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The Potential Effect of Autologous Injectable Platelet Rich Fibrin (I-PRF) on Healing of Experimentally Induced Full-Thickness Skin Wound in Donkeys

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ABSTRACT

The aim of this study is to evaluate the potential effect of autologous I-PRF on healing of experimentally induced full-thickness skin wound in donkeys. Six adult healthy donkeys were subjected to two full thickness skin wounds on the back region. Hence, 2 groups were evaluated; control group (wounds at the left side) treated with local daily dressing with povidone iodine and I-PRF group (wounds at the right side) treated with I-PRF solution at day 0 and repeated at day 7 PW. Wounds were clinically evaluated for determination of the wound surface area, percentages of wound area and contraction. Histopathological evaluation was performed at days 21 and 42PW. Immunohistochemical evaluation was performed at 21 days PW for detection of CD31 and alpha smooth muscle actin (α-SMA) markers. The wound surface area was significantly lower in I-PRF group than the control group from 7 up to 42 days post wounding, the wound contraction percentage was significantly higher in I-PRF group than the control group from 14 up to 42 days PW. Better histopathological findings were demonstrated in I-PRF group compared to control group. Immunohistochemical evaluation revealed high immunreactivity against both CD31 and α-SMA antigens in I-PRF group and low immunreactivity against CD31 antigen and no immunreactivity against α-SMA antigen in control group. In conclusion, I-PRF improvesthe healing of experimentally induced full thickness skin wounds in donkeys evidenced by faster wound contraction as well as well-formed granulation tissue with complete re- epithelization.

Key words: Donkey, Healing, I-PRF, Wound.

INTRODUCTION

Wounds are the most common surgical disorders in equine species and often treated hesitantly as a result of misunderstanding or unfamiliarity. Therefore, the treatment is usually ineffective and may end the career of the animal (El-Sayad et al., 2004).

Wound healing is one of the most complex biological processes that occurs during mammalian life (Gurtner et al., 2008). It requires a coordinated interplay between cells, growth factors, and extracellular matrix proteins (Maxson et al., 2012). The process of wound healing has been divided into three overlapping phases; The inflammatory phase which involves hemostasis and acute inflammation, the proliferative phase during which tissue formation occurs and the remodeling phase in which the healing tissue regains its strength (Franz, 2007). Acute wounds proceed through these steps in a timely manner and achieve functional and

anatomic integrity. while, chronic wounds will similarly begin the healing process, but will have prolonged inflammatory, proliferative or remodeling phases, resulting in tissue fibrosis and non-healing ulcers, which is not an uncommon outcome in horses (Wilmink et la., 2002).

Studies on platelets revealed that they are responsible for releasing numerous biomolecules that are capable of stimulating the proliferation and activation of cells involved in healing process. These biomolecules include platelet-specific proteins and number of growth factors including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and epidermal growth factor (EGF), cytokines and angiogenic factors (Khan, et al., 2000; Choukroun, et al., 2017).

Over the last decades, platelet concentrates had travelled a long way from introduction of platelet concentrates as a source of blood proteins rich with growth factors to promote wound healing to introduction of fibrin glue, platelet rich plasma (PRP), platelet rich fibrin (PRF), advanced - platelet rich fibrin(A-PRF), titanium - platelet rich fibrin (T-PRF), injectable platelet rich fibrin (I-PRF) in recent years (Agarwal, 2017).

Injectable platelet rich fibrin (I-PRF) is a new generation of the platelet concentrates resulting from low-speed centrifugation whole blood sample without using anticoagulant (Mourao et al., 2015).It obtained in a liquid formulation that can be used for injectable purposes and can remain in liquid state for about 15 minutes after centrifugation (Masuki et al., 2016; Miron et al., 2017; Abd El Raouf et al., 2019). However, several previous studies described the effect of I-PRF on wound healing in human (Fotani et al., 2019;Giudice et al., 2020;Ozsagir et al., 2020)and in rats (Elsherbini & Ezzat, 2020), there was little information in the literature about the use of I-PRF in management of wounds in equine. Therefore, the aim of the present study is to evaluate the potential effect of I-PRF on healing of experimentally induced full-thickness skin wounds in equine.

MATERIALS AND METHODS

Animals

The present study was approved by Animal Welfare and Ethics Committee, Faculty of Veterinary Medicine, University of Sadat City.

