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The Effect of Autologous and Homologous Platelets-Rich Plasma Gel on Cutaneous Wound Healing In Rescued Donkeys: A Comparative Study

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BACKGROUND: Cutaneous wound healing in equine is frequently delayed and complicated. Therefore, a new therapy is needed to achieve rapid and satisfactory healing. **Aims:** This study aimed to investigate whether the use of autologous PRP gel in cutaneous wounds enhances wound repair and alters oxidative stress cascades than homologous PRP-gel in the rescued donkeys. **Methods**: 10 rescued donkeys were selected and allocated into two groups A and B. Group-A donkeys (n=5) were treated with APRP-gel, while group-B donkeys (n=5) were treated with HPRP-gel. A full-thickness (4 mm^2) skin wounds were selected in each donkey. Wound re-epithelialization was assessed by H&E staining and collagen re-establishment was assessed by Masson's trichrome staining. The CAT activity and MDA concentration were assessed in blood samples on days 7, 21, and 36. **Results**: We observed a significant increase in re-epithelialization, fibroblasts and angiogenesis from days 21 ($P < 0.05$) to 36 ($P < 0.01$) in APRP-wounds than HPRP-wounds. Collagen organization in APRP-wounds was good ($P<0.05$) from days 21 to 36 than HPRP-wounds. Malondialdehyde (MDA) concentrations in APRP-wounds were significantly decreased from day 21 to day 36 compared with HPRP-wounds ($P<0.01$). There was no difference in catalase (CAT) activity between APRP and HPRP wounds ($P>0.05$). **Conclusion**: APRP-wounds improved cutaneous wound healing in rescued donkeys by reducing oxidative stress, speeding wound epithelialization, and developing more structured tissue with interlocking collagen bundles than HPRP wounds. However, further ultrasonography study is required to investigate the effect of PRP gel on cutaneous wound healing in rescued donkeys.

Keywords: Cutaneous Wound Healing, Donkeys, Masson's trichrome, Oxidative Stress, Platelet-Rich Plasma Gel.

Introduction

Cutaneous wound healing is a physiological response that occurs when skin integrity is compromised [1-3]. A cascade of local responses generated by tissue injury initiates and orchestrates this complicated and dynamic response to physical trauma, which consists of three overlapping phases (inflammation, proliferation, and remodelling) [2,

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4- 6]. In equine, surgical or traumatic wounds take longer to heal, and the cost often restricts the wound's recovery. Hence, successful healing of cutaneous wounds requires the use of a combination of drugs. Platelets are a rich source of a complex group of growth factors (GFs) that are important for wound healing. In haemostasis, wound healing, and re-epithelialization, Platelets release a variety of growth factors that promote angiogenesis, vascular fibroblast proliferation, and collagen synthesis [1].

The PRP has been extensively studied as a new biological matrix able to promote wound healing across a variety of tissues, including skin, muscle, bone, cartilage, and even tendon lesions, with promising results [7]. PRP is a plasma component that contains 3-7 times as many platelets as whole blood [8-11]. It also holds supraphysiological growth factors GF [insulin-like growth factors I, II, epidermal GF, connective tissue GF, platelet derived GF, neural GF, vascular endothelium GF, hepatocyte GF, interleukin 8 (IL8), fibroblasts GF, and transformed GF] as well as histamine, serotonin, calcium, zinc, superoxide dismutase (SOD) and adenosine triphosphate (ATP). Because of these significant factors, it is effectively used in dermatology, mammalian reproduction and orthopaedics [8, 12, 13]. PRP can be made from the patient's own blood (autologous PRP) or from multiple donor blood (homologous PRP) [14]. Homologous-PRP from healthy, approved, routine blood donors has several advantages, including ease of preparation, platelet counts above therapeutic values, and the ability to secure a greater quantity at one time [15].

Oxidative stress results from an imbalance in the body's pro- and antioxidant defenses. The oxidative stress process raises the levels of reactive oxygen species (ROS) which are essential for wound healing. The low levels of ROS regulate many signal transduction pathways in cells as well as give phagocytes the energy to phagocytose bacteria [16, 17]. Although wound healing requires an adequate amount of active oxygen, active oxygen causes more adverse biological effects in many cases. High levels of ROS directly react with lipids, proteins, and DNA of cells, leading to cell death [18]. For instance, lipid peroxidation, or the generation of malondialdehyde (MDA), is an indication of ROS damage to cell membranes and organelles' plasma membranes [19]. In response to excessive oxidation, the body produces enzymatic antioxidants, of which catalase (CAT) is the most significant. Antioxidants can prevent oxidative damage and promote wound healing for wounds that are difficult to heal [20].

