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ORIGINAL ARTICLE

Healing Potential of Single Dose of Inactivated Autologous PRP, Laser, and PRP/Laser Combination on Full-Thickness Skin Defect in Dogs

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ABSTRACT

It is worth considering the regenerative capacity of platelet-rich plasma (PRP) and laser for challenging skin wounds. Therefore, this study aimed to investigate the healing potential of a single dose of inactivated autologous PRP, Laser, and PRP/Laser on full-thickness skin defects in dogs. Three mongrel dogs were subjected to 4 circular full-thickness skin defects on the thoracic region. Hence, 4 groups were evaluated: The control group (conventional treatment); the PRP group (single subcutaneous infiltration of inactivated autologous PRP); the laser group (laser for one session), and the PRP/laser group (single subcutaneous infiltration of autologous PRP followed by laser for one session). Measured variables were the percentage of wound size, catalase activity, malondialdehyde concentration, and expression of vascular endothelial growth factor A and collagen I alpha 2 genes. Tissue biopsies were also harvested for histopathologic and immunohistochemistry assessments. The percentage of wound size was significantly lower in all groups than in the control group with a greater reduction in the PRP group. Histopathologic findings were better in PRP and PRP/laser groups with superiority for PRP. Other variables were significantly different among groups at some time points. In conclusion, PRP has a greater potential than laser and PRP/laser for accelerating and improving the quality of healing of acute full-thickness skin wounds in dogs.

Introduction

Full-thickness skin defect is a critical condition that represents a challenge in human and animal surgical repairing practice. This could be attributed to the concomitant damage of the subdermal blood plexus, disturbed circulation, and difficult approximation of wound edges.[1] For rapid and acceptable healing of full-thickness skin defects , innovative therapeutic modalities such as regenerative bioproducts could be involved.[2-4] Platelet-rich plasma (PRP) is an important bioproduct that emerged as an innovative approach to wound management due to its mitogenic, angiogenic, and chemotactic properties.[5] In experimental acute full-thickness skin wounds in dogs, single and repeated administrations of locally injected autologous PRP have been associated with better quality of the newly regenerated skin.[6-9] Repeated application of PRP has also accelerated the healing of acute excised skin wounds in dogs.[10] The healing outcome of using autologous PRP gel has been also stated in a dog suffering from a necrotic lesion that occurred after a surgical biopsy of a dorsal tail mass.[11] In another report, topically applied PRP hasn't ameliorated the healing of canine skin wounds.[12]

Laser phototherapy has also emerged as an innovative modality for enhancing tissue regeneration and suppressing inflammation,[13] based on some

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photobiomodulation and photobiostimulation mechanisms.[14,15] Low level laser therapy has been effective to promote the healing of skin wounds in dogs following hemilaminectomies as well as the healing of distal limb excisional skin wounds in horses.[13,16] Skin wounds with tissue loss in both dogs and cats have also shown faster closure rates following laser therapy.[17] Further, the laser has positively regulated the healing of tears and contusions of different tissues rather than skin-like muscles, tendons, and ligaments.[18,19] Occasionally, the stimulating effect of laser on the healing process of skin wounds hasn't been demonstrated.[20]

Due to the lack of beneficial effects of laser and PRP on the healing of skin wounds in some studies and the need for an in-depth understanding of pathways involved in their actions in different animals and different wound models. A necessity is still present to conduct several studies for effectively judging their therapeutic values under different conditions and intensifying the understanding of the characteristics and mechanisms underlying their effects in repairing tissues. Further to the authors' knowledge, the combined effect of PRP and laser on the healing of skin wounds in dogs has been yet investigated. For this, this study aimed to determine the healing potential of PRP, laser, and PRP/laser combination on full-thickness skin defects in dogs. We hypothesized that all of these therapies would promote the healing process with greater efficacy for PRP/laser combination as a result of a synergistic biological action of both therapies.

Materials and Methods

Animals

Three male adult mongrel dogs weighing (range) 15–20 kg and aged 1-3 years were used in this study. Dogs were judged to be healthy based on physical examination, complete blood count, and serum biochemistry analyses. During the study period, dogs were kept in their kennels where they were fed balanced food and allowed free access to water. The present study was approved by the Institutional Animal Care and Use Committee of Faculty of Veterinary Medicine, University of Sadat City, Egypt (protocol number: VUSC-031-1-20). All methods were performed in accordance with the relevant guidelines and regulations of Institutional Animal Care and Use Committee of Faculty of Veterinary Medicine, University of Sadat City. This study followed ARRIVE guidelines on the use of experimental animals. Throughout the study, all efforts were exerted to minimize dogs' stress. Upon completion of the study, animals were transported to appropriate shelter of dogs.

Experiment Design

This study was an experimental, blinded study. In the present study, each of the studied dogs (3 dogs) was subjected to 4 full-thickness skin defects (circular /3 cm in diameter) after accomplishment of general anesthesia. For this, studied dogs were premedicated with xylazine HCl (Xyla-Ject® 2%, Adwia Co., Egypt) given intravenously at a dose of 1 mg/kg. Anesthesia was induced later by intravenous injection of ketamine HCl (Sigmatic, Egypt) at a dose of 10 mg/kg. To maintain surgical anesthesia, intermittent boluses of ketamine HCl were given as needed.[21]

After complete aseptic preparation of the proposed area, in each of the studied dogs, the four circular full-thickness skin wounds were performed on the thoracic region; two wounds on the left side and two on the right side using a sterile template. After adequate hemostasis, each wound was subsequently treated with one of the four tested treatments in this study. Hence, four groups were evaluated as follows:

- Control group: Full-thickness skin defect located caudally at the left side of the thoracic region was treated with conventional treatment (local daily dressing with povidone-iodine antiseptic solution (Betadine® 10%, Mundipharma, Germany) and application of sodium fusidate cream (Fucidin®, Minapharm, Egypt) twice daily.[8]
- PRP group: Full-thickness skin defect located cranially at the left side of the thoracic region was treated with single subcutaneous infiltration of inactivated (no exposure to physical or chemical activation methods) autologous PRP.
- Laser group: Full-thickness skin defect located caudally at the right side of the thoracic region was treated with the laser for one session.
- PRP/laser group: Full-thickness skin defect located cranially at the right side of the thoracic region was treated with single subcutaneous infiltration of autologous PRP directly followed by laser for one session.

