore, promoting rapid and high-quality skin healing is a challenge in medical and surgical equine clinics.

The wound healing process occurs in at least four overlapping phases: hemostasis, inflammation, proliferation, and finally remodelation (HARPER et al., 2011). Another authors subdivided the third stage into

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migration and proliferation and the fourth into contraction and remodeling (SCHULTZ et al., 2011).

Platelet-rich plasma (PRP) is an autogenous source of growth factors, and has been widely used to promote faster healing of skin wounds, although results obtained from controlled studies are contradictory. Among the bioactive factors contained in PRP with important therapeutic potential, transforming growth factor-β1 (TGF-β1), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (bFGF) are prominent (BOSWELL et al., 2012). Although some studies demonstrate the effectiveness of growth factors in accelerating the healing process of skin wounds in horses (MACIEL et al., 2012), other studies have not shown this effect (MONTEIRO et al., 2009; SOUZA et al., 2014a). However, a previous report has described a better response in some constituents of the wound treated with leucocyte-poor PRP (LP-PRP) with respect to the control group (SOUZA et al., 2014b) along with better healing quality (SOUZA et al., 2015).

We hypothesized that skin wounds treated with LP-PRP have a better quality healing process. The objective of the present study was to macroscopically and microscopically evaluate the process of healing by second intention of skin wounds located in the gluteal region of horses treated (T) or untreated (UT) with one application of LP-PRP 12 h after surgical wound induction.

MATERIAL AND METHODS

This work was approved by the Ethics Committee of Animal Use of the Universidade Federal de Viçosa (Process number 96/2011).

Eight healthy gelding crossbred horses, aged 16–17 years (average, 16.37 ± 0.52) were used. Only seemingly healthy animals checked during general physical examination and without dermatological disorders were included in the research. The animals were housed in individual 20 m^2 masonry stalls 21 days before the onset of the experiment, in which they were fed Tifton 85 hay and elephant grass in addition to a diet proper for horses. Water and mineral salt were offered *ad libitum*. The horses were left free in a *Brachiaria decumbens* paddock for 2 h in the morning every day. Appetite, mucosal color, body temperature, and intestine movements were evaluated daily. This management was maintained throughout the trial. During the adaptation period, which was three weeks, the animals were weighed, bathed with acaricide solution containing deltamethrin, and dewormed with moxidectin administration (0.2 mg/kg).

Three square-shaped skin injuries were created in the right and left gluteal region of all horses, as described previously (FERREIRA et al., 2007; SOUZA et al., 2014a). The injury was created with a square-shaped plastic mold (6.25 cm^2), and the epidermis, dermis, and subcutaneous tissue were removed. The wounds were identified in both gluteal regions as A, B, and C in the craniocaudal direction and were allowed to heal by second intention. Complete closure of the injury was evaluated in wound C. Injuries A and B were biopsied for histomorphometric evaluation. Antitetanus serum was applied on the day of cutaneous wound induction. Pain was reduced using a single dose of butorphanol tartrate (0.08 mg/kg/IV). Anti-inflammatory drugs or antibiotics were not used.

LP-PRP was manually obtained based on the protocol described previously (ZANDIM et al., 2013). The concentration of platelets and leukocytes was determined manually in LP-PRP and in the blood collected with EDTA. Quantification was conducted in a Neubauer chamber, and Türk solution was used to count leukocytes and Brecher's method to count platelets. Twelve hours after surgery, 0.5 mL of LP-PRP was administered to each of the four edges of the wound (T group/wounds) in one of the gluteal regions, left or right (randomization), totaling to 2 mL per wound. The contralateral injury was used as control (UT group/wounds). All wounds (T and UT) were cleaned daily with Milli Q water.

For histomorphometric analysis, six fragments were obtained, the first being injured skin (T0), using samples obtained with number 23 scalpel blade while inducing the surgical wound. The remaining samples were obtained by biopsies using a 6-mm diameter *Punch* after 24 h (T1), and at two (T2), seven (T3), and 14 (T4) days. The sixth biopsy (T5) was performed after clinical healing. The fragments were placed in buffered formalin for at most 48 h and subsequently in 70% alcohol. Next, they were processed in a routine manner: 5-µm-thick sections were collected and stained with hematoxylin–eosin (HE) and Masson's Trichrome staining. The related histological features, viz., inflammation, angiogenesis, new-epithelium thickness, and re-epithelialization, were scored from 0 to 3 based on the semi-quantitative scale of Lepault et al. (2005) in addition to the thickness of new epithelium as studied by Monteiro et al. (2009) (Table 1). Three researchers evaluated the histological fragments in a blinded manner. Moreover, morphometric analysis was performed. For this, 10 photographic images were obtained at random using a light microscope (Olympus BX50) coupled to a photographic camera (Olympus QColor 3) connected to a computer for image capture using the QCAPTURE PRO 6:00: 6.05 program. The images were placed on a grid with 400 intersections made on Power Point software, using 48 intersections. Cell types and structures quantified by morphometry were total leukocytes, neutrophils, eosinophils, macrophages, blood vessels, collagen, fibroblasts, fibrocytes, and myofibroblasts.

Table 1. Classification of the histological characteristics of inflammation, angiogenesis, neoepithelial thickness, and re-epithelialization according to the mentioned criteria.

Variables	Scores	Criteria
Inflammation	0	- Absent;
	1	- Diameter of the inflammatory focus measured less than twice the thickness of the adjacent intact epidermis;
	2	- Diameter between two and five times the thickness of the intact epidermis;
		- Diameter more than five times the thickness of the intact epidermis.
	3	
Angiogenesis	0	- No new capillaries were apparent within the wound bed;
	1	- New capillaries were present between one and five;
	2	- New capillaries were present between six and 15;
	3	- More than 15 new capillaries were present.
Neoepithelial	0	- Lower than the adjacent intact epidermis;
thickness*	1	- Equal to the adjacent intact epidermis;
	2	- Higher than the adjacent intact epidermis.
Re-epithelialization	0	- No new epithelium was observed at the wound margin;
	1	- New epithelium had advanced to cover one third of the granulation tissue present in the biopsy;
	2	- New epithelium had advanced to cover two third of the granulation tissue present in the biopsy;
	3	- Epithelialization was complete.

*Based on the classification described by Lepault et al. (2005) and Monteiro et al. (2009).