Six adult healthy donkeys (5-6 years old with average body weight180-220 kg) were used in this study. The animals considered normal based on physical examination (not suffering from any skin diseases) and hematological analyses. During the experiment, the animals were housed in a covered stable containing several partitions and they were fed twice daily with wheat straw and a one kilogram of concentrates and allowed free choice of water.

Experiment design

In the present study, each animal was subjected to two full thickness skin wounds (one on each side of the back region). Hence, 2 groups were evaluated as follows:

- Control group: wounds at the left side of the back region treated with conventional treatment by local daily dressing with povidone iodine antiseptic solution (Betadine® Mundipharma, Germany).
- I-PRF group: wounds at the right side of the back region treated with I-PRF solution at day 0 and repeated at day 7 post wounding.

Creation of skin wounds

On the day of surgery, all animals received aprophylactic dose of antitetanic serum. The skin of the back region was clipped and aseptically prepared with povidone-iodine on both sides then the animals were sedated using xylazine hydrochloride at a dose of 1.1 mg/kg, IV with local infiltration of Lidocaine HCL at dose of 1ml/cm. In each animal, one full-thickness excisional square skin wound (3 x 3 cm) (Sadek et al., 2020)was created bilaterally on the back area using sterile template. Then, the wounds were dissected from the underlying tissue using scissors and tissue forceps and bleeding was controlled by placing sterile

gauze on wound surface. Each wound was subsequently treated with one of the two tested treatments in this study. The day of wound creation was designed as day 0.

Preparation and application of I-PRF

I-PRF was prepared according to the protocol developed by Miron et al. (2017). Ten ml of venous blood was withdrawn from the jugular vein in a plain plastic tube without anticoagulant. The tubes were rapidly transferred to the centrifuge and centrifuged at 700 rpm for 3 min. After centrifugation, the RBCs were separated at the bottom of the tube and the I-PRF is formed at the top of the tube as orange colored solution. The prepared I-PRF solution (total dose of 3 ml) was then withdrawn by a sterile syringe and injected subcutaneously and intradermally at the induced wound margins at day 0 and repeated again at day 7 post wounding (figure 1).

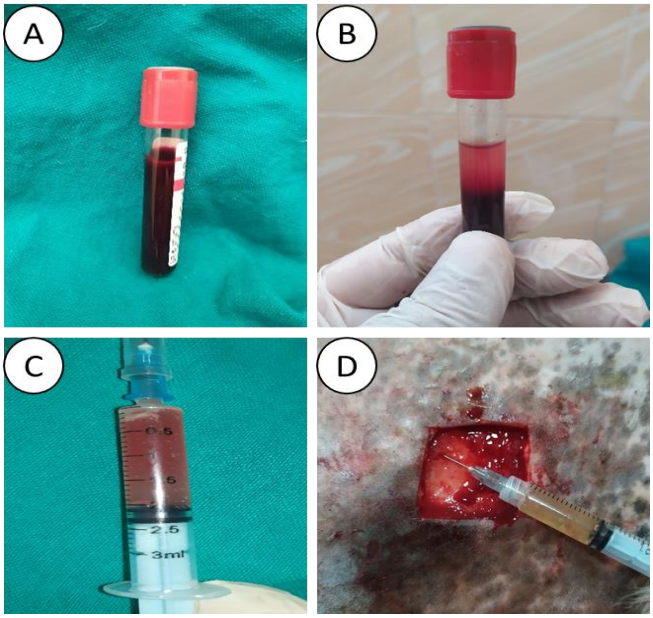

Figure (1): Preparation and application of I-PRF; A) venous blood sample before centrifugation, B) blood components after centrifugation at 700 rpm for 3 min and formation of I-PRF at the top and RBCs at the bottom of the tube, C) I-PRF after aspiration by syringe, D) injection of I-PRF subcutaneously and intradermally around the wound area.

Estimation of wound healing

Clinical evaluation of the wounds

Control and I-PRF groups were clinically evaluated for 42 days through determination of the wound surface area, percentage of wound area and contraction. The width and the length of wounds were measured using digital caliber after the wound had been carefully cleaned by saline to visualize wound margins. The wound surface area, percentage of wound size, and wound contraction percent were calculated using the following equations (Zaid et al. 2017):

Wound surface area = Length of the wound (mm) x Width of the wound (mm) =…..... mm2.