For PRP and PRP/laser groups: Autologous PRP was obtained using the double spin method as previously described.[8,22,23] Briefly, in a ten ml vacuum tube containing one 1 ml of sodium citrate (3.8% solution), about 9 ml of each animal's venous whole blood (WB) (obtained from the left jugular vein) was added. The citrated WB was exposed to soft spin at 250 g for 10 min from which three layers were obtained using a tabletop centrifuge (sigma 3 - 01k, Germany). The top two layers were collected and exposed to hard spin at 2000 g for 10 min. After the second spin, the upper two-thirds portion recognized as PPP (platelets-poor plasma) was discarded softly and then about 1.5 to 2 ml PRP was collected. The prepared inactivated autologous PRP (total dose of 3 ml) was injected only at day 0 with subcutaneous infiltration at the induced wounds (margins and over the wounded area) (Figure 1).

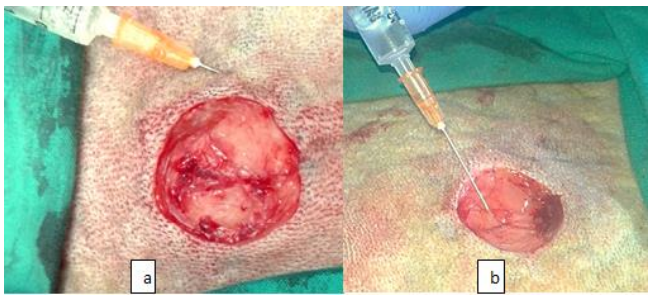

Figure 1. Subcutaneous infiltration of autologous PRP at the margins of full-thickness skin defect (a) and at the center of the wound (b).

Figure 2. Laser application on a full-thickness skin defect.

For Laser and PRP/laser groups: A short-wave infrared 808 nm diode laser (MLL-III-808/1~2500mW®, Changchun New Industries Optoelectronics Technology Co., Ltd., China) was used with the following stimulation parameters: the power of 5.8 W/cm^2 and a spot size of around 5.6 mm^2. The laser was applied to the corresponding wound groups (at margins and over the wounded area) for 5 seconds at a constant distance of 20 cm for only one session on day 0.[24] (Figure 2).

In all groups, postoperative analgesia was provided using meloxicam for three subsequent days.

Clinical Evaluation

All studied groups were clinically evaluated (for 21 days) through determination of the percentage of wound size. For this, digital photographs were taken for all groups in presence of a metal ruler near the wounds at 0, 7, 14 and 21 days and analyzed using Digimizer image analysis software (Version 5.4.7© 2005-2021 Med-Calc Software Ltd). Based on the data obtained from Digimizer, the percentage of wound size on the specified days was calculated using the following equation:

$$\text{Percentage of the wound size on the day (x)} = \frac{\text{Wound size at the day (x)mm}^2}{\text{Wound size at the day (0)mm}^2} \times 100$$

For blinding, an evaluation was conducted by an assessor who was unaware of the treatment given.

Biochemical Evaluation (Antioxidant/Oxidant Biomarkers)

Assessment of catalase activity. For determination of catalase activity, catalase (CAT) reacted with a known quantity of (H_2O_2) and the reaction was stopped after 1 min with a catalase inhibitor. In the presence of peroxidase, the remaining (H_2O_2) reacted with 3,5-Dichloro-2-hydroxybenzene sulfonic acid, and 4-aminophenazone to form a chromophore with a color intensity inversely proportional to the amount of catalase in the sample. The absorbance was measured at 510 nm.

Assessment of lipid peroxidation. Malondialdehyde (MDA) concentration was used as the index of lipid peroxidation.[25] The concentration of MDA was determined by measuring the thiobarbituric acid reactive species. The absorbance of the resultant pink product was measured at 534 nm.

Both catalase activity and MDA concentration were evaluated in wound fluid samples at 0, 7, 14, and 21 days. A typical wound fluid sampling method was done at wounds as previously defined.[8] Briefly, washing of the skin wounds was performed using sterile water followed by the application of an occlusive dressing over the wound. The exudate that accumulated under the dressing after 30 minutes to 1 hour was recovered by washing with 1 ml of saline. Afterward, the collected wound fluid samples were centrifuged at 14000 g for 10 minutes. Aliquots were made from samples and stored at -80 °C until analyzed.

Molecular Evaluation (Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) of Vascular Endothelial Growth Factor A (VEGFA) and Collagen I Alpha 2 (COL1A2) Genes)

Total RNA in skin biopsies (on 0 day (for the excised skin disc) and 7, 14, and 21 days) were extracted using QIAamp RNA mini kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Total RNA purity and concentration were obtained using a nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany). The isolated RNA was used for cDNA synthesis using reverse transcriptase (Fermentas, EU). Real-time PCR (qPCR) was performed in a total volume of 20-µl using a mixture of 1 µl cDNA, 0.5 mM of each primer (Table 1), iQ SYBR Green Premix (Bio-Rad 170-880, USA). PCR amplification and analysis were achieved using Bio-Rad iCycler thermal cycler and the MyiQ real-time PCR detection system. Each assay includes triplicate samples for each tested cDNAs and no-template negative control, the expression relative to the control is calculated using the equation 2-ΔΔCT.[26]

Table 1. Primer sequences of reference, VEGFA, COL1A2 genes of *Canis lupus* familiars.