Macroscopic characteristics (wound C; T and UT) such as exudation, presence of crust, and granulation tissue as well as evolution of the wound closure until apparent healing were evaluated daily. Daily measurements of the retraction on the edge of wound C were also performed, which were then scanned, and the cross-sectional area (cm^2) was determined using QUANT v 1.0.0.28 software (VALE et al., 2003), as previously described (SOUZA et al., 2015). Finally, before and weekly after the wound induction, hematological (hemoglobin, hematocrit, erythrocyte, total platelets, and leukocytes) and biochemical (plasma total protein and fibrinogen) characteristics were evaluated in whole blood obtained by jugular venipuncture in vacuum tubes containing EDTA as the anticoagulant. The reference values used were based on those described by Kaneko et al. (2008) and Robinson; Sprayberry (2009).

Macroscopic variables (exudation, presence of crust, presence of granulation tissue, and wound closure) were subjected to Student's *t* test for two dependent or paired means. Histological characteristics (inflammation, angiogenesis, thickness of new epithelium, and re-epithelialization) were evaluated by non-parametric Friedman test. The normality and homogeneity of the parametric variables were analyzed by the Shapiro–Wilk and Bartlett tests, respectively.

The comparison between T and UT wounds at each time, considering the animal effect, was performed using ANOVA. The same analysis was used to evaluate the result within each group and to determine the relationship between the final wound healing time and the higher or lower quantity of leukocytes in LP-PRP. Means were compared by Tukey's test. The significance level adopted was 5%.

RESULTS

LP-PRP was administered immediately after laboratory processing and confirmation of the number of platelets and leukocytes. In whole blood and LP-PRP, the values of platelets ranged from 100,000 to 150,000 platelets/μL and from 320,000 to 390,000 platelets/μL, respectively. The mean values of leukocytes ranged from 5600 to 10900 cells/μL and from 50 to 900 cells/μL in total blood and LP-PRP, respectively. The mean values for platelets/μL in whole blood were 116.25 ± 7.50, 212.13 ± 7.77, 210.88 ± 52.49, 180.75 ± 22.36, and 204.88 ± 58.97 at T1, T2, T3, T4, and T5, respectively.

There was no difference ($p > 0.05$) between the maximum time required for wound closure and the highest (900, 800, 450, and 350) or lowest (100, 150, 150, and 50) amounts of leukocytes/μL in LP-PRP for all eight animals. In addition, no significant correlation was found ($r = -0.08$; $p > 0.05$) between the time required for wound healing and the concentration of leukocytes in LP-PRP from each horse on the day of treatment.

None of the eight horses showed extreme variations in rectal temperature, loss of appetite, or other physical changes that could compromise data collection. Variables pertaining to hematological characteristics are shown in Table 2. The values remained within the normal range or were slightly elevated for the specie.

Table 2. Mean values and standard deviations of hematological and biochemical variables evaluated during the experimental period.

Tempo	0	1	2	3	4	5
Hematocrit (%)	30.4 ± 3.9	28.6 ± 3.4	30.3 ± 3.1	28.4 ± 4.7	28.9 ± 3.1	28.5 ± 3.6
Hemoglobin (g/dL)	10.0 ± 1.1	9.7 ± 1.1	9.8 ± 0.8	9.5 ± 1.4	9.7 ± 1.0	9.7 ± 1.0
Erythrocyte (cells/μL)	6.3 ± 0.9	6.0 ± 1.0	5.8 ± 0.7	5.9 ± 1.1	5.8 ± 0.7	5.8 ± 0.8
Leukocytes (cells/μL)	7.9 ± 1.8	7.9 ± 1.5	8.1 ± 2.3	7.9 ± 1.9	7.7 ± 1.3	6.6 ± 1.1
PTP (g/dL)	7.8 ± 0.5	7.7 ± 0.5	7.6 ± 0.5	7.8 ± 0.4	7.6 ± 0.4	7.6 ± 0.4
Fibrinogen (g/dL)	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.1	0.3 ± 0.2

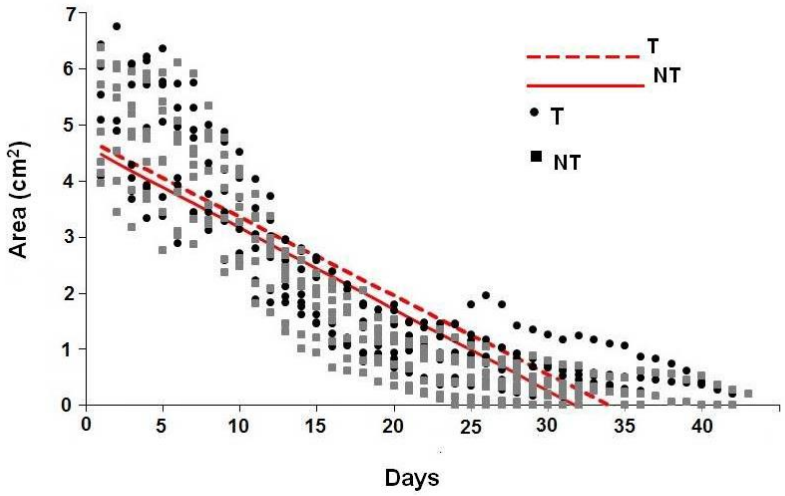
Times: 0, before; 1, 24 h; 2, 48 h; 3, 1 week; 4, 2 weeks; 5, final (wound closure). PTP: plasma total protein.

Macroscopic evaluation

During wound closure visualization, performed during cleaning, discreet initial bleeding and a wound bed completely filled with a clot crust were observed, and when the latter was removed, it revealed a hyperemic region with small sinuosities and serous exudate. The edges were beginning to show irregular contours around the seventh day after the wounds were induced. The mean values and standard deviation of the maximum permanence period referring to the macroscopic characteristics evaluated in the wound of both groups were as follows: exudation, 3.2±0.2 days (T) and 3.1±0.3 (UT); crust, 13.7±14 days (T) and 15.6±1.1 (UT); granulation tissue, 15.7±0.6 days (T) and 16.1±0.6 (UT); and cicatrization, 38.7±6.5 days (T) and 36.9±7.4 (UT).

The maximum healing time, monitored by measuring the wound area, is shown in Figure 1. After two weeks, there was a presence of a reddish brown crust covering the entire wound bed, regardless of the treatment. The characteristic was similar to that of the healing tissue, with the damaged area initiating an edge-rounding process.