Percentage of wound area at day (x) = Wound surface area at day (x) mm2 x 100/ wound surface area at day (0).

Percentage of wound contraction at day (x) = 100 – Percentage of wound area at day (x).

Histopathological evaluation

For histopathological evaluation, tissue biopsies from skin wounds (at days 21 and 42) were fixed in 10% neutral-buffered formalin overnight, then washed with 70% ethanol, and processed with several processes of paraffin sectioning. Sections were then stained with hematoxylin and eosin to evaluate morphological features of tissues and were stained with Masson's trichrome to

highlight the formation collagen tissue during proliferation and remodeling phases of wound healing.

Immunohistochemical evaluation

Samples were taken from wound margins at day 21 post wounding. CD31 and alpha smooth muscle actin (α-SMA) markers were detected by immunoperoxidase technique and evaluated on light microscope. For immunoperoxidase staining, skin samples from the wound sites were fixed in 10% formalin, dehydrated with different grades of alcohol series, embedded in paraffin, and sectioned into 4-µm-thick sections on positive slides.

Statistical analysis

Statistical analysis was performed with SPSS 20 software (SPSS Inc., Chicago, IL, U.S.A.). Different variables were analyzed by Student's t-test. P values <0.05 were considered statistically significant.

RESULTS

Clinical evaluation of the wounds

In the present study, throughout the observation period, there was a gradual decline in the wound surface area in both control and I-PRF groups. At 42 days post wounding, the I-PRF group showed complete wound healing evidenced by complete disappearance of the open wound area while at the same time point, wounds of the control group were not completely healed (Figure 2). Moreover, from 7 up to 42 days post wounding, the wound surface area was significantly lower in I-PRF group than the control group (Figure 3).

The percentage of open wound area in relation to day 0 was significantly lower in I-PRF group than the control group from 14 up to 42 days post wounding (Figure 4).

The wound contraction percentage progressively increased in both control and I-PRF groups whereas it was significantly higher in I-PRF group than the control group from 14 up to 42 days post wounding (Figure 5).

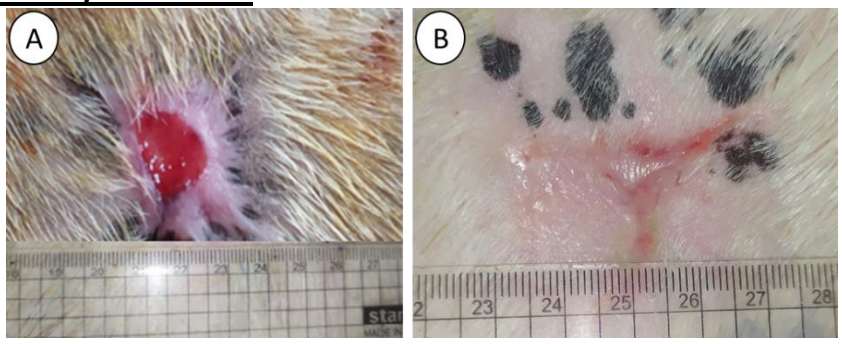

Figure (2): Photographs showing the wound area in the two groups at 42 days post wounding: (A) wound area from control group compared to (B) wound area from I-PRF treated group.