Target genes	Accession no.	Sequence (5' to 3')	Product size
GAPDH (Reference gene)	XM_038448971.1	F: 5'- ATGGGCGTGAACCATGAGAA -3' R: 5'-CAGTGGAAGCAGGGATGATGT-3	238bp
VEGFA	NM_001003175.2	F: 5'-TCTGACTAGGAGTTCGGGGA-3' R: 5'-CCCTTCCTCCACCAATGTCT-3'	214bp
COL1A2	NM_001003187.1	F: 5' CGGTCTCAGAGGCGAAATTG 3' R: 5' CTCCCTTAGCACCAGGTTGA 3'	239bp

Histopathology and Immunohistochemistry

For histopathologic examination, tissue biopsies were taken from the corners of each wound (on days 7, 14, and 21) and fixed in 10% neutral buffered formalin. Tissues were dehydrated using ascending grades of ethanol, cleared in xylene, embedded in paraffin, sectioned by rotatory microtome, and stained with hematoxylin and eosin stain.[27] Tissue sections were examined using an Olympus BX43 light microscope and photographed using an Olympus DP27 camera.

Immunohistochemistry of Tumor necrosis factor-α (TNF-α) and nuclear factor κβ (NF-κβ) was performed in Paraffin-embedded tissue sections (tissue biopsy taken on day 7). Citrate buffer (pH = 6) was used for antigen retrieval. Primary antibodies against TNF-α (sc-52746, Santa Cruz, USA) and NF-κβ (sc-8008, Santa Cruz, USA) were applied to slides followed by the secondary (HRP) labeled antibody according to manufacturer protocol (Universal poly HRP DAB kit for mouse and rabbit, Genemed, Sakura, USA). Primary antibodies were not applied in negative control slides. The area percentage of positive NF-κβ and TNF-α was measured in tissue biopsies of day 7 using Image J software (National Institutes of Health, Maryland, USA). For obtaining tissue biopsies (dissected by scalpel) for molecular, histopathology, and immunohistochemistry assessments, the studied dogs were generally anesthetized.

Statistical Analysis

Statistical analysis was performed with SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). Different variables were analyzed using a one-way analysis of variance (ANOVA) with Tukey's test to compare groups at each assessment time. Results were expressed as a mean ± SE. The level of significance was set at $p < 0.05$.

Results

Throughout the entire observation period, there was a gradual decline in the percentage of wound size in all treatment groups (control, PRP, laser, and PRP/laser groups) with being significantly higher in the PRP, laser, and PRP/laser groups compared to the control group. From 7 up to 21 days, the percentage of wound size was significantly different between PRP, laser, and PRP/laser groups. At 7 and 21 days, the greatest reduction in the percentage of wound size was detected in the PRP group while at 14 days, the greatest reduction was evident in PRP/laser group (Figures 3 and 4).

The activity of the catalase enzyme was significantly different among all groups from day 0 till day 21. During this period, it was significantly higher in PRP, laser, and PRP/laser groups compared to the control group. Further, in the PRP group, it was significantly higher compared to the laser and PRP/laser groups from day 0 till day 14. In PRP/laser group, it was also significantly higher compared to PRP and laser groups on day 21 (Table 2).

Regarding the concentration of MDA, it was significantly lower in PRP and PRP/laser groups compared with control and laser groups from day 0 up to day 21. From day 7 till day 21, its concentration was lower in the PRP group compared with all other groups (control, laser, and PRP/laser groups). At some time points, a significant difference was detected between the laser and control groups (Table 2).

At day 0, there was no significant difference in gene expression of VEGFA between groups while significantly greater expression was demonstrated in PRP and PRP/laser groups relative to control and laser groups from day 7 till day 21. At 7 days and 14 days, gene expression of VEGFA was also significantly higher in PRP compared to PRP/laser group. A significant difference between the laser and control groups was only detected at 21 days (Table 2).

The expression of the COLIA2 gene did not vary significantly between groups at day 0. From 7 days to 21 days, gene expression was significantly higher in PRP and PRP/laser groups relative to control and laser groups. At 7 days, a significant difference was detected between the laser and control groups. At 7 days and 14 days, the PRP group showed significantly higher expression of the COLIA2 gene compared to PRP/laser group (Table 2).

Microscopy of the skin biopsy after 7 days revealed marked polymorphonuclear leukocytic cells (PMNL) infiltration with no evidence of re-epithelization in the control wound group (Figure 5a). However, the PMNL infiltration was reduced and re-epithelization and granulation tissue formation was observed in the PRP wound group (Figure 5b). Severe PMNL infiltration was seen in the laser wound group (Figure 5c). Re-epithelization and granulation tissue formation were seen in PRP/laser wound group (Figure 5d).