Despite the frequent use of PRP-related healing, there are limited data on the efficacy of autologous and homologous PRP gels in cutaneous wound healing. Moreover, there is a dire need to compare the efficacy of autologous and homologous PRP that has not been reported yet in rescued donkeys in Pakistan. Therefore, the aim of this study was to investigate whether the use of autologous PRP gel in wounds enhances wound repair and alters oxidative stress cascades compared to homologous PRP gel in the rescued donkeys.

Material and Methods

Ethical statement

All procedures for this study were approved and performed at the Department of Veterinary Surgery and Pet Science, University of Veterinary and Animal Sciences, Lahore, Pakistan, under Ethical Approval No. DR/458, as requested by the Ethical Review Board; Date: July 10, 2020.

Animals

A study was conducted on rescued donkeys with skin wounds in the Lahore region of Pakistan's Punjab province. Animals were housed during the experiment using the University indoor stables and the indoor stables of the Society for the Prevention of Cruelty to Animals in Lahore. During the trial period, donkeys were provided with adequate water, grass and concentrated feed.

Preparation of platelet-rich plasma (PRP) gel

The day before surgery, donkey platelet-rich plasma was made from two tubes containing 10 ml of donkey whole blood. Blood was collected using two Falcon tubes (15 ml each) containing 10% sodium citrate anticoagulant. Spin the tube at 300 g/10 m for 5 minutes in a swing centrifuge (SCILOGEX) to separate plasma red blood cells. The midzone is a thin layer of white blood cells that exists between these two layers and contains mostly white blood cells and the largest platelets. The upper section of each tube was then removed, and 500 uL of plasma was transferred to Tube A. This fraction was utilized in the production of autologous thrombin. The remaining plasma and intermediate region were transferred to a new tube labelled B for PRP. Incubate the tubes at room temperature. Fill tube A with 300 uL of 10% calcium gluconate, mix, and incubate at 37 °C for 15 minutes. The two tubes (A and B) were then

centrifuged at room temperature at 640 g/10m. Tube A, which contained thrombin-rich substrate, was fully utilized. After homogenization, add tube A thrombin to half the volume of tube B in a 2:1 ratio (2 mL PRP:1 mL thrombin). After 40 minutes at room temperature, PRP gels formed [1]. For the homologous PRP, the donkey was manually restrained and blood was obtained from the external jugular vein, which was processed according to the previously described methodology [1].

Experimental design and treatment

In this study, 10 donkeys were used, weighing between 200 and 250 kg and aged between 4 and 8 years. The animals were housed in the indoor stables of the Society for the Prevention of Cruelty to Animals in Lahore. All experimental animals had adequate water and dry food. Mineral salt was also provided to all the donkeys throughout the experiment. Donkeys were divided into two groups, each consisting of five animals: Group A (APRP-treated) and Group B (HPRP-treated). In group A (n=5), the wound of each animal was treated with autologous PRP gel, while in group B (n=5) animals were treated with homologous PRP gel.

Each full-thickness skin wound (4 mm^2) was depilated and shaved from the margins before being removed from the subcutaneous tissue with 25 cm/10 cm scissors (Noorani Surgical). After washing with sterile normal saline (NaCl 0.9%, Geofman), a thick layer of autologous PRP gel was applied to group A, then wrapped with sterile gauze and protected with a dressing. After two days, the bandage was removed, the area was washed with sterile saline, covered with PRP gel and bandaged. Treatments were given every four days until day 26. Semi-occlusive gauze was placed with APRP gel over the skin wound with a soft bandage to provide fresh air to prevent overgrowth of granulation tissue. After the first 26 days, the PRP gel was applied to the wound every 8 days. After washing with physiological sterile solution, a thick layer of homologous PRP gel was applied to the cutaneous wound in Group B animals, and the bandage was adapted using the same method as in Group A animals. The wounds were dressed with sterile non-adherent semi-occlusive gauze. The wounds were bandaged (Surgitex, Rehman Rainbow (PVT.) LTD.). For animal welfare, prophylactic systemic antibiotics such as Biocon 5gm Inj. containing benzyl penicillin, procaine penicillin, and streptomycin sulphate (Vetcon Pharma) were administered intramuscularly twice a day, and the donkeys were placed in hygienic stables with limited exercise and no anti-inflammatory drug administration during the course of the study.