Figure 1. Reduction of the injury area in both wounds treated and untreated with LP-PRP. A similar behavior was observed among the groups, which was confirmed by linear regression analysis ($p > 0.05$).



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Regardless of the wound group (T and UT), from the fourth day onwards the entire wound bed was filled with granulation tissue, which was easily visible macroscopically from the fourth to the sixteenth day in both T and UT wounds with LP-PRP. The maximum time required for wound healing was 47 days in both groups, with mean values of 36.87 ± 7.45 and 38.75 ± 6.47 days for the UT and T wounds, respectively. There was no difference ($p = 0.59$) between the groups.

Histopathological evaluation

Intact skin fragments (T0) showed epidermis with the stratum corneum in its entire extension, and dermis with hair follicles, sebaceous glands, blood vessels, and rare lymphoplasmacytic inflammatory cells. After 24 h of wound induction (T1), inflammatory infiltrate, which was predominantly neutrophilic in nature, was observed in the dermis (Figure 2a); severe and extensive epidermal necrosis with neutrophilic inflammatory infiltrate (Figure 2b), with the diameter being 2–5 times

higher than the thickness of the intact epidermis classified as score 2 for inflammatory reaction, was observed in both T and UT wounds. At this stage of the injury, it was not possible to score for re-epithelialization of the epidermis or dermal angiogenesis or fibroplasia.

In fragments obtained at 48 h (T2; Figure 2c, d), a markedly high fibrinopurulent exudate with a predominance of neutrophils and eosinophils covering the epidermis and superficial dermis and dermal angiogenesis along the wound bed were observed. Both characteristics were scored as 2, with the diameter being 2–5 times the thickness of the intact epidermis for the inflammatory infiltrate and 6–15 for new capillaries during angiogenesis. Furthermore, there was a reduction ($p > 0.05$) in the number of neutrophils relative to the time of 24 h in both groups.

In the samples obtained after one week (T3), epidermal re-epithelialization was observed with hyperplasia foci (Figure 2e, f). In the dermis, the granulation tissue was very expressive, characterized by small vessels lined by tumescent endothelial cells, edema or amorphous fundamental substance, intense fibroblast proliferation, and discreet organization of the collagen fibers. The disorganized fibroblasts were best visualized with Masson's Trichrome staining (Figure 3). The population of total leukocytes ($p = 0.8835$) associated with the injury was mixed with random predominance of neutrophils ($p = 0.8358$) and eosinophils ($p = 0.8619$), with no difference among groups. Lymphocytes were also observed in a lower amount.

Wounds biopsied at 14 days showed similarity in histologic findings between groups. Marked hyperplasia of the epidermis was observed (Figure 2g, h), and there was no difference between the quantity of neutrophils ($p = 0.2088$), eosinophils ($p = 0.3506$), number of blood vessels ($p = 0.9728$), collagens ($p = 0.4836$), fibroblasts ($p = 0.8182$), fibrocytes ($p = 0.8853$), or myofibroblasts ($p = 0.6234$) associated with wound repair. Significantly high total leukocyte ($p = 0.0179$) and macrophage ($p = 0.0596$) count as well as increased angiogenesis was observed in the T wounds, compared to the UT wounds.

Histological sections performed after skin closure (Figure 2i, j) in both groups were scored as 2 for re-epithelialization, with the new epithelium covering 2/3 of the epidermis, and 3 for fibroplasia (parallelism between fibroblasts). Overall, a more regular arrangement of fibroblasts and fibrocytes and the parallelism of collagen fibers were observed in 75% of the histological fragments of the T wounds, suggesting better healing in this group (Figure 2i) than in UT wounds (Figure 2j), but there was no statistical difference, except for the number of fibrocytes, which was higher in the UT group seven days after wound induction.

Masson's Trichrome staining (Figure 3) used for a detailed examination of the collagen fibers revealed other characteristics of the wound. In MT-stained samples, marked necrosis of the epidermis on wound edges and presence of dried crust covering the injury (Figure 3a, b) were observed at 48 h after wound induction.

After one week, fibroblasts were prominently observed by Trichrome staining and had started to acquire the appearance of myofibroblasts, cells with intermediate morphological characteristics between fibroblast and smooth muscle cell with actin aggregates and myosin in the cytoplasm. The new capillaries could be observed as small shoots. The scores for angiogenesis were 2–3 in both groups. There was a smaller amount of amorphous substance, with extracellular matrix consisting predominantly of thick collagen fibers in initial stage of organization (Figure 3c, d).

Within two weeks, a large number of vessels were observed, culminating in the maximum expression of angiogenesis, i.e., more than 15 new capillaries were present and alignment of collagen fibers was improved (Figure 3e, f). At this time, there was a higher ($p = 0.0034$) amount of blood vessels in the T wound. The transition between organized and aligned collagen fibers was also more noticeable.

In the final phase (T5), an accentuated organization of the collagen fibers (Figure 2g, h) was observed, arranged in parallel-aligned bundles, and this alignment was better visualized in the T group (Figure 3g).

Figure 2. Photomicrograph of the equine skin healing process. Image showing fragments obtained after 24 h of wound induction in T (a) and UT (b) wounds. Image (a) shows neutrophilic infiltrate, which is predominantly inflammatory, in the dermis (arrows); (b), epidermal necrosis area at the top left edge (arrows) is associated with polymorphonuclear inflammatory infiltrate and is compatible with the time of wound induction. Images c (T) and d (UT) represent 48 h after wound induction (T2), with more accentuated inflammatory infiltrate in the T wound (arrows). One week after wound induction was performed (Time 3), epidermis with irregular borders in both groups (e, f) and onset of collagen organization (arrows) are observed. In the second week (T4), the leucocyte infiltrate is almost imperceptible, but there is a moderate organization of fibroblasts and hyperplastic epidermis (g, T; h, UT; arrows). At T5, consistent with macroscopic closing of the injury, a thinner epidermis was observed in the UT wound (i), but the T wound (j) showed a healing process, seemingly more organized than the UT wound (arrows). Hematoxylin–Eosin staining. Bars = 50µm.