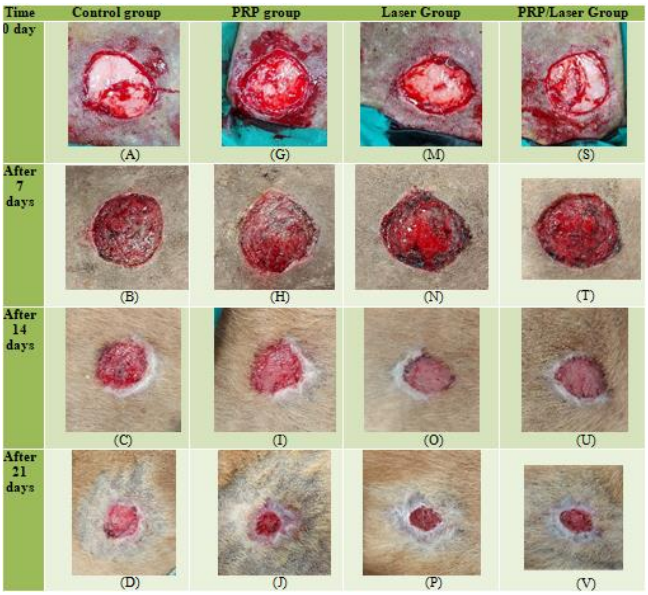

Figure 3. Digital photographs of induced full-thickness skin defect of the control group (A to D), PRP group (G to J), Laser group (M to P), and PRP/Laser group (S to V).

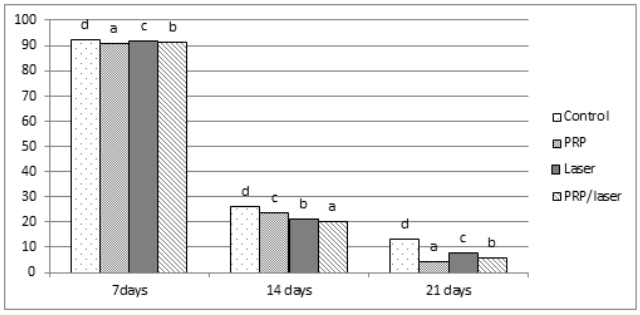

Figure 4. The percentage of wound size in control, PRP, laser, and PRP/laser groups. For each time point, values with different superscripts (a, b, c, and d) are statistically different ($p \leq 0.05$).

Histopathologic evaluation of the skin at the site of the wound after 14 days revealed an increased thickness of the epidermis at the cut edges, moderate PMNL infiltration in the dermis, and marked fibroblast migration in the control wound group (Figure 6a). On the other hand, The PMNL infiltration was minimal and the granulation tissue and neoangiogenesis were well observed in PRP wound group (Figure 6b). No sign of re-epithelization, marked PMNL cell infiltration and fibrous connective tissue formation was observed in the laser wound group (Figure 6c). However, the wound treated with laser and PRP (PRP/laser wound group) revealed marked PMNL infiltration, epithelial migration, and moderate granulation tissue formation (Figure 6d).

Microscopy of the skin biopsy after 21 days showed epithelial migration, moderate PMNL infiltration, and marked granulation tissue formation in the control wound group (Figure 7a). The PRP group revealed epithelium bridging the wound (prominent epithelization), minimal PMNL infiltration, and well-formed granulation tissue (Figure 7b). The laser wound group showed epithelial migration, moderate PMNL infiltration, and well-formed granulation tissue (Figure 7c). The wounds of PRP/laser showed re-epithelization, minimal PMNL infiltration, and fibrous connective tissue formation (Figure 7d).

TNF-α was moderately expressed in the leukocytes in the control wound group while it was mildly expressed in the leukocytes in PRP wound group at day 7. High expression was observed in leukocytes in the laser wound group and moderate TNF-α expression in leukocytes infiltrating wounds of the PRP/laser group (Figure 8). Also on day 7, the expression of NF-κβ was marked in cells infiltrating wounds of the control group. It was also expressed in keratinocytes in wounds of the PRP group. On the other hand, in wounds of the laser group, NF-κβ expression was restricted to a few leukocytes. In wounds of the PRP/laser group, moderate NF-κβ expression was observed in keratinocytes and cells infiltrating wounds (Figure 9).

Table 2. Activity of catalase enzyme, concentration of malondialdehyde and gene expression of vascular endothelial growth factor A and collagen I alpha 2 in control, PRP, laser and PRP/laser groups.

Variables	Groups	Time			
		Day 0	7days	14 days	21 days
Activity of catalase enzyme (U/l)	Control	35.1 ±1.9^{a}	29.5±2.9^{a}	27.7±1.5^{a}	19.7±1.5^{a}
	PRP	45.2 ±1.5^{d}	59.5±2.9^{d}	56.0±2.9^{d}	31.7±1.5^{c}
	Laser	38.5 ±2.9^{b}	35.5±2.9^{b}	31.8±1.7^{b}	24.7±1.5^{b}
	PRP/laser	41.0 ±2.8^{c}	48.8±1.7^{c}	43.5±1.7^{c}	37.8±1.7^{d}
Concentration of malondialdehyde (mM/gm)	Control	61.7±1.7^{b}	77.3±1.5^{d}	49.7±1.5c	36±2c
	PRP	55.3±1.5^{a}	49±0.6^{a}	24.3±0.7a	17.7±1.5a
	Laser	62.3±1.5b	61±1^{c}	47.7±1.5c	42.3±1.5d
	PRP/laser	56.3±0.7^{a}	55±1.2^{b}	32.7±1.5^{b}	29±2b
Gene expression of vascular endothelial growth factor A	Control	1±0.0^{a}	1±0.0^{a}	1±0.0^{a}	1±0.0^{b}
	PRP	1.2±0.08^{a}	2.3±0.1c	4.8±0.4^{c}	1.2±0.1^{c}
	Laser	1.1±0.03^{a}	0.8±0.05a	0.6±0.05^{a}	0.3±0.05^{a}
	PRP/laser	1.1±0.06^{a}	1.9±0.03^{b}	2.7±0.1^{b}	1.2±0.03^{c}
Gene expression of collagen I alpha 2 genes	Control	1±0.0^{a}	1±0.0^{a}	1±0.0^{a}	1±0.0^{a}
	PRP	1.1±0.05^{a}	11.5±0.2^{d}	8±0.2^{c}	3.5±0.2^{b}
	Laser	1±0.03^{a}	2.4±0.2^{b}	0.8±0.1^{a}	0.7±0.05^{a}
	PRP/laser	1.1±0.05^{a}	4.5±0.2^{c}	4.8±0.1^{b}	3.5±0.03^{b}

Values are presented as mean ± SE. Different superscripts (a, b, c, and d) in the same cell are statistically different ($p \leq 0.05$).