Histomorphometric examination

H&E stain

All donkeys were sedated intravenously with Xylazine hydrochloride (Xylaz® Farvet Holland) at a dose rate of 1.1 mg/Kg prior to the procedures [21], and the biopsy sites were scrubbed with saline solution and gauze. Full-thickness specimens were collected using a 6-mm surgical biopsy punch (Kai medical® Japan). The wound was biopsied from a 4-to-5 mm skin edge and a 3-to-4mm intact skin portion. During the early stages, the sample was preserved for 24 hours in 10% neutral-buffered formalin. Later, the sample was changed to a 70% alcohol fixative. Following fixation in various concentrations of alcohol, tissues were embedded in paraffin after being cut into 1.5 mm slices. Moreover, tissue was stained by H & E for tissue morphology analysis using standard light microscopy techniques. Various semi-quantitative factors such as the extent of re-epithelialization, and vascularization were observed in the histopathology of biopsy samples. The semi-quantitative scoring system was used in this blind study [22]. According to the semi-quantitative system, zero denoted no vascularization, no fibroblast observation, no cutaneous epithelium formation, absence of fibroblastic cell, and one denoted enlargement of cutaneous epithelium depth and width, presence of limited fibroblastic cell, newly created vasculature, two denoted epithelial cell relocation, moderate numbers of fibroblastic cells, or recently created vasculature, and three denoted epithelial incision apposition, moderate numbers of fibroblastic cells, or recently created.

Masson's trichrome stain

Collagen fiber staining was achieved in the Laboratory of the Department of Pathology, University of Veterinary and Animal Sciences, Lahore. Staining was performed following the procedure developed by the Center for Musculoskeletal Research (CMSR) at the University of Rochester Medical Center. Tissues were deparaffinized and rehydrated. At 58 ˚C for 15 minutes, Bouin's Fixative (Fisher Scientific) was used. The slides were then cleaned in distilled water for 10 minutes after cooling. The biopsy tissue was then stained for 5 minutes with (C.I. 26905; C.I. 42685) Biebrich Scarlet Acid Fuchsin

(Fisher Scientific). The sample was then stained for 2 minutes with a 1 percent phosphomolybdenum-phosphotungstic acid solution (Fisher Scientific). Tissues were counterstained with Aniline blue solution (C.I. 42775) for 5 minutes before being rinsed with distilled water. Biopsied tissue samples were also washed with a 1 percent acetic acid aqueous solution. At the end, the slides were dried, cleansed of debris, and mounted. Observation and photomicrographs were obtained at each biopsy sampling to determine improvement in cutaneous wound healing and regenerative cell production. In the current study, a simple descriptive scale of 0-3 was used to assess collagen abundance and collagen organization for each feature in trichrome-stained slides. A sample with a score of 0 indicates a lack of collagen bundles or ordered collagen fibril formation. A score of 3 indicates that collagen fibers are sufficient and the collagen fibers are formed in an orderly manner. Comparable fluctuating collagen fibers with uniform blue color were more structured than collagen fibers with chromatic aberration and comparable fiber damage or infiltration.

Blood sampling

A blood sample of 10 mL was collected from the Juglar vein of each animal on days 7, 21, and 36, using a plain vacutainer. To analyze the blood samples, the samples were sent to the Department of Physiology, Faculty of Biosciences, University of Veterinary and Animal Sciences, Lahore, Pakistan. The blood samples were centrifuged at 3000 rpm for 15 minutes at 4 C with a temperature-controlled centrifuge (HARRIER 18/80 UK). The supernatant was separated and subsequently stored at -20 C for future analyses.

Oxidative stress examination

MDA concentration

Serum MDA concentration (µmol/mL) was measured according to the method described by Ohkawa et al. [23]. The reaction mixture was made from 375 l of 20.0% acetic acid (pH 3.5), 375 l of 0.8% thiobarbituric acid, and 50 l of 8.1% sodium dodecyl sulfate mixed with 100 l of plasma. The samples were heated for 1 hour at 95 °C and centrifuged for 10 minutes at 3000 g. Using an Epoch Reader Microplate spectrophotometer (UV-2800, Biotechnology Medical Services, USA), the absorbance was measured at 532 nm, and the MDA content was expressed in mol/mL (ε = 1.56 × 105 mmol/L/cm).