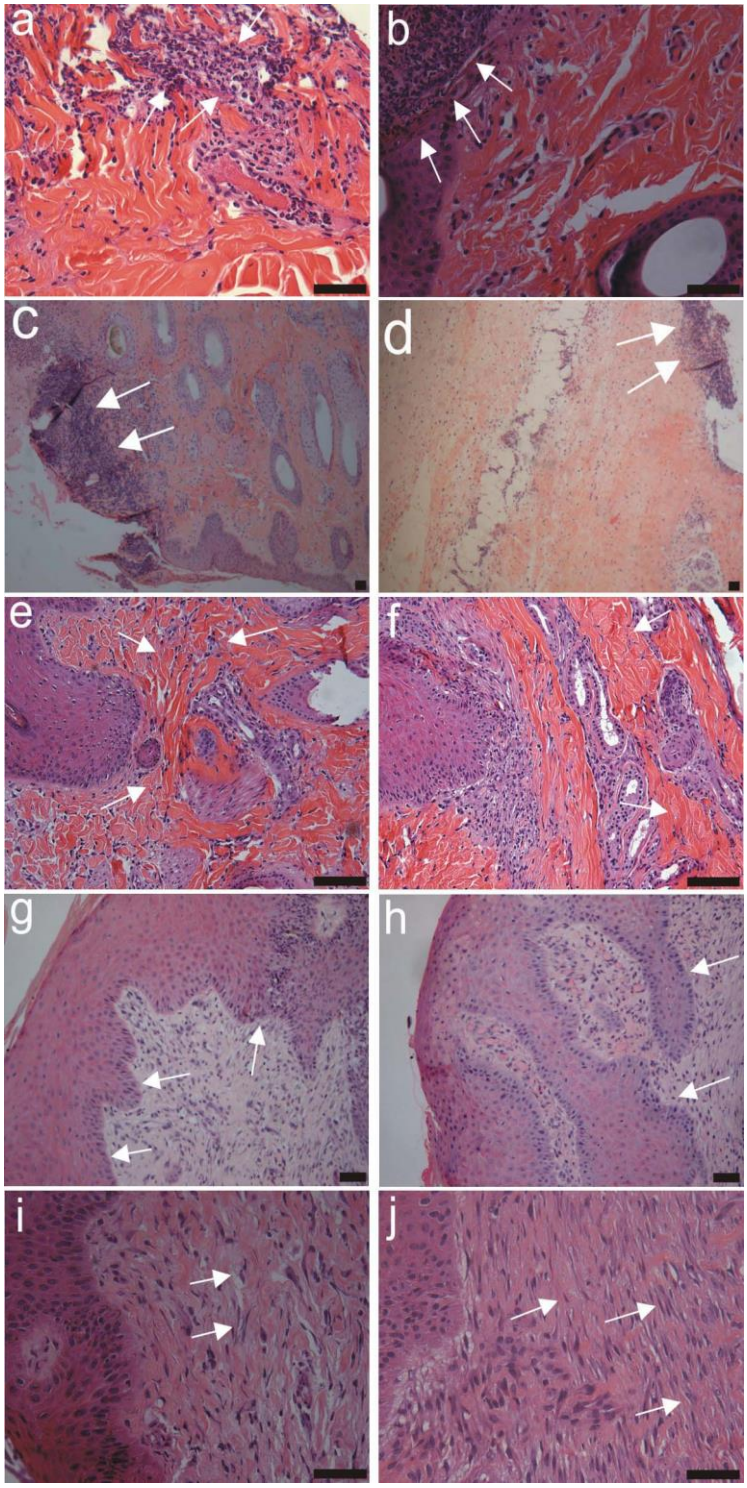

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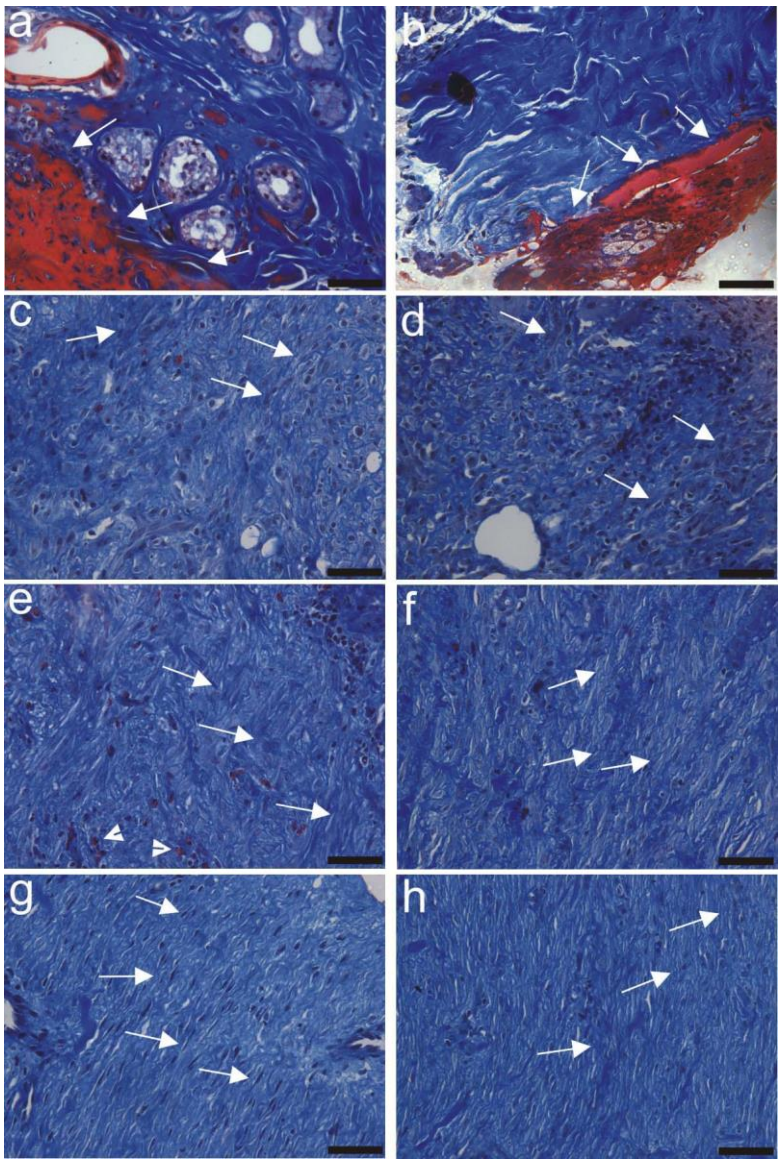
Figure 3. Photomicrograph of equine cutaneous wound healing. In T (a) and UT (b) groups at the time of 48 h (T2), an area of hemorrhage and necrosis in the epidermis and superficial dermis is highlighted in red color (arrows), contrasting in the dermis with compact collagen fibers, in parallel arrangement, of dark blue color. In the findings at one week (T3), sparse collagen fibers with empty spaces, moderate alignment of fibroblasts (arrows), and few vessels are observed in both groups (c, T; d, UT). In injuries after two weeks (T5), the collagen fibers are in a compact arrangement, there are few empty spaces filled with collagen (arrows), a higher number of blood vessels in the T wound (arrow heads) (e) compared with the UT wounds (f), and a higher amount of angiogenesis at 14 days after the injury are observed. In figures g (T wound) and h (UT), obtained when the injury has macroscopically healed, collagen fibers are arranged in a less compressed manner in the UT wound (arrows). The alignment of fibroblasts and fibrocytes is better evidenced in T wounds (arrows). Masson's Trichrome. Bars = 50µm.