Regarding the area percent of TNF-α and NF-κβ staining, at day 7, the area percent of TNF-α was significantly lower in PRP and PRP/laser groups compared to other groups while no significant difference was detected between PRP and PRP/laser groups. Meanwhile, on day 7, the area percent of NF-κβ was significantly lower in the laser group compared to other groups. No significant changes were detected between the control, PRP, and PRP/laser group (Figure 10).

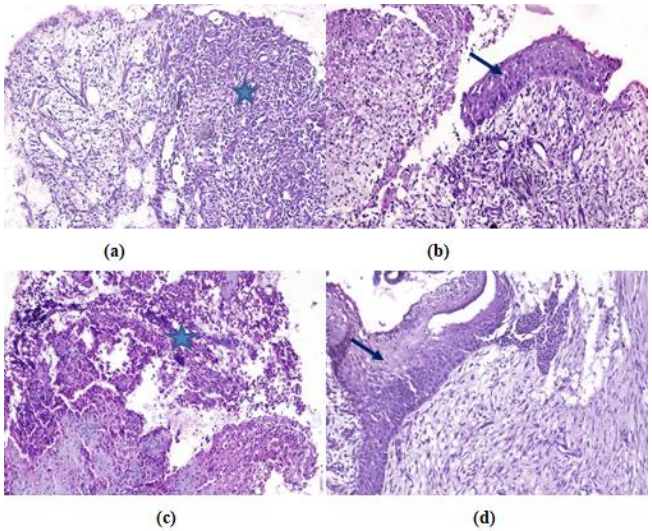

Figure 5. Histopathologic findings of skin wound of a dog at 7 days (H&E stain): (a) marked inflammatory cells (Star) and fibroblasts infiltration in control wound (×200), (b) re-epithelization (arrow) and few inflammatory cells infiltration in PRP treated wound (×200), (c) severe polymorphonuclear leukocytes infiltration (Star) in the laser-treated wound (×100) and (d) re-epithelization (arrow) and granulation tissue formation in PRP/laser-treated wound (×200).

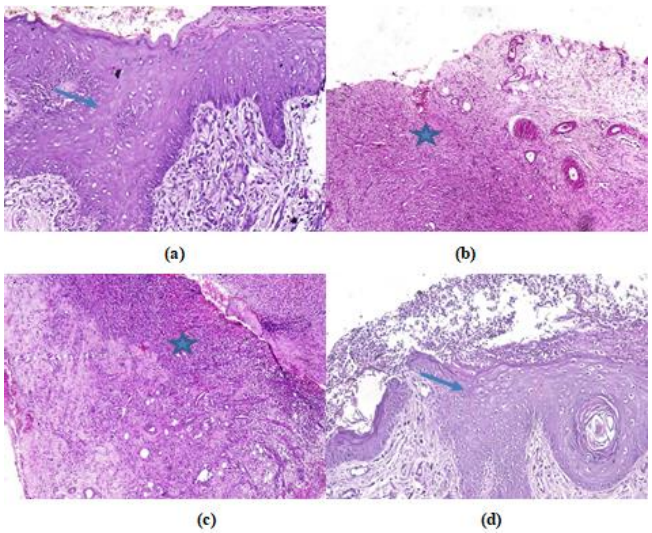

Figure 6. Histopathologic findings of skin wound of a dog at 14 days (H&E stain): (a) increased thickness of the cut edges of the epidermis (arrow) and moderate PMNL cells infiltration in the dermis in control untreated wound (×200), (b) granulation tissue (star) and neoangiogenesis in PRP treated wound (×100), (c) severe polymorphonuclear leukocytes infiltration and fibrosis in the laser-treated group (×100), and (d) re-epithelization (arrow) and granulation tissue in PRP and laser treated wound (×200).

Discussion

Under the conditions of the present study, a greater reduction in the percentage of wound size and consequently accelerated healing were evident in PRP, laser, and PRP/laser groups compared to the control

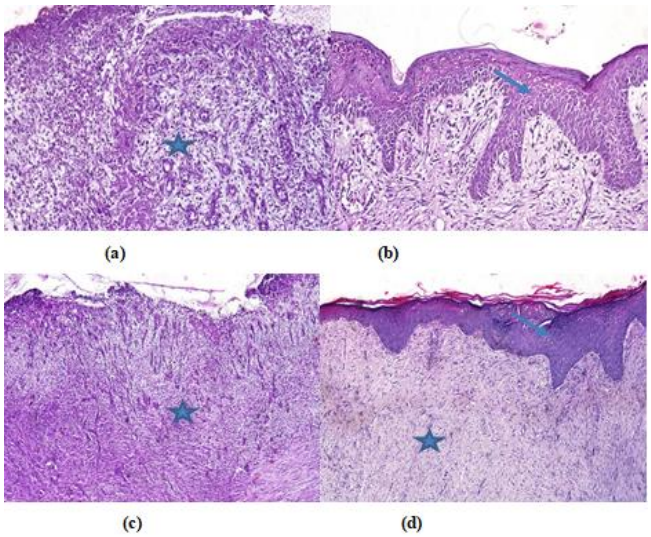

Figure 7. Histopathologic findings of skin wound of a dog at 21 days: (a) granulation tissue formation (Star) and inflammatory cells infiltration in control untreated wound, (b) re-epithelization (arrow) and granulation tissue underneath in PRP treated the wound, (c) granulation tissue (star) and polymorphonuclear leukocytes infiltration in the laser-treated group, and (d) fibrous connective tissue formation (star) and re-epithelization (arrow) in PRP and laser-treated wound. (H&E stain, ×100).

