

## Effects of Autologous Platelets Rich Plasma on Full-thickness Cutaneous Wounds Healing in Goats

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**Abstract:** This investigation was designed to evaluate the role of Platelet-Rich Plasma (PRP) on healing of experimentally wounded skin in ten adult bucks, aged 2-3 years and weighing 25-30 kg. The animals divided randomly and equally into (control and treatment groups). Four of 3×3 cm of full-thickness square cutaneous wounds was induced on both sides of the lateral thoracic region of each animal under the effect of local anesthetic proceeding by xylazine hydrochloride as a sedative. A pair of left wounds was treated by injection with 5 mL of autonomous PRP (treatment group), 2 mm lateral to the wound edges and in the wound center. While, the right wound were injected by 5 mL of sterile saline by the same procedure (control group). Each group was divided into five subgroups (four wounds of each), for morphometrical and histopathological evaluations of wound healing process represented by percent of wound contraction, epithelialization and total healing at 3, 7, 14, 21 and 28 days post-wounding. The morphometrical appearance of the wounds which treated with PRP, showed that the contraction, re-epithelialization and healing percent were statically significant ( $p<0.05$ ) in comparison with control wounds during four weeks study. Based on histopathological results, there was re-epithelialization of epidermis, with highly cellular granulation tissue, well differentiated keratinocytes of epidermis with scar formation in the dermis of the sectioned skin. We conclude that local injection of PRP leads to accelerate and improvement of wound healing in comparison to control wounds.

**Keywords:** Goats, platelets rich plasma, skin, wound healing

### INTRODUCTION

The skin healing is the aim of studies and researches due to its clinical, scientific and economic interest, especially due to the great frequency of wounds caused by injury in goats. The wound's healing is a physiological phenomenon that initiates from the loss of integrity of the skin generating a solution of continuity that reaches the underlying layers in diverse degrees and depends on a series of chemical reactions classically divided in four phases: inflammation, a proliferative phase, remodeling and maturation (Kairuz *et al.*, 2007). Appropriate wound care is critical and various treatment modalities are used to improve the wound bed (Beckert *et al.*, 2007). The aim of wound care is to promote wound healing in the shortest time with minimal pain, discomfort and scarring to the patient and must occur in a physiologic environment conducive to tissue repair and regeneration (Priya *et al.*, 2007). Despite numerous treatments available for deteriorated cutaneous wound healing, there is still the need for more effective therapy (Lee *et al.*, 2011). In recent years, advanced treatment modalities, such as cell-based therapies and PRP (Azari *et al.*, 2008) are used. A topical treatment with platelet derived growth factor as its active ingredient was approved in 1997 to

treat diabetic foot and leg ulcers that extend into the subcutaneous tissue or beyond which receive inadequate vascularization. The Plasma Rich in Growth Factors (PRGFs) is a mixture of autologous proteins concentrated from a determined volume of PRP. The later stimulates angiogenesis, promoting vascular in growth and fibroblasts proliferation. In addition, it acts as hemostatic effect by forming a fibrin clot. Also application of PRP enhances wound healing in both soft and hard tissue. PRP is 100% biocompatible and safe. It poses absolutely no infectious risk to the patient because it is made from the patient's own plasma. A literature to assess the current clinical experience and the possible effects of (PRP) on wound-healing yields recorded by few reports (Kimura *et al.*, 2005), thus the present study was designed to detect the role of PRP in healing of experimentally wounded skin in goats.