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Morphometric evaluation

Of the analyzed morphometric variables, the total leukocyte and fibrocyte counts showed differences among groups, with respective higher values in the samples obtained two ($p = 0.017$) and one ($p = 0.023$) weeks after the injury in T (total leukocytes) and UT (fibrocytes) wounds with LP-PRP. Results referring to morphometric analysis are shown in Tables 3 and 4.

A considerable increase in neutrophils ($p = 0.0012$) coincided with the time observed for total leukocytes, i.e., in the sample obtained during the first 24 h compared with time zero. Similarly, the increase was higher ($p < 0.05$) in the T group than that in the UT

group. Eosinophils showed a peak of maximum expression after 48 h in the UT group, which reduced one week after injury induction when values close to baseline were observed for both groups (Table 3).

The macrophage count increased by more than five cells from T0 to T3, with a peak on the seventh day after the injury was induced in both the T and UT groups. From one week onwards, the number of macrophages reduced in the UT group, but in the T group it still remained high up to two weeks (Table 3), showing a statistical difference regarding the UT group at 14 days (p = 0.059). After two weeks, there was a discreet decline in these cells, approaching baseline values at T5 in both groups.

Table 3. Morphometric evaluation (mean ± standard deviation) of total leukocytes, neutrophils, eosinophils, macrophages, and blood vessels obtained by the scoring system to evaluate the skin healing process of horses treated or untreated with LP-PRP

Time (days)	Treated	Untreated
	Leukocytes	
0	0.9 ± 2.4 ^{Ad}	1,9 ± 2.3 ^{Ab}
1	33.0 ± 20.0 ^{Aa}	26.9 ± 13.2 ^{Aa}
2	28.5 ± 20.7 ^{Aab}	22.6 ± 8.2 ^{Aa}
7	21.0 ± 6.5 ^{Aabc}	20.4 ± 9.9 ^{Aa}
14	11.5 ± 4.4 ^{Abcd}	6.5 ± 1.4 ^{Bb}
37	3,4 ± 4.3 ^{Acd}	2.1 ± 1.2 ^{Ab}
Mean ± SD	16.7 ± 16.6^{A}	13.4 ± 12.7 ^{A}
	Neutrophils	
0	0.1 ± 0.3 ^{Ab}	0.1 ± 0.3 ^{Ab}
1	25.7 ± 20.2 ^{Aa}	18.9 ± 14.7 ^{Aa}
2	18.4 ± 18.3 ^{Aab}	10.9 ± 7.1 ^{Aab}
7	10.0 ± 6.3 ^{Aabc}	9.1 ± 11.7 ^{Aab}
14	2.1 ± 3.4 ^{Abc}	0.4 ± 0.5 ^{Aab}
37	2.2 ± 3.6 ^{Abc}	0.4 ± 0.7 ^{Ab}
Mean ± SD	9.8 ± 14.5 ^{A}	6.6 ± 10.5 ^{A}
	Eosinophils	
0	0.0 ± 0.0 ^{Ab}	0.0 ± 0.0 ^{Aa}
1	0.7 ± 0.7 ^{Aab}	1.5 ± 2.0 ^{Aa}
2	0.2 ± 0.7 ^{Aab}	16.6 ± 0,3 ^{Aa}
7	1.0 ± 0.9 ^{Aa}	1.1 ±1.5 ^{Aa}
14	0.2 ± 0.7 ^{Aab}	0.0 ± 0.0 ^{Aa}
37	0.0 ± 0.0 ^{Ab}	0.1 ± 0.3 ^{Aa}
Mean ± SD	0.3 ± 0.7 ^{A}	3.2 ± 1.1 ^{A}
	Macrophages	
0	1.7 ± 2.0 ^{Ac}	1.7 ± 1.9 ^{Ac}
1	6.5 ± 2.5 ^{Aab}	6.5 ± 1.8 ^{Aab}
2	9.9 ± 3.6 ^{Aa}	11.6 ± 4.7 ^{Aa}
7	10.0 ± 3.1 ^{Aa}	10.1 ± 6.0 ^{Aab}
14	9.1 ± 3.9 ^{Aa}	6.1 ± 1.5 ^{Abc}
37	2.1 ± 2.3 ^{Abc}	1.6 ± 1.3 ^{Ac}
Mean ± SD	6.6 ± 4.5 ^{A}	6.3 ± 5.0 ^{A}
	Blood Vessels	
0	7.4 ± 6.5 ^{Ab}	7.2 ± 6.1 ^{Ab}
1	16.0 ± 11.1 ^{Aab}	14.1 ± 8.3 ^{Aab}
2	15.0 ± 6.4 ^{Aab}	13.6 ± 6.8 ^{Aab}
7	27.2 ± 13.3 ^{Aa}	19.0 ± 8.1^{Aa}
14	10.6 ± 6.6 ^{Ab}	10.5 ± 5.1 ^{Aab}
37	7.6 ± 4.2 ^{Ab}	10.6 ± 6.3 ^{Aab}
Mean ± SD	13.9 ± 10.6 ^{A}	12.5 ± 7.5 ^{A}

Different uppercase and lowercase letters in the same row and column, respectively, differ ($p < 0.05$) by Tukey's test.