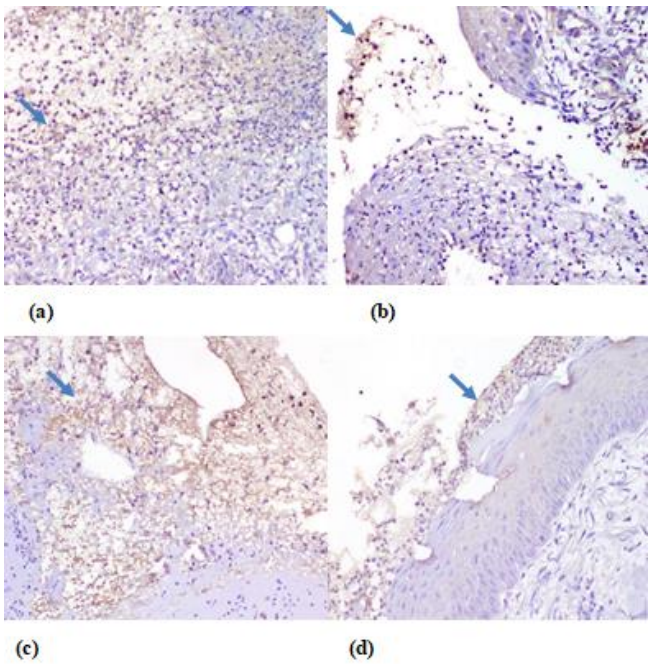

Figure 8. Immunohistochemical findings of tumor necrosis factor-α (TNF-α) expression in skin wounds at 7 days: (a) moderate expression of TNF-α (arrow) in the leukocytes in the control untreated wound, (b) mild expression of TNF-α (arrow) in the leukocytes in PRP treated the wound, (c) High expression (arrow) in leukocytes in the laser-treated group, and (d) moderate expression of TNF-α (arrow) in leukocytes infiltrating PRP and laser-treated wound. TNF-α Immunoperoxidase and hematoxylin counterstain, ×400.

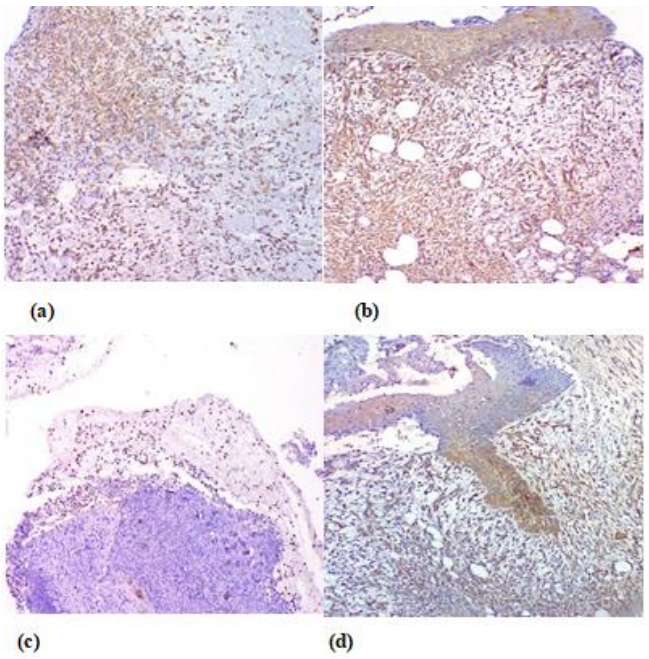

Figure 9. Immunohistochemical findings of nuclear factor-kappa Beta (NF-κβ) expression in the skin wound of a dog at 7 days: (a) marked NF-κβ expression in cells infiltrating the wound in control untreated wound, (b) in keratinocytes in PRP-treated wound, (c) in few leukocytes in the laser-treated group, and (d) moderate NF-κβ expression in keratinocytes and cells infiltrating PRP and laser-treated wound. NF-κβ Immunoperoxidase and hematoxylin counterstain, ×200

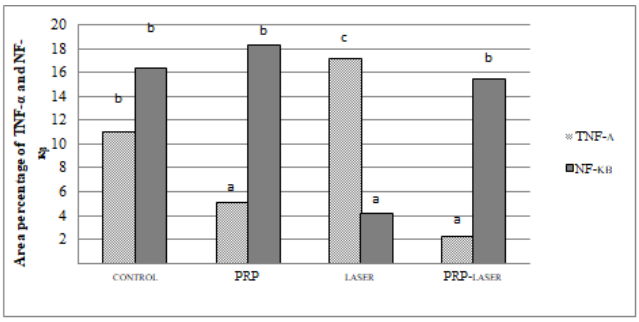

Figure 10. The area percent of tumor necrosis factor-α (TNF-α) and nuclear factor-kappa Beta (NF-KB) in control, PRP, laser, and PRP/laser groups. For each variable among groups, values with different superscripts (a, b, c, and d) are statistically different ($p \leq 0.05$).

group. Concerning PRP, these results could denote that using inactivated PRP did not prevent the release of growth factors and other bioactive components from platelets and their subsequent stimulating effect on wound healing. For this, we postulated that these components might be partly released from platelets by mechanical activation during wound infiltration. We also postulated that intrinsic activation that occurs naturally during the physiological wound-healing process,[28] might be a contributing factor. In agreement with our results regarding PRP, in dogs,[8] and mice,[29] with acute full-thickness skin wounds, wound size was greatly reduced in the PRP group compared to the control group. Despite this, in the previous study in dogs,[8] this difference was only significant at day 14 while in our study it lasted longer as it was evident from day 7 up to day 21. Further, in the present study, a single dose of inactivated PRP was used compared to multiple doses of activated PRP in the other study.[8] Better results in our study might be attributed to a better regenerative effect of inactivated injectable PRP compared to the activated one. This assumption could be supported by the reported ability of endogenous activation of PRP (via contact with elements of injured endothelium such as collagen and endothelial cells) to result in a slower release of bioactive proteins that better benefits wound healing.[30-32] Better results in our study might be also attributed to the injection of inactivated PRP subcutaneously at wound margins and over the wounded area not only at wound margins as in aforementioned study.[8] This application method might benefit wound healing by inducing a better distribution of PRP and the associated growth factors and increasing the number of exposed cells in situ.