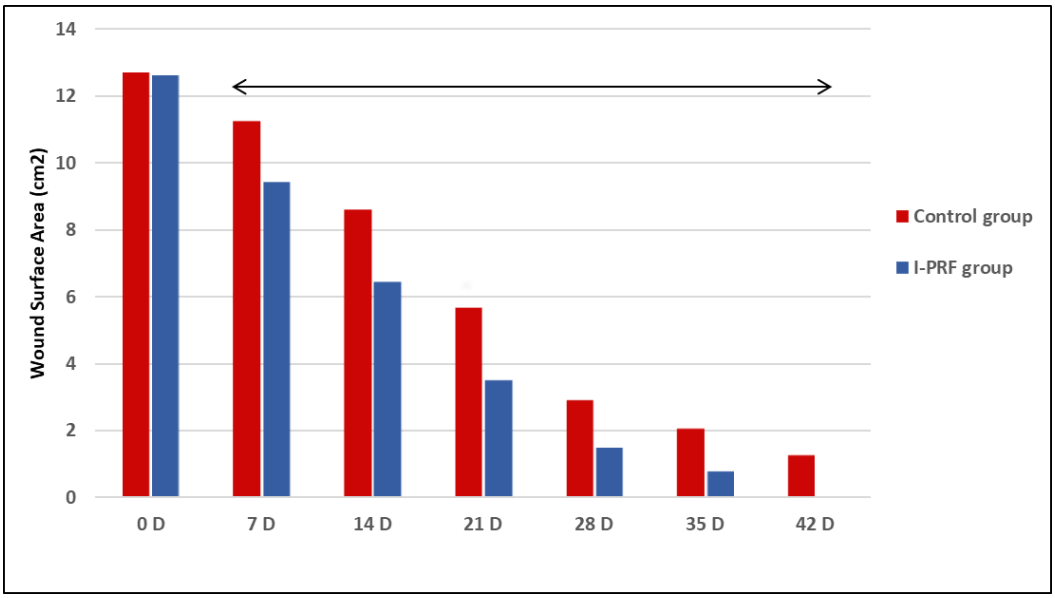

Figure (3): The mean of wound surface area in control and I-PRF groups. ⟷ Significant difference between groups.

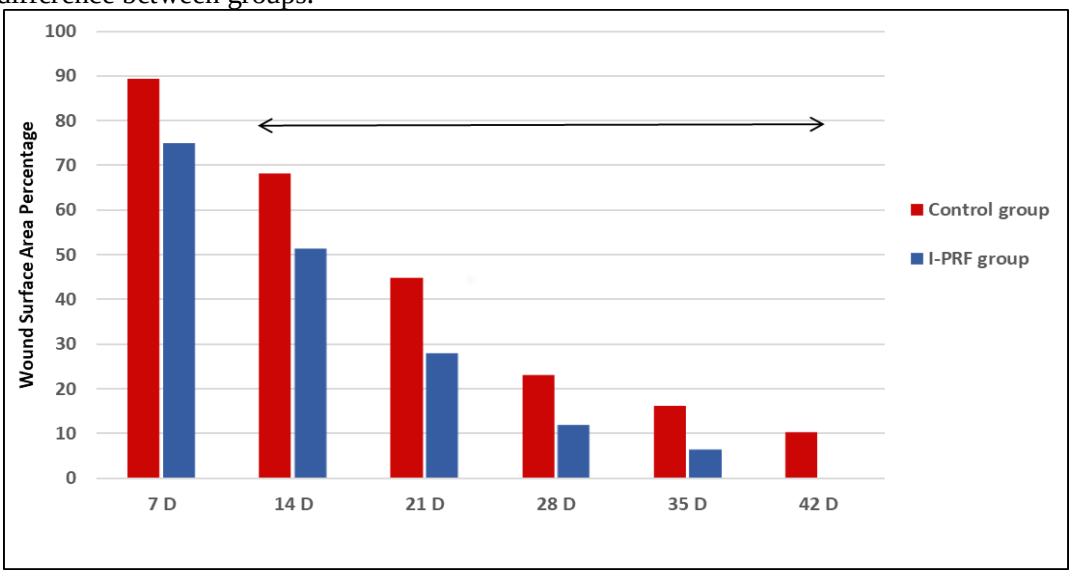

Figure (4): The mean of wound surface area percentage in control and I-PRF groups. ⟷ Significant difference between groups.