Higher numbers of blood vessels were observed in the skin samples evaluated after one week, particularly in the T group (Table 3), and the reduction in vessels was higher in this group (p = 0.0672) than that in the UT group at the moment of wound closure. In fact, in both groups there was a considerable, but not statistically significant (p = 0.120), increase in the number of vessels. However, from the seventh day onwards, there was a gradual decrease until wound closure, when the count returned to values close to the physiological limits found

at time zero. Regarding collagen fibers, a decrease (p = 0.0115) in T1 compared with the healthy skin (T0) was observed. Thereafter, there was a gradual increase until the value was almost five times more than that found in healthy skin (T0), which occurred at T5 in both the groups.

The maximum increase in number of fibroblasts (Table 4) was observed in T3, maintaining a similarity of values between the groups. The increase relative to time T0 was 2.4 times for both the groups. From one week until the final evaluation, there was a progressive reduction with similarity maintained between groups (p = 0.5623). Fibrocytes reduced significantly at T1 in relation to T0 but increased progressively in both groups until the final evaluation (Table 4), with differences observed at time T3 (one week) when the UT group exhibited higher values (p = 0.0230), as previously mentioned. Finally, myofibroblasts increased progressively similar between both the groups, with the values being more than 18 times from time T0 until the final time with no statistical differences. Myofibroblasts reached its peak value at T5 for both groups, with higher values (p = 0.389) in the UT group.

Table 4. Morphometric evaluation (mean ± standard deviations) of collagens, fibroblasts, fibrocytes, and myofibroblasts obtained by the scoring system to evaluate the skin healing process of horses treated or untreated with LP-PRP

Time (days)	Treated	Untreated
	Collagens	
0	1.631,7 ± 304.4 ^{Abc}	1.631,4 ± 304,4 ^{Abc}
1	498.0 ± 237.0 ^{Ac}	432,0 ± 223,70 ^{Ad}
2	1.223,5 ± 377.4 ^{Ab}	1.190,38 ± 502,08 ^{Ac}
7	1.644,6 ± 433.4 ^{Aab}	1.661,2 ± 226.7 ^{Abc}
14	1.850,7 ± 552.4 ^{Aa}	2.018,4 ± 308.9 ^{Aab}
37	2.192,7 ± 258.7 ^{Aa}	2.260,7 ± 332,6 ^{Aa}
Mean ± SD	1.506,8 ± 647.9 ^{A}	1.532,3 ± 684,5 ^{A}
	Fibroblasts	
0	36.1 ± 12.3 ^{Ac}	36.1 ± 12.3 ^{Ac}
1	38.2 ± 16.5 ^{Ac}	37.6 ± 17.7 ^{Ac}
2	51.4 ± 25.1 ^{Abc}	46.2 ± 25.7 ^{Ac}
7	87.9 ± 17.3 ^{Aa}	89.5 ± 14.5 ^{Aa}
14	76.4 ± 21.6 ^{Aab}	74.6 ± 12.1 ^{Aab}
37	52.7 ± 12.2 ^{Abc}	53.4 ± 11.1 ^{Abc}
Mean ± SD	57.1 ± 25.7 ^{A}	56.2 ± 25.1 ^{A}
	Fibrocytes	
0	22.6 ± 6.8 ^{Abcd}	20.9 ± 6.5 ^{Ac}
1	12.4 ± 5.3 ^{Ad}	12.1 ± 4.5 ^{Ac}
2	17.7 ± 6.9 ^{Acd}	14.1 ± 8.4 ^{Ac}
7	27.9 ± 7.9 ^{Bbc}	38.5 ± 9.4 ^{Aab}
14	34.2 ± 16.8 ^{Ab}	35.1 ± 9.2 ^{Ab}
37	51.2 ± 6.7 ^{Aa}	48.4 ± 5.5 ^{Aa}
Mean ± SD	27.7 ± 15.5 ^{A}	28.2 ± 15.3 ^{A}
	Myofibroblasts	
0	4.9 ± 2.5 ^{Ac}	5.4 ± 3.9 ^{Ac}
1	11.7 ± 5.8 ^{Ac}	12.5 ± 4.0 ^{Ac}
2	17.1 ± 5.1 ^{Ac}	16.6 ± 9.3 ^{Ac}
7	40.0 ± 8.4 ^{Ab}	43.2 ± 19.5 ^{Ab}
14	68.7 ± 16.2 ^{Aa}	74.1 ± 17.9 ^{Aa}
37	80.4 ± 17.4 ^{Aa}	86.4 ± 13.4 ^{Aa}
Mean ± SD	37.1 ± 30.8 ^{A}	39.7 ± 33.7 ^{A}

Different uppercase and lowercase letters in the same row and column, respectively, differ (p < 0.05) by Tukey's test.

DISCUSSION

As mentioned previously, the maximum time for macroscopic closure of injuries was 47 days, with the mean values being lower in wounds UT with LP-PRP. This average healing time is similar to the period mentioned for non-infected wounds located in the thoracic region, which is approximately four weeks (SCHWARTZ et al., 2002) as well as in injuries performed by the same mode and region as those reported in a previous study (FERREIRA et al., 2007). The shorter closure time of the wound UT with PRP was also reported by Monteiro et al. (2009) in injuries induced on the dorsolateral surface of the metacarpal. However, a faster macroscopic healing may not result in better quality tissue (SOUZA et al., 2015). This was also observed in the present study, wherein the T wounds with LP-PRP demonstrated a superior feature of the scar

tissue in the sixth biopsy, primarily with respect to the alignment of collagen fibers and fibroblasts, concluded after the assessment of the three evaluators.

The macroscopic findings observed during the daily examination corroborate with the findings of Ferreira et al. (2007), who observed formation of a dark crust from the clot in the site, followed by formation of granulation tissue, contraction, and re-epithelialization. The observation period of serous secretion in wounds was quite short, approximately three days after the induction of wounds. The wounds remained reddish and shiny in appearance, primarily after daily cleaning but with very little secretion.