For laser group, laser has been applied for only one session. Unlike this, in previously related reports in both dogs and horses,[13,16] laser has been repeatedly applied for several sessions. We selected this approach to investigate whether single application of laser would have a beneficial effect on wound healing in dogs in similarity to the previous findings in rat model.[33]

Greater reduction in wound size and accelerated healing in the laser group compared to the control group is consistent with a previously related report,[16] in which low-level laser irradiation (at 8 J/cm^2 applied once daily for 7 days) has accelerated healing of surgical skin wounds in dogs. Likewise, skin wounds with tissue loss in both dogs and cats have shown faster closure rates following laser therapy (4-20 J/cm^2).[17] Photobiomodulation therapy produced by low-level laser irradiation (830 nm applied once at 3-4.2 J/cm^2) has similarly improved wound healing in mice.[34,35] Despite the efficacy of laser in accelerating wound healing in our study, it seemed less efficacious compared to PRP (greater reduction in wound size). Consistently, PRP has been suggested to be a superior treatment to laser due to its ability to induce marked expression of cyclin A and cyclin-dependent kinase 4 proteins,[36] for their role in initiating proliferation and migration of dermal fibroblasts during wound-healing phases.[37]

In PRP/laser group, no synergistic effect was elaborated by combining PRP and laser. This might be caused by slightly higher stimulation of different cells to a degree that negatively limits their biological effects. This assumption might be supported by a previous report where it was reported that excessive effect associated with an excessively high platelet concentration could potentially inhibit the healing process.[38]

Catalase can modulate organelle's antioxidative stress system in intact cells.[39] Results revealed that catalase activity was significantly higher in the PRP group

compared to the control group. On contrary, in a previous study,[8] no significant difference in catalase activity was detected between control and PRP-treated wounds in dogs. In the laser group, catalase activity tended to be significantly higher compared to the control group. This finding might partly explain accelerated healing in this group compared to the control group due to lower oxidative stress possibly presented in wounds of the laser group. Comparing laser with PRP, higher catalase activity was observed in the PRP group. This could further explain accelerated healing in the PRP group relative to both control and laser groups.

Lipid peroxidation induced by reactive oxygen species results in the formation of several end-products including MDA. Therefore, MDA can be considered a marker for free radicals that potentially inhibit the migration and proliferation of different cells at the wound site.[40,41] The concentration of MDA was significantly lower in the PRP group relative to the control group at most time points. Similar findings were reported in a previous study.[8] Its concentration also tended to be significantly lower in PRP/laser group compared to the control group. Decreased concentration of MDA in both PRP and PRP/laser groups relative to the control group might refer to some antioxidant properties for these therapies and might partly explain accelerated healing in these groups compared to the control. Also, in the PRP group, the concentration of MDA tended to be lower at some points compared to PRP/laser group. This could denote a higher degree of lipid peroxidation and the presence of a less favorable microenvironment for the healing process in this group compared to the PRP group at some time points which might refer to one of the factors that limit a synergistic action to be evident in PRP/laser group. This might also partly explain slower healing in PRP/laser group than PRP group.

Gene expression of VEGFA was determined in this study as an indicator of the angiogenic effect of tested treatments "5". Higher expression of VEGFA was detected in the PRP group compared to the control group. Similarly, enhanced secretion of VEGF has been demonstrated in PRP-treated wounds compared to control wounds in mice.[29] Elevated expression of the VEGFA gene in the PRP group could be attributed to the availability of this growth factor and PDGF in the used platelets.[5] The role of PDGF in increasing the expression of VEGF could be explained by its role in promoting the proliferation and migration of epidermal keratinocytes, in which expression of VEGF is up-regulated in both early and later stages of wound healing.[42,43]

The results of the present study revealed significantly higher expression of the COLIA2 gene in the PRP group relative to the control group. In the same way, in a previous study in dogs,[8] the expression of COLIA2 was higher in PRP-treated wounds compared to control wounds. The expression of the COLIA2 gene tended also to be higher in PRP/laser group compared to the control group and in both PRP and PRP/laser groups relative to the laser group. These findings might indicate greater maturation and better quality of regenerated tissues in PRP and PRP/laser groups whereas replacement of type III collagen by type I collagen is one of the main features of the remodeling phase of wound healing.[43]

Skin biopsies at different time points revealed lower PMNL cells infiltrating wounds of the PRP group compared to the control group. Similarly, in another study, PMNLs were less observed in the PRP-treated wounds compared to control wounds.[8] Lower PMNL cells in the PRP group could denote a reduced inflammatory response in this group as well as platelets associated anti-inflammatory effects.[45] In contrary to the PRP group, greater PMNL cells and subsequently greater inflammatory response were evident in the laser group compared to the control group. This might support the previous assumption of greater sensitivity of canine inflammatory cells to the energy emitted by low-level laser therapy.[46]