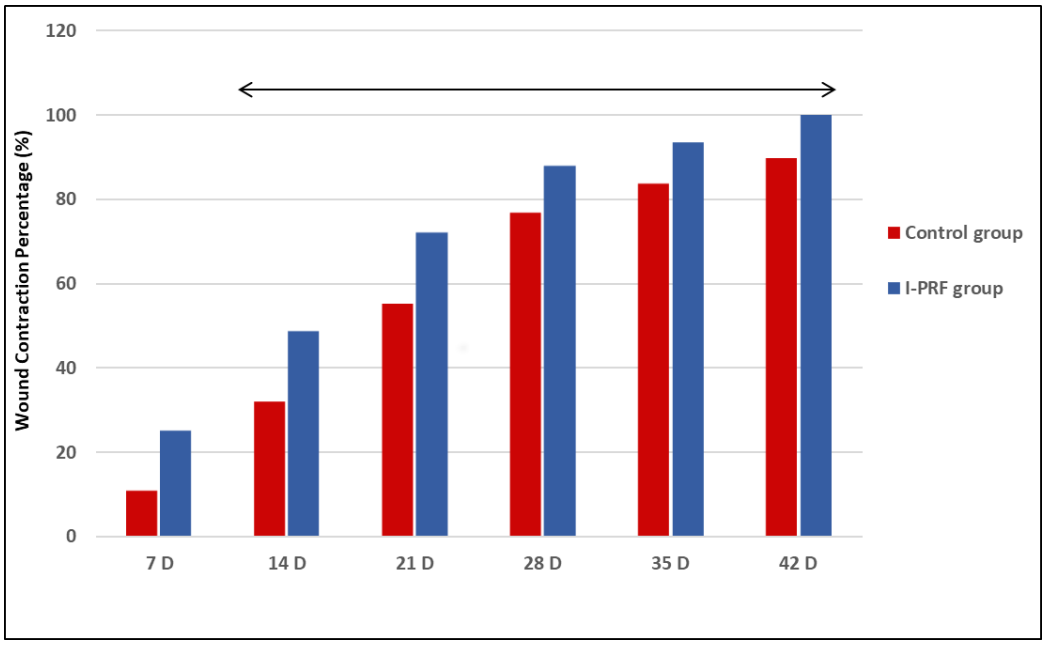

Figure (5): The mean of wound contraction percentage in control and I-PRF groups. ↔ Significant difference between groups.

Histopathological Evaluation

Histopathological examination of control wound samples at day 21 post wounding revealed high proliferation of fibrous tissue with very congested and hemorrhagic granulation tissue and scab on top while at 42 days the wounds showed high proliferation of mature fibrous tissue with detached surface and no epithelization was observed (figure 6). I-PRF treated wound at day 21 post wounding showed closure of the wound surface with epithelized epidermis with high cellularity in epidermis and proliferation of immature collagen bundles while at 42 days post wounding, the wounds showed thickening of the epidermal layer with a thin layer of keratin and increased production of collagen in the dermis layer (figure7).