The reddish brown crust, observed in the wound bed two weeks after the surgical injury was induced, is consistent with the proliferative phase of healing, as observed previously in skin wounds induced due to burning by electrocautery in the dorsal region of rabbits (RIBEIRO et al., 2013). Two weeks after surgical removal of the rabbit skin, the authors observed resected wounds, revealing the presence of thin and yellowish crusts a little rougher and more difficult to be removed. Similar thin crusts were also observed in the wound beds in this study until about 14 days after wound induction in both the T and UT groups. The drying of the crust observed on the surface two weeks after wound induction has been previously mentioned in the right and left hemithorax and in the right and left lumbar region of horses (HUSSNI et al., 2010). In their study, thick and dark red colored crusts were still easily removable until the 12th day after the injuries were induced.

The amount of leukocytes found in LP-PRP was 50–900 cells/μL. Although this topic has already been discussed previously (SOUZA et al., 2014b), it is interesting to emphasize another study (EL-SHARKAWY et al., 2007) that compared the stimulus to the release of cytokines (marker of inflammation) in tissues T with whole blood, LP-PRP, and platelet-poor plasma (PPP). The exposure to PRP with leukocytes, according to the authors, was associated with a reduction in the release of pro-inflammatory cytokines. Recently, products resulting from culture of tendon fragments of the superficial digital flexor muscle for 96 h in LP-PRP generated from venous blood of eight horses were investigated (BOSWELL et al., 2014). The authors concluded that the lower number of leukocytes in PRP can be more important than a high number of platelets to reduce expression of pro-inflammatory molecules and increase the expression of extracellular matrix molecules. Therefore, the use of LP-PRP in this study, whose wounds were not contaminated, was a proper decision.

The chemotactic activity exerted by platelets is well documented (BOSWELL et al., 2012; WASTERLAIN et al., 2012). Therefore, we expected similar observation in this study, i.e., the wound with the highest number of platelets would display a greater flow of leukocytes. This situation is also compatible with the considerable increase in neutrophils in the first 24 h after inducing injuries in both the groups ($p > 0.05$), with a higher amount of neutrophils observed in T wounds. As previously mentioned, the neutrophil value remained high until macroscopic wound closure. The first inflammatory cell to respond to the mediators released by platelets and the coagulation cascade is the leukocyte. This increase in leukocytes was followed by a discreet reduction up to one week, with a more sudden decrease occurring until the macroscopic closure of the surgical wound. This marginal increase observed the T group was not statistically significant and may be related to the platelet chemotactic effect, thereby determining the increase in neutrophils in the T group. However, it is important that the neutrophil value should not be high for a long duration, as it can compromise wound closure, which, coincidentally, is slower in T wounds. In addition, under physiological conditions, neutrophil value increases, as evidenced by the high values in the UT group (Table 3).

Macrophages release various biologically active substances that are considered essential for recruiting more inflammatory cells to the site of injury. Schwartz et al. (2002) studied the healing of wounds induced in the metacarpal and pectoral regions of six horses. According to the authors, neutrophils indicating acute inflammation were found in all skin samples one week after the injury was induced. However, in the samples evaluated at two weeks, macrophages were present, indicating chronic inflammation. These cells were the predominant cell type in the tissue samples evaluated from two weeks until macroscopic wound healing was observed. In the present study, maximum values of macrophages were found seven days after the injury was induced ($p > 0.05$) in both T and UT groups. This period coincided with the maximum tissue derangement in the wound. The presence of macrophages indicates the transition from the inflammatory to the proliferative phase during the skin healing process, and these cells execute their optimum action to phagocytize the wound area and devitalize tissues as well as ingest bacteria and neutrophils. Macrophages secrete and synthesize growth factors that stimulate fibroblast migration in wounds (SCHULTZ et al., 2011). Therefore, the response seems to vary according to the site of the skin injury in horses.

The skin at the gluteal region of horses exhibits focal inflammatory infiltrate in the surface of the wound, with a predominance of pleomorphism and a moderate amount of eosinophils accompanied by vascular reactions, 10 days after the induction of the surgical wound as observed in this study. The evaluation of factor VIII in tendon of horses treated with LP-PRP and eosinophilic infiltrate in the tendons treated with PRP by immunohistochemistry (ZANDIM et al., 2013) is possibly

due to the chemotactic effect of histamine released by platelets, which is in agreement to the study by Chandra et al. (2007) who studied rabbit skin treated with platelet gel.

Although morphometric analysis did not show differences ($p > 0.05$) for the number of blood vessels (Table 3), higher angiogenesis ($p = 0.03$) was observed in T wounds with LP-PRP two weeks after wound induction (Figure 3). Histological results were obtained using the classification of Lepault et al. (2005) by a rather subjective evaluation based on histological scoring from 0 to 3. These results demonstrate the risk of comparing different techniques to evaluate a histological characteristic. Some techniques can be considered more reliable, and would thus be the primary choice while conducting a controlled and standardized study. Nevertheless, the subsequent reduction observed, with values being close to the UT group, is a positive factor because persistence of angiogenesis is an undesirable aspect as it can change the mechanical properties of the extracellular matrix through proteolytic activity, thereby slowing down the entire physiological mechanism involved in tissue regeneration (PUFE et al., 2005). Therefore, it is necessary that anti-angiogenic factors (endostatin, angiostatin, thrombospondin, and pigment epithelium derived factor) be present in sufficient amounts to allow the regulation and control of angiogenesis.

Angiogenesis can be stimulated by factors that act in the wound microenvironment, including low pH, oxygen tension, and high quantities of lactate (BHUSHAN et al., 2002). One of the major angiogenesis modeling proteins is thrombospondin (BORNSTEIN, 2009). However, angiogenin acts through activation of endothelial vessels and smooth muscle cells, resulting in biological processes such as cell migration, invasion, proliferation, and formation of tubular structures (GAO; XU, 2008). In addition, TGF-β, VEGF, bFGF, and connective tissue (CTGF) growth factors are potent angiogenic markers for endothelial cells. The increase in the number of blood vessels observed in the T group cannot be associated with ischemic processes because the wounds were healed by second intention and evaluated and cleaned daily, showing a macroscopically excellent clinical aspect.

Skin flaps stained with HE, obtained from intact skin (T0), showed characteristics of a healthy skin, as previously described (SCHWARTZ et al., 2002). A discrete organization of the collagen fibers was mentioned by these authors, with abundant presence of collagens (type I and III) using Picrosirius Red staining, in the first week of the healing process in skin samples of healthy horses. The increase in the amount of collagens occurs progressively until the fourth week, which was when the researchers concluded the study.