In the PRP group, granulation tissue was formed earlier compared to the control group. Consistently, PRP was effective in inducing similar results in mice.[47] On contrary to our findings, in a previous study in dogs, the presence of granulation tissue did not vary between PRP and control wounds.[48] Different results could be partly attributed to different methods of PRP application, subcutaneous injection in our study versus topical in the other study,[48] which might maintain different levels of growth factors over a certain time period. Differences might also be induced by using inactivated PRP in our study compared to activated one in the other study.[48] Different results might also be induced by prior administration of dexamethasone in the later study,[47] which might ameliorate the effect of PRP on wound healing. This assumption could be supported by glucocorticoids associated reduction in circulating monocyte levels and inhibition of procollagen synthesis in fibroblasts.[49,50]

Also in the PRP group, granulation tissue did not only appear earlier compared to the control group but it was also well-formed. The granulation tissue formation is dependent on angiogenesis and collagen synthesis. Earlier and organized formation of granulation tissue in the PRP group relative to the control group might be induced by greater expression of VEGF in PRP group considering the correlation between the activity and the level of VEGF and the amount of granulation tissue formed in animals.[51] On the other hand, in the laser group, the time for the appearance of granulation tissue was similar to that in the control group. Inconsistent with

these findings, in a previous study in dogs, there was no difference in the first appearance of granulation tissue between laser and control wounds.[46]

Concerning epithelization, histopathologic findings revealed more pronounced epithelization in the PRP group compared to the control group. Similar findings were demonstrated following PRP application on excised wounds in dogs,[10] and mice.[29] The positive impact of PRP on epithelization could be attributed to the elevated level of VEGF and earlier angiogenesis being detected in the PRP group compared to the control group. This could be based on the vital role of angiogenesis in providing a sufficient amount of oxygen and nutrients to the wound bed which is known to promote granulation tissue formation and re-epithelialization.[52] Another contributing factor for PRP-associated epithelization might be the reported ability of PRP to increase the expression of insulin-like growth factor-1 (IGF-1) that promotes epidermal cell proliferation.[29] Better epithelization in the PRP group might be further induced by the presence of a copious amount of transforming growth factor alpha (TGF-α) which persuades differentiation of suprabasal cells with subsequent stimulation of epidermal regeneration.[53] Another possible explanation might be the reported ability of PRP to significantly increase the activity and expression of matrix metalloproteinase-9 (MMP-9) which plays an essential role in re-epithelization during skin wound healing.[8]

Laser did not seem to improve epithelization. This was evidenced by no difference in epithelization between the laser and control groups. Consistently, in another study in dogs,[20] with surgical closed and open wounds (15 mm biopsy punch), epithelial healing did not differ between control wounds and those treated with low-level laser therapy (once daily for 5 days with a 980-nm laser and a total energy density of 5 J/cm2). Contrary to our findings, compared to control wounds, complete re-epithelization was demonstrated in equine distal limb skin wounds received low-level laser therapy (635 nm and energy output of 17 mW per diode applied for 5 minutes every other day for 80 days).[13] Different results might be caused by different stimulation parameters used in both studies as well as using different species that could have different skin properties and healing processes.[54] For PRP/laser group, epithelization was more prominent compared to control and laser groups. This could be mainly attributed to the active constituent of PRP.

In the current study, the area percent of TNF-α was significantly lower in PRP and PRP/laser groups relative to control and laser groups. Consistently, in rats with infected excisional wounds, TNF-α was significantly lower in PRP treated wounds relative to control wounds.[55] Reduced level of TNF-α in both PRP and PRP/laser groups could reveal a lower inflammatory response in wounds of these groups and consequently might be an important factor for enhanced healing in these groups relative to others. Such an assumption could be strengthened by the documented role of TNF-α in initiating the inflammatory phase,[56] and the ability of the intense inflammatory reaction to negatively impact wound healing.[57] Considering the previously reported role of TNF-α in promoting the recruitment of inflammatory leukocytes into the wounded tissues "55", elevated levels of TNF-α in both control and laser groups could be an important pathway for marked infiltration of PMNL cells in these groups.

The NF-κβ is a transcription factor that regulates the expression of multiple genes and cellular functions including migration and survival.[58] Further, NF-κβ pathway has been reported to modulate the expression of different growth mediators.[59] Hence, this factor was evaluated in our study to assist explanation of the results from different groups.

In the present study, the area percent of NF-κβ was significantly lower in the laser group compared to other groups (control, PRP, and PRP/laser groups). Based on this finding along with the documented role of enhanced activities of NF-κβ in accelerating wound healing,[60] accelerated healing in the laser group relative to the control group wasn't mediated by this factor. In the same way, the absence of a significant difference in the area percent of NF-κβ between each of the PRP and PRP/laser groups and the control group could also exclude the contribution of this factor in accelerated healing in both groups compared to the control group.

In conclusion, laser, PRP, and PRP/laser accelerated healing (greater reduction in wound size) of acute full-thickness skin wounds in dogs with a greater potential for PRP. PRP and PRP/laser seemed effective in improving healing quality considering histopathologic findings and expression of the collagen I alpha 2 gene with greater efficacy for PRP. The effect of PRP and PRP/laser on skin healing in dogs is mediated partly by favorable changes in the expression of TNF-α. Up-regulation of catalase enzyme activity is one of the pathways for accelerated healing following laser, PRP, and PRP/laser applications. Down-regulation of the concentration of MDA and greater expression of the VEGFA gene denote some mechanisms of accelerated healing following PRP and PRP/laser applications and even faster healing following PRP than PRP/laser. Due to accelerated and improved healing quality following PRP. The adopted PRP application method could be a promising method for management of acute full thickness skin defects in dogs.

Conflict of Interest

The authors declare they have no competing interest.

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