### MATERIALS AND METHODS

**Preparation of platelet-rich plasma:** Blood sample 50 mL was collected under a septic technique from the jugular vein of each buck via a 21 gauge needle and deposited in 3.2% sodium citrate tube with capacity for 10 mL. Then, placed in a centrifuge at 3200 rpm for 15 min. During centrifugation, blood is separated into



Fig. 1: Preparation of PRP. Plasma of total blood (upper arrow), buffy coat represented the leukocytes and platelets of total blood (middle arrow) and erythrocytes (lower arrow)



Fig. 2: Show's, the positions of squared wounds on lateral thoracic regions

three different fractions: platelet-poor plasma (superior layer), white blood cells (intermediate layer) and red blood cells (inferior layer) (Fig. 1). The first supernatant plasma fraction (50%), adjacent to the buffy coat, was obtained under aseptic conditions in a laminar flow chamber. This fraction was centrifuged at 3200 rpm for another 15 minutes in order to obtain two part: the upper part which is platelet poor plasma PPP and the lower part was the PRP (25%). Then the PRP was aspirated with another pipette and placed in a sterile tube and activated with calcium chloride (4.5 mEq/5 mL), using 50 µL/mL of PRP, to provide a gel matrix for a PRP to adhere to the site of injection (Kevy and Jacobson, 2004). An average of 5ml of PRP was obtained from every 50 ml of whole blood. A fraction of 1ml of each animal was analyzed for platelet count. On the day of PRP obtainment, the platelets count in PRP were 200.000 to 350.000 cells/µL.

**Experimental animals and management:** Ten apparently healthy adult local breed bucks, aged (2-3) years with body weights ranging from (25-30) kg., were used for the present clinical trial. The animals source were the herd of Veterinary Medicine College, Baghdad University. They housed in semi-opened stall at the farm animal along the period of the experiment, receiving free water and food (concentrated and alpha-alpha hay). The bucks were dewormed with Ivermectin in a dose rate of 0.2 mg/kg administered subcutaneously, month prior to wounding.

**Pre-operative considerations:** The dorsal surface of thoracic regions was clipped free hair and prepared aseptically for the wounding. Thirty minutes prior to wounding, Penicillin-Streptomycin was administered

I.M., in a dose of 20000 IU/kg and 10 mg/kg B.W., respectively. The bucks were premedicated by intramuscular administration of 2% Xylazine hydrochloride 0.05 mg/kg.; anesthesia was accomplished by linear subcutaneous infiltration of lidocain hydrochloride 2% at the intended incision sites at a dose rate of 1 mL/1cm<sup>3</sup>.

**Technique of wounding:** Four square full-thickness skin wounds (3×3 cm) were created on the dorsal thoracic sides of each buck (two wounds on each side), 10-15cm apart (Fig. 2). The total number of the wounds is (40 wounds). The animals submitted to two equal groups, the control group and the treatment groups. All wounds were not sutured (opened wounds). The control wounds were injected by 5 mL of sterile saline, 2 mm lateral to the wound edges and within the wound bed, immediately after wounding. While the treatment wounds were injected intradermal by 5 mL of PRP at different sites, by the same technique mentioned for the control group. After completion of wounds treatment in both groups, the wounds were bandaged by use of a protective, non-pressure and non-adherent dressing. The bandages have been changed, two days intervals and wounds were gently cleaned with gauze sponges soaked in normal saline, without debridement of the wound bed.

**Clinical evaluations:** A complete clinical examination was performed on all animals daily during the studied period. Wounds photographed on the day were it made and then twice a week till day 28<sup>th</sup>. The scab of each wound was carefully removed by using saline for better visualization of the epithelialization and granulation tissue area. The percent of epithelialization, wound contraction and total wound healing were calculated for each wound, depending on the method mentioned by Eppley *et al.* (2004).

**Morphometrical and histopathological evaluations:** The morphometrical and histopathological evaluation were performed at days (3, 7, 14, 21 and 28) days for both groups (four wounds for each period). For biopsies collection full-thickness skin (1 cm<sup>3</sup>) were harvested and fixed in (10%) neutral buffer formalin solution. The tissue specimens were processed in a tissue processor, paraffin blocks were mad at (5-6) µm thick sections which were cut with a microtome and stained with Hematoxylin and Eosin dyes (Luna, 1968). Then stained slides were examined under light microscope.

**Statistical analysis:** Data are expressed as Mean±Standard Error (M±SE). Statistical analysis was carried out on the load bearing data using two-ways, Analysis of Variance (ANOVA) in addition to Least Significant Difference (LSD). p-value <0.05 was considered to indicate a statistical differences (Snedecor and Cochran, 1973).