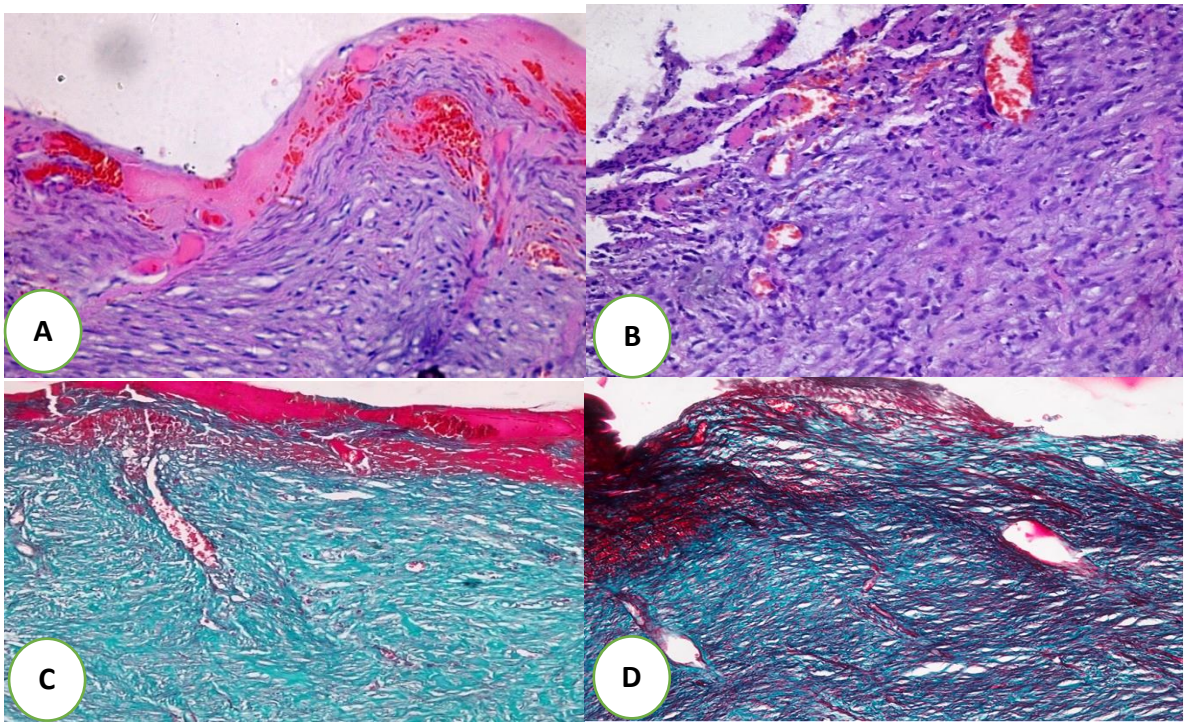

Figure (6): Photomicrograph showing the wound healing site of control group; A) at 21 days post wounding showing high proliferation of fibrous tissue with very congested and hemorrhagic granulation tissue and scab on top. H&E 200X. B) At 42 days showing high proliferation of mature fibrous tissue with detached surface and no epithelization was observed H&E 200X. C) At 21 days showing high proliferation of fibrous tissue with very congested and hemorrhagic granulation tissue and scab on top. MTC 100X. D) At 42 days showing high proliferation of mature fibrous tissue with detached surface and no epithelization was observed MTC 200X.

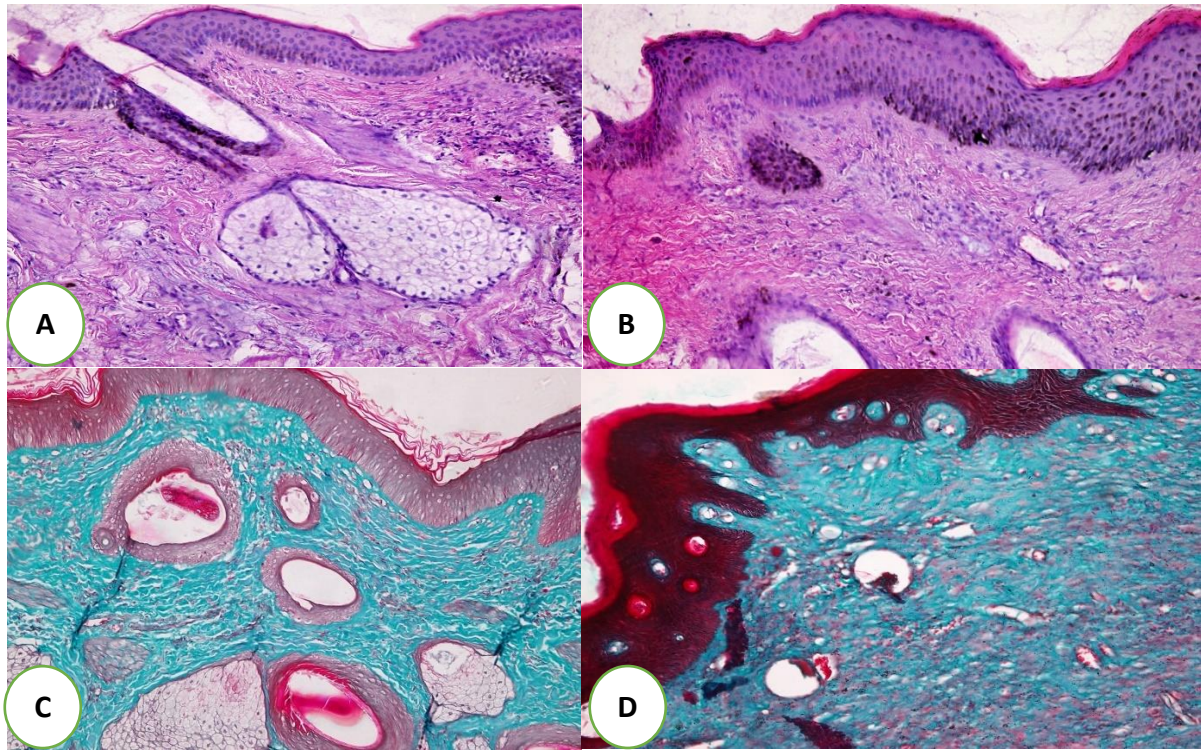

Figure (7): Photomicrograph showing the wound healing site of I-PRF group; A) at 21 days post wounding showing closure of the wound surface with epithelized epidermis with high cellularity in epidermis and proliferation of immature collagen bundles. H&E 200X. B) At 42

days showing thickening of the epidermal layer with a thin layer of keratin and increased production of collagen in the dermis layer H&E 200X. C) At 21 days showing closure of the wound surface with epithelized epidermis with proliferation of condensed collagen bundles. MTC 200X. D) At 42 days showing thickening of the epidermal layer with a thin layer of keratin and increased production of collagen in the dermis layer MTC 100X.