Analysis of CAT activity

According to the procedure described by Aebi, [24], the rate of degradation of substrate H_2O_2 evokes the catalytic activity of catalase. The rate of decomposition of hydrogen peroxide was measures by a drop absorbed at 240 nm every 30 seconds for 3 min. The CAT levels were measured as mmol/min. CAT activity was defined as the amount of catalase enzyme required to degrade 1 µmole of hydrogen peroxide per second at 25°C.

Statistical analysis

All data were statistically analyzed by two-ways ANOVA using Graph pad Prism (Version 7.04, Graph Pad Software Inc., San Diego, CA). All values were expressed as (Mean± SE). The level of statistical significance were ($P<0.01$) and ($P< 0.05$).

Results

Histomorphometric evaluation

Re-epithelization

The results of H&E staining indicated that APRP-treated wounds obtained on day 7 showed increased thickness of epithelium, presence of few fibroblasts, new blood vessels, while HPRP-treated wounds indicated limited epidermal differentiation, absence of fibroblast and angiogenesis (Fig. 1A, B). The amount of re-epithelization, drift fibroblast ($P<0.05$) and angiogenesis ($P<0.05$) were higher in the APRP-treated wounds compared with the HPRP-treated wounds (Fig. 2A, B, C).

On day 21, APRP-treated wound showed epidermal proliferation, kerato-hyaline granules were seen in many keratinocytes, and moderate number of fibroblasts and angiogenesis (Fig. 1C). In contrast, HPRP-treated wound showed undifferentiated keratinocyte, no kerato-hyaline granules, few fibroblasts and angiogenesis on day 21 (Fig. 1D). Re-epithelization ($P<0.05$), number of fibroblast ($P<0.05$) and angiogenesis ($P<0.01$) recordings were significantly increased in the APRP-treated wounds than the HPRP-treated wounds on day 21 (Fig. 2A, B, C).

On day 36 of wound healing, APRP-treated wound showed marked mature epithelial cells growth, excessive number of fibroblasts and new blood vessels formed, while HPRP-treated wound showed moderate number of fibroblasts, some dead tissue mass along with less re-epithelialization (Fig. 1E, F). Compared with HPRP wounds, all skin wounds healed, and APRP wounds had higher fibroblast numbers and angiogenesis ($P<0.01$) (Fig. 2A, B, C).