The beginning of wound bed filling by granulation tissue, which was independent of the T or UT group, was observed at only four days after wound induction, similar to a previously mentioned case (THEORET et al., 2002), in which it was observed five days after the induction of 1 cm^2 wounds in the skin corresponding to the lateral surface of the third metacarpal.

The differential increase in leukocytes and angiogenesis at 14 days after the injury was performed in the T group is a relevant result because leukocytes stimulate the action of growth factors. Furthermore, a marked decrease in leukocytes and angiogenesis was observed at the final time (T5), which is a very important finding. The permanency of the inflammation process stimulates the formation of an exuberant granulation tissue (THEORET et al., 2002).

During the stage of migration and proliferation, a high quantity of fibroblasts is observed, and the proportion is high during complete healing (SCHULTZ et al., 2011). In the present study, fibroblasts reached the peak value in the week the injury was induced in both groups, and then decreased subsequently. Collagen fibers, fibroblasts, and myofibroblasts were the most numerous histological findings during all the periods of microscopic analysis (Table 4). However, this occurred in both groups. In fact, once an injury occurs, fibroblasts are attracted to the site of the inflammatory process and begin to produce extracellular matrix components. This is possibly due to the considerable amount of these cells observed during morphometric analysis performed for skin flaps obtained 48 h after the wounds were induced.

The differentiation of fibroblasts into myofibroblasts in the newly formed granulation tissue is fundamental to wound contraction. When fibroblasts acquire biochemical and morphological aspects similar to smooth muscle fibers, they are called myofibroblasts (DESMOULIÈRE; GABBIANI, 1996). These cells may also originate from fibrocytes, by the action of TGF-β and other chemokines. Their effect on wound contraction occurs by centripetal movement of the injury margin (CLARK, 1993) caused by the tensile strength generated (SILVER, 1982). Despite the importance of myofibroblasts, in the present study, a progressive increase (16 times) was observed in both the groups.

There is a mutual relationship between collagen fibers and fibroblasts. Fibroblasts bind to fibronectin and incite collagen production during the proliferation stage of the skin healing process (BOSWELL et al., 2014; SCHULTZ et al., 2011; SOUZA et al., 2014a). Higher quantities of collagen were observed at the last two evaluation times, i.e., at 14 days and when the wound was macroscopically healed (Table 4), regardless of the group. Souza et al. (2014a) also observed no difference between the treated and control wounds regarding the expression of type I and III collagens by qRT-PCR, although the authors

observed a more rapid stabilization of the collagen in injuries that received a single application of PRP.

When the wound was macroscopically closed, fibroblast parallelism and a nearly complete re-epithelialization were observed, with the new epithelium covering almost all the adjacent intact epidermis. This finding was observed in both the groups. According to Balbino; Pereira; Curi (2005), the scar remodeling process occurs in successive stages, from production and processing to orientation of collagen fibers. Initially, collagen is deposited randomly, following the organization of fibronectin for orientation. This process depends on the nature and direction of the tensions to which the tissues are subjected.

Here, only the value of fibrocytes differed ($p = 0.0014$) between the groups, with higher values observed for UT wounds only when evaluated one week after wound induction. The lower amount of fibrocytes observed a week after wound induction in the T group suggests that fibroblasts remained for longer than seven days after the treatment with LP-PRP was effected. As previously mentioned, these cells can generate myofibroblasts, but one of their main functions is providing stimulus for collagen formation. In fact, according to Pagnano et al. (2008), they also participate in the formation of antigenic activity. In the evaluation at 14 days, fibroblasts level increased considerably, which was similar to that observed in the UT group ($p > 0.05$).

Histomorphometric analysis revealed that some cells, such as macrophages, eosinophils, and fibroblasts as well as blood vessels, reached the peak level on the seventh day after induction of the surgical wound, with a discreet decline to baseline levels at 37 days, whereas the number of collagen fibers, fibrocytes, and myofibroblasts increased linearly from T3 (7 days) to T5 (37 days). The form of evolution of these characteristics over time, determined by histomorphometry, is compatible with a physiological healing process.

In recent times, the use and the perspectives of therapeutic use of PRP or its variables are challenging. Hessel et al. (2014) reported data from a study wherein they compared the concentration of platelets and leukocytes as well as TGF-β1 and PDGF-BB in platelet autologous concentrates obtained by the manual method and four different systems (Angel, ACP, E-PET, and GPS III) used as semi-automatic methods. The results were rather discrepant and were more unfavorable for the manual methods. According to the authors, these differences between PRP-obtaining procedures can influence the bioactivity of platelet-rich components and consequently the quality of tissue regeneration. The authors also commented on the increased possibility of infection by the manual method, which was used in this study. However, none of the seven animals in the study showed unexpected inflammatory reaction after the administration of LP-PRP. It is assumed that a lower number of leukocytes may be more important for the effect of the platelet-rich component than the number of platelets itself. It is also known that excess platelets in PRP may have a lower response than when it contains only 2.5 times the number of platelets present in whole blood. Finally, it is important to stress that quantitative and semiquantitative methods have a higher cost, and most times an easily obtainable and inexpensive product is sought.

The results obtained in this study were derived from a controlled and randomized investigation and contribute to the permanence of uncertainty regarding the effectiveness of LP-PRP. This uncertain aspect about the suitable effect on skin healing process has been previously mentioned by Baksh et al. (2013), Brossi et al. (2015), Witte et al. (2016), and Bennell; Hunter; Paterson (2017), in horse or human beings. Perhaps, different results could be found if other histomorphometric evaluations are performed after wound macroscopic healing because according to Bosch et al. (2011), the effect of PRP is long-term. In addition, Yamauchi; Mechanic (1988) affirmed that the skin continues remodeling after apparent injury closure. In this regard, in the opinion of the authors of this study, further investigations should be conducted with a longer evaluation period of the skin healing process, regardless of the healing presented by the injury.

In conclusion, this experiment confirmed that LP-PRP applied 12 h after skin wound induction does not accelerate the healing process in horses, but histologic evaluation suggests that the treated scar tissue exhibits apparent better microscopic quality during the final phase of the clinical healing process. Moreover, vascularity is more intense after two weeks of administration of the therapy, but a reduction occurs subsequently. However, inflammatory cells such as neutrophils increase in the wound site within 48 h, intensifying the inflammatory stage of the skin healing process, without interfering with the final time required for wound healing.

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