Immunohistochemical evaluation

Immunohistochemical examination of skin wound sections at 21 days post wounding revealed low immunreactivity against CD31 antigen and no immunreactivity against α-SMA antigen in control group (figure8) while the I-PRF group showed high immunreactivity against both CD31 and α-SMA antigens (figure9).

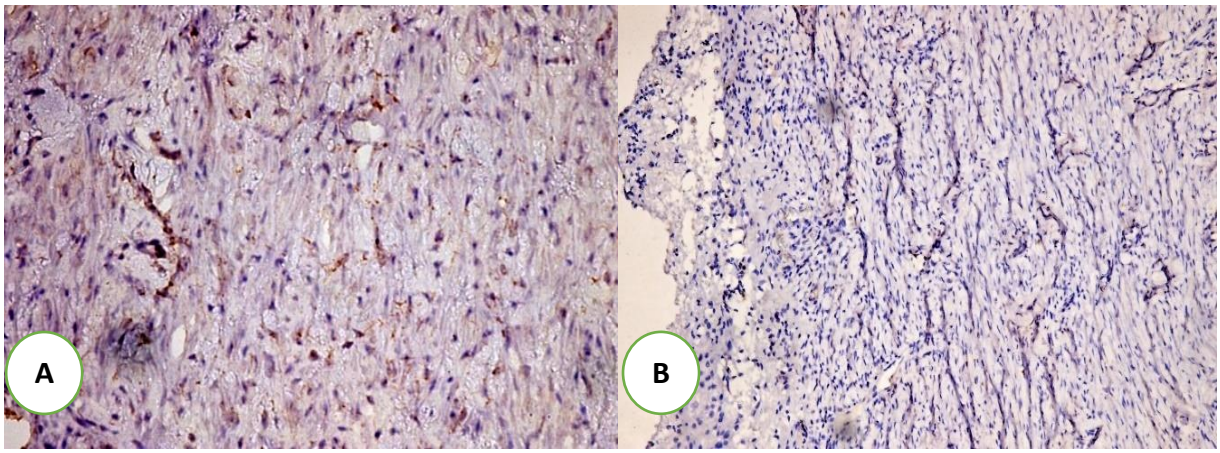

Figure (8): Immunohistochemistry of skin wound section of control group 21 days post wounding; A) low immunreactivity against CD31 antigen. Avidinbiotin immunoperoxidase complex x400. B) no immunreactivity against α-SMA antigen. Avidinbiotin immunoperoxidase complex x200.