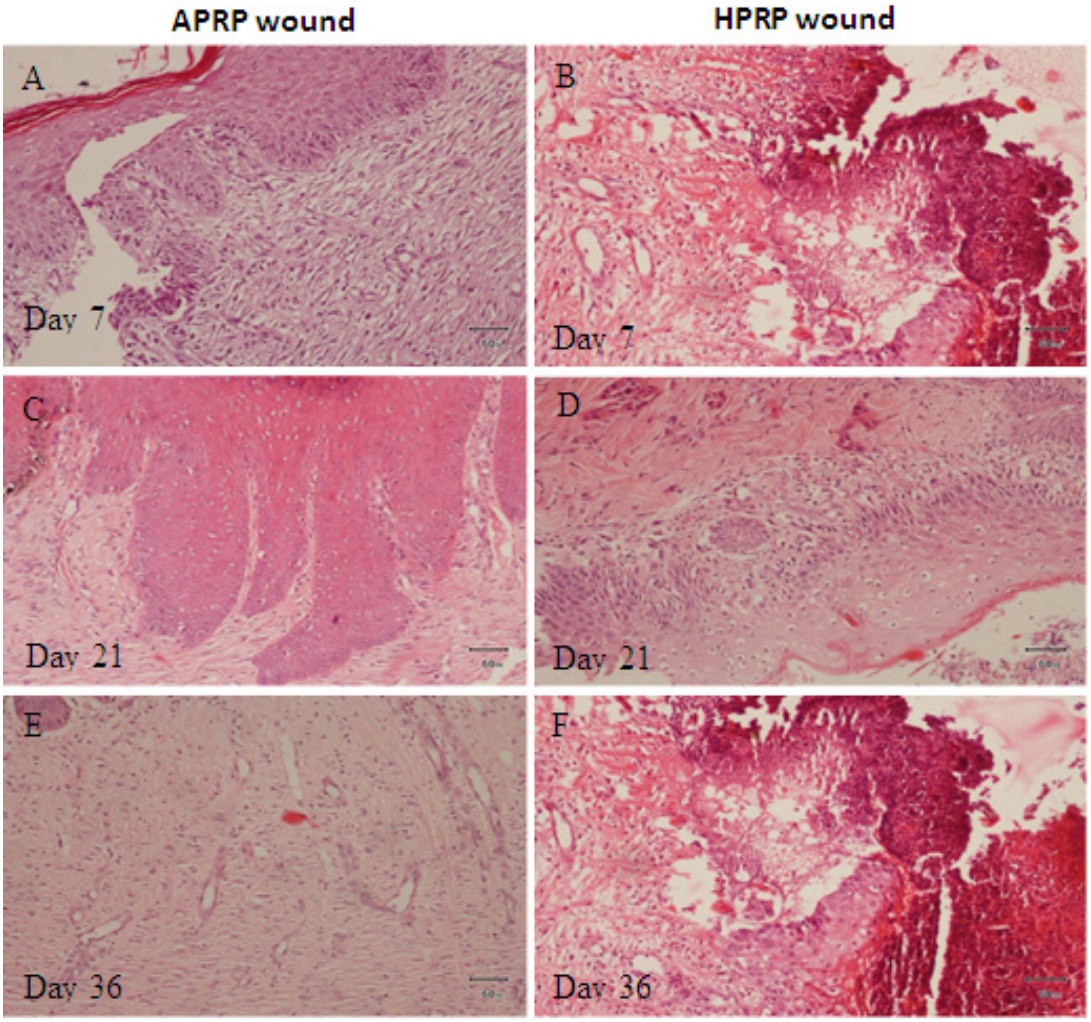

Fig. 1. H&E stains results of Re-epithelization in the cutaneous wound of both groups. Bar=50nm; A; APRP wound showed improved thickness of epithelium, existence of few fibroblasts, new blood vessels on day 7. B; HPRP wound on day 7 post treatment indicated limited epidermal differentiations, lack of fibroblast and blood vessels. C; APRP wound obtained on day 21 indicated epidermal proliferation, kerato-hyaline granules are seen in many keratinocytes, presence of moderate number of fibroblasts and angiogenesis. D; HPRP wound indicated undifferentiated keratinocyte, no kerato-hyaline granules, few fibroblasts present at day 21. E; APRP wound obtained on day 36 showed marked mature epithelial cells, excessive number of fibroblasts and new blood vessels formed. F; HPRP wound showed moderate number of fibroblasts, some dead tissue mass along with less re-epithelialization on day 36.

Collagen fibers

The results of Masson's trichrome staining pathological section indicated that the collagen fibers in APRP wounds increased significantly over time than HPRP wounds. At day 21 of wound healing, APRP wound showed minimal and organized collagen fiber, while HPRP wound showed disorganized collagen fibers (Fig. 3A, B). The amount and organization of collagen fibers significantly increased ($P<0.05$) in the APRP wounds than the HPRP wounds on day 21(Fig. 4A, B) ($P<0.05$). At 36 day of wound healing, collagen fibers were well organized also fibroblast perpendicular to the epidermis in APRP-treated wounds (Fig. 3C). In contrast, less dense and unorganized collagen fibers were found in HPRP wounds (Fig. 3D). Compared with HPRP wounds, the number ($P<0.01$) and organization ($P<0.05$) of collagen fibers were higher in APRP wounds than HPRP wounds (Fig. 4A, B).

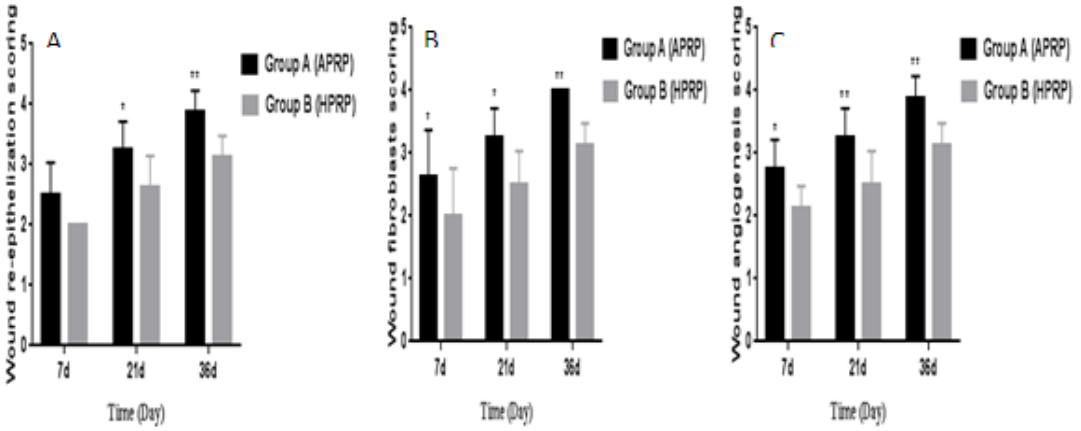

Fig. 2. Lesion score of wound healing in APRP and HPRP treated wounds (HE stains). A; Lesion score of wound re-epithelization; B; Lesion score of wound fibroblasts; C; Lesion score of wound angiogenesis; "" shows that the difference is more significant (P<0.01) between the PRP treatment group and the HPRP group; "*" indicates that the PRP treatment group has a significant difference compared with the HPRP group (P<0.05).**

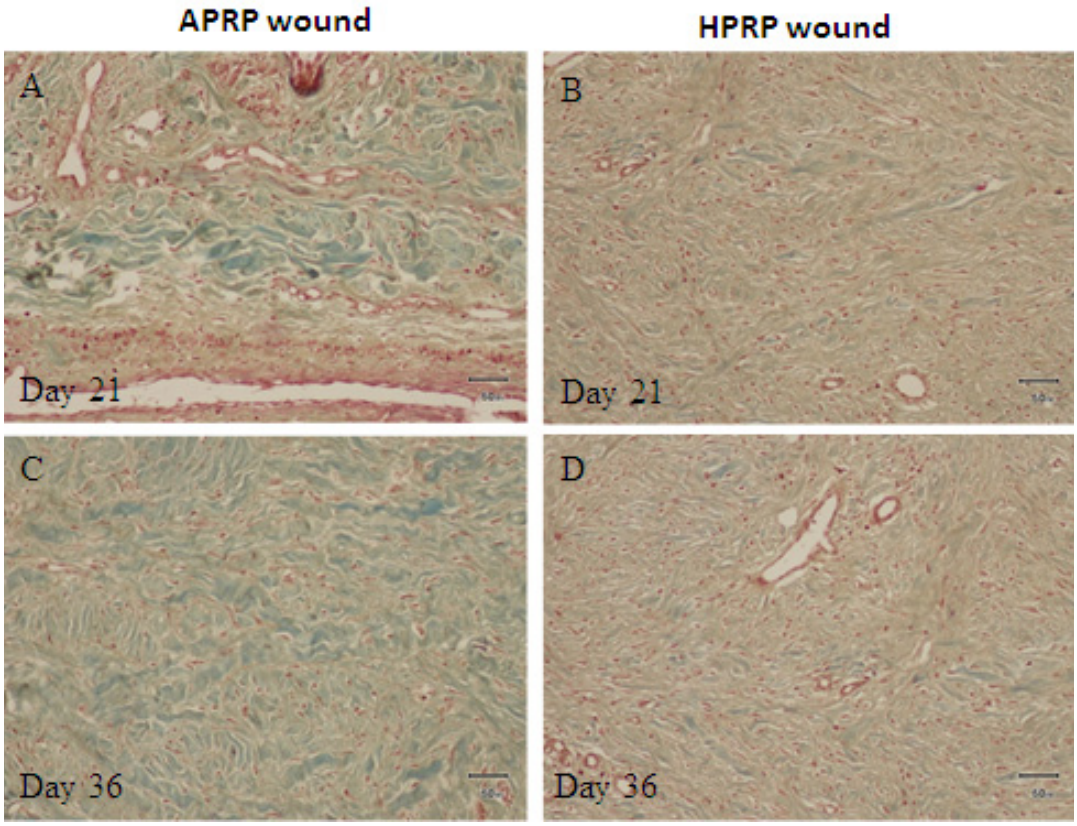

Fig. 3. Masson's trichrome stain results of collagen fiber in the cutaneous wound of both groups. Bar=50nm; A; APRP wound showed minimal and organized collagen fiber on day 21 post treatment. B; HPRP wound showed disorganized collagen fibers on day 21 C; APRP wound showed well organized collagen fibers also fibroblast perpendicular to the epidermis on day 36. D; HPRP wound showed less dense and unorganized collagen fibers on day 36.