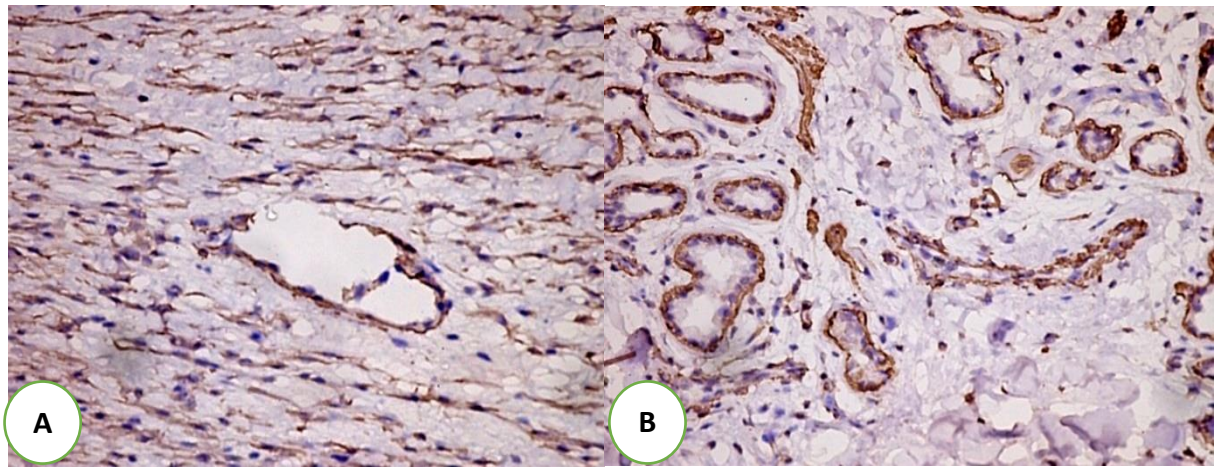

Figure (9): Immunohistochemistry of skin wound section of I-PRF group 21 days post wounding; A) High immunreactivity against CD31 antigen. Avidinbiotin immunoperoxidase complex x400. B) High immunreactivity against α-SMA antigen. Avidinbiotin immunoperoxidase complex x400.

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DISCUSSION

Wound healing is a complex process occurs in response to a disruption of the normal structure and function of the tissue (Gopinath et al., 2004). Equine species commonly manifest a debilitating difficulty with the healing process of wounds especially in the lower parts than the upper parts of the body (Jacobs et al., 1984).

Injured horses usually lose their value for prolonged periods of time therefore, any mechanisms which accelerate the process of wound healing would have a significant impact (Knottenbelt, 1997).

Injectable platelet rich fibrin (I-PRF) is a new alternative to the platelet concentrate to different areas of regenerative medicine. In this study I-PRF is prepared according to the method mentioned by Miron et al., (2017) in which blood is drawn without anticoagulant in plastic tubes and centrifuged at 700 for 3 minutes. This time is considerably shorter than the protocols for other platelet concentrates (such as PRP& A-PRF). This can be attributed to the fact that the separation of blood components during preparation of I-PRF occurs in the first 2-4 minutes. Moreover, the plastic tubes have a hydrophobic surface that do not efficiently activate the coagulation process. Hence, all the blood components that are required to form a good platelet concentrate (plasma containing all clotting factors & platelets) reach the top of the tube under the low centrifugation force in the first 2-4 minutes.
In the current study, the wound contraction percentage progressively increased in both control and I-PRF groups and it was significantly higher in I-PRF group than the control group from 14 up to 42 days post wounding. The faster contraction in I-PRF group may be attributed to its platelets content which induces a reduction in wound size by increases the fibroblast to macrophage ratio and increases proliferating fibroblast (Strukova et al., 2001), and presence of Vascular endothelial growth factor (VEGF) which is a mediator of angiogenesis that stimulates endothelial cell proliferation and also promotes fibroblast proliferation (Kliche & Waltenberger, 2001).
Concerning the histological evaluation, control wound samples at day 21 post wounding revealed high proliferation of fibrous tissue with very congested and hemorrhagic granulation tissue covered by scab on top and high proliferation of mature fibrous tissue with detached surface and no epithelization was observed at 42 days. I-PRF treated wound at day 21 showed closure of the wound surface with epithelized epidermis with high cellularity in epidermis and proliferation of immature collagen bundles and thickening of the epidermal layer with a thin layer of keratin and increased production of collagen in the dermis layer at 42 days. The formation healthy granulation tissue and the complete re-epithelialization of the wound site in I-PRF treated wound could be explained by the transforming growth factors (TGF: TGF-ß1 and TGF- ß2) which activate fibroblasts for formation of pre-collagen, which induces the deposition of collagen which lead to wound healing as explained by Carter et al., (2003) and Varela et al., (2019) , also the platelets aid in hemostasis and re-epithelialization by releasing growth factors that stimulate the angiogenesis and promoting fibroblast proliferation which in turn provide an increase in the collagen synthesis this also reported by Marx (2004).
Immunohistochemical staining of skin wound sections of I-PRF group at 21 days post wounding revealed high immunreactivity against both CD31 and α-SMA antigens while the control group showed low immunreactivity against CD31 antigen and no immunreactivity against α-SMA antigen. These findings indicate quicker angiogenesis and proper collagen deposition in I-PRF group than the control one.

CONCLUSION

In conclusion, I-PRF improves the healing of experimentally induced full thickness skin wounds in donkeys. This was evidenced by faster wound contraction as well as well-formed granulation tissue with complete re- epithelization when compared with the control group.

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