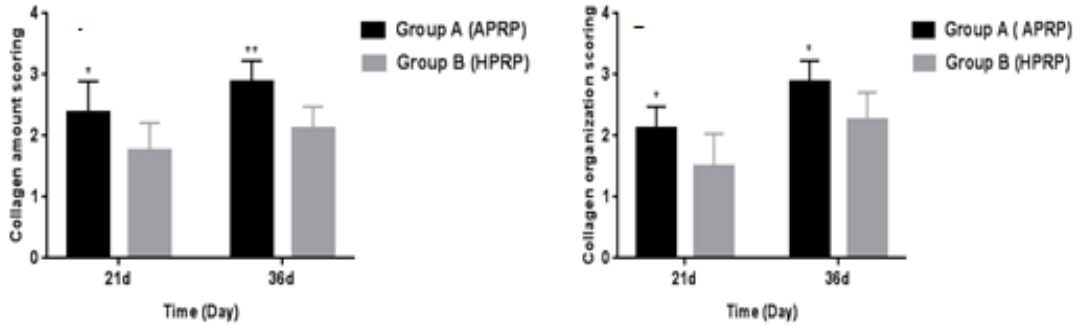

Fig. 4. Lesion score of collagen amount and organization of wound healing in both groups. A; Lesion score of collagen amount (MST stain) B; Lesion score of collagen organization (MST stain). "" Indicates that the difference between the APRP wounds and the HPRP wounds is more significant (P<0.01); "*" indicates that the APRP wounds has a significant difference than the HPRP wounds (P<0.05).**

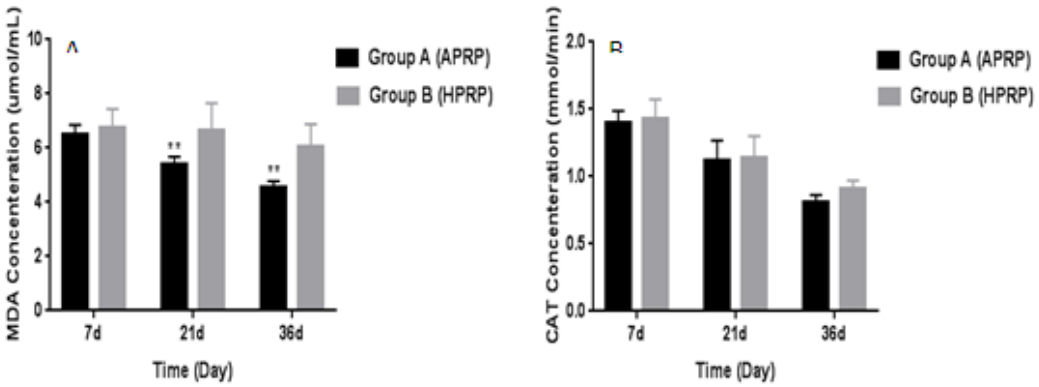

Fig. 5. Oxidative stress markers: A; shows MDA concentrations in APRP and HPRP wounds; B; indicates CAT activity in APRP and HPRP wounds. '' indicated the differences were significant (P<0.01).**

Oxidative stress markers

There was no significant difference (P>0.05) in CAT activity between the two groups at any points (Fig. 5B). In contrast, MDA was significantly decreased (P<0.01) in autologous PRP-treated wounds than the homologous PRP-treated wounds on day 21 and day 36 (Fig. 5A).

Discussion

The wound healing process involves a series of cellular and molecular events, as well as different cell types interacting for remodeling and wound healing [25]. According to Naude (2010), the healing process is divided into three stages: inflammation, proliferation and remodeling [26]. Ding and Tredget (2015) suggested that hemostasis may be specified as the initial stage preceding inflammation [27]. During the hemostatic phase, vasoconstriction, platelet release responses, clot formation, and release of proinflammatory cytokines and growth factors dominate [17]. An inflammatory phase follows, characterized by the infiltration of leukocytes, neutrophils, and macrophages, the latter responsible for phagocytosis of microorganisms and wound debridement [27]. During the proliferative phase, myofibroblasts undergo fibroproliferation, angiogenesis, granulation tissue formation, and wound contraction, overlapping with the previous phase [25]. According to Guo and DiPietro [17], the remodeling phase is the period when wound vascularization returns to normal, followed by collagen remodeling, an event that can last for several years.

Wang and Nirmala (2016) reported that the action of PRP may promote tissue healing by providing ample growth mediators (containing platelets and a fibrin matrix) as a natural support to progenitor cells, replacing tendon stem cells in deformities of tendon connective tissue [28]. Bottegoni et al. (2016) described the better effect of APRP as compared to HPRP in humans infected with osteoarthritis but they also described short time clinical by APRP may be due to its multiple growth factors that may be missing in HPRP. Moreover, as APRP contains antibodies from the same host this may be another factor in attracting immune cells at injury site and fasting the healing process [29]. This study reported that the thickness of epithelial cells increased in wounds treated with autologous PRP gel and all full-thickness cutaneous wounds healed with a significantly higher number of fibroblasts and newly established blood vessels compared to HPRP treated wounds. These results are in agreement with the studies of some authors [28, 29].

Collagen fibers are part of the supercellular extracellular network and serve as an underlying framework in tissues, directing cell proliferation and relocation during skin wound healing [30]. The increased wound elasticity that occurs at the fibroblast stage is associated with increased collagen levels within the wound [31]. During the healing process, collagen deposition is a clinically important factor in determining scar quality. Collagen is the most abundant component of the dermal matrix and plays a structural role because of its mechanical properties, which give tissues shape and structure [32]. DeRossi et al. (2009) observed that injuries treated with platelet-rich plasma (PRP) showed faster cellular differentiation in the epithelium and improved cutaneous collagen binding [1]. In the recent investigation, collagen fibers were arranged as thick, comparable, undulating bundles in case of treated with APRP gel cutaneous wounds, but less in HPRP-treated wounds, implying that APRP accelerated granulation tissue development which were consistent with the previous study described by Xue and Jackson [33]. According to Kumar et al. (2011), oxidative stress is defined as a shift in cellular oxidation-reduction reactions in which reactive oxygen species (ROS) are produced in excess and their removal is complicated by insufficient antioxidant enzyme activity [34]. Free radicals are produced during normal cellular function; however, upregulation and inadequate removal of free radicals can result in irreversible cell damage [35]. Recent research has shown that reactive oxygen species (ROS) are primarily correlated with wound healing and is also involved in different developmental phases of wound healing [36]. ROS-induced lipid peroxidation produces a variety of end products, including MDA. MDA is produced as an end product of lipid peroxidation by reactive oxygen species. MDA measures the degree of ROS degradation [30]. Sezer and Keskin (2014) suggest that MDA is one of the main biochemical markers for determining the degree of cellular damage in tissues [37]. In our study, there was a remarkable decline in MDA quantity in donkeys administered HPRP in contrast to the HPRP wound that were consistent with the previous study described by some researchers [38]. As a result, the findings of this recent study confirm that biomarkers involved in tissue oxidation significantly support platelets' role in the anti-inflammatory process during wound healing, repair, and restoration [39]. In contrast to HPRP wounds, CAT levels in APRP wounds found no significant reduction. This non-significant decrease in serum concentration antioxidant catalase (CAT) levels could be explained by increased CAT activity due to cutaneous wounds, which is similar to the findings described by Iuchi et al. [40].

Conclusion

Our study concluded that in comparison to HPRP-wound treatment, APRP gel-treated wounds accelerated skin wound healing in donkeys, increased wound epithelialization, and the formation of tissue with symmetrically arranged, interconnected collagen fibers of dermal collagen. The pathogenesis of wounds is aided by oxidative stress. Therefore, this study suggests that APRP gel be used as a safer treatment for cutaneous wounds in rescued donkeys. To further explore the effect of PRP gel on wound repair in rescued horses, an ultrasonographic analysis level study is required.

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Funding statement

This study has no funding.

Conflicts of Interest

None

Author's Contribution

Hamid Akbar, Muhammad Arif Khan and Muhammad Hassan Mushtaq conceptualized the hypothesis of this manuscript. Muhammad Talha Sajjad conducted the research. Muhammad Abid Hayat and Ghulam Mustafa statistically analysed the data. Muhammad Talha Sajjad performed the experiments. Muhammad Talha Sajjad wrote the manuscript. Hamid Akbar and Muhammad Abid Hayat critically reviewing and editing the manuscript. Muhammad Talha Sajjad and Shehla Gul Bokhari participated in the biochemical analysis and collect the data. All authors read and approved the final manuscript.

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