Table 1: Show's, the percent of wound epithelialization (%) in both groups

Days	Control group M±SE	Treatment group M±SE	LSD value
3	0.00±0.00	0.00±0.00	0.00 NS
7	28.20±3.77	32.35±2.37	7.142
14	46.40±3.54	57.20±2.86	6.733*
21	60.10±2.60	69.00±1.90	6.904*
28	74.28±1.85	85.10±1.30	8.893*
LSD value	10.265*	11.382*	---

\*(p<0.05), NS: Non-Significant

Table 2: Show's, the percent (%) of wound contraction in both groups

Days	Control group M±SE	Treatment group M±SE	LSD value
3	0.00±0.00	0.00±0.00	0.00 NS
7	30.44±3.70	38.00±3.25	4.155*
14	46.00±3.12	57.33±2.78	4.209*
21	61.00±2.75	68.7±2.20	5.215*
28	78.66±2.00	87.44±1.10	6.316*
LSD value	13.602*	9.403*	---

\* (p<0.05), NS: Non-Significant

Table 3: Show's, the percent (%) of total wound healing in both groups

Days	Control group M±SE	Treatment group M±SE	LSD value
3	0.00±0.00	0.00±0.00	0.00 NS
7	32.77±4.30	41.77±3.45	7.319*
14	47.44±3.70	56.44±3.30	7.277*
21	60.55±2.69	75.55±2.18	7.946*
28	75.40±1.90	88.44±1.14	8.163*
LSD value	12.188*	10.452*	---

\*(p<0.05), NS: Non-Significant

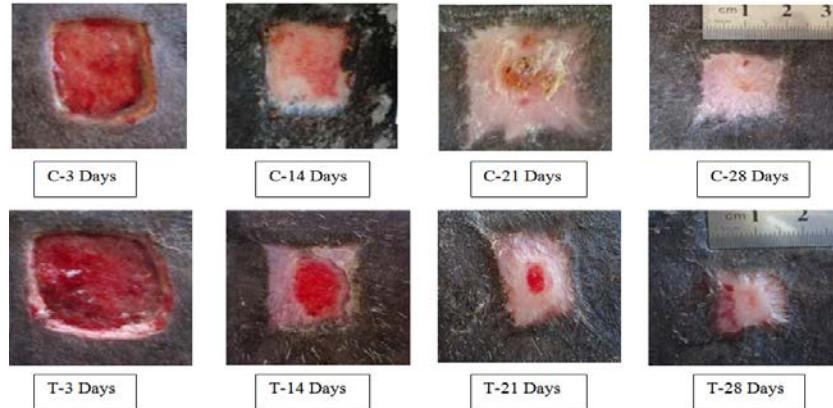


Fig. 3: Steps of wound healing in control (C) and treatment (T) groups according to the time

## RESULTS

**Clinical observations:** During 4 weeks study, no any complications such as wound infection or exuberant granulation tissue formation in the wound sites of both groups were noticed. Animals reflected normal appetite, urination and defecation within the first 24 h post-wounding. The results of vital parameters such as temperature, respiratory and heart rates during the first week post-wounding were within the normal rang as mentioned in the most dependent references.

### Wound morphometric analysis:

- Woundepithelialization:** The progress of epithelialization in treatment wounds was statistically significant (p<0.05) in comparing with

control wounds, started from second week. The percent of epithelialization in control wounds was (74.28±1.85) and in treatment wounds was (85.10 ±1.30) at day 28 (Table 1).

- Wound contraction:** There was significant differences (p<0.05) in speed of wound contraction between control and treatment groups started from first week. It was (78.66±2.00) and (87.44±1.10), respectively, at the end of the study (Table 2).
- Wound healing:** The rate of wound healing showed similar result as mentioned in wound contraction phenomena i.e., that the significant differences (p<0.05) between both group started from first week post-wounding. The highest rate was recorded in treatment group (88.44±1.14 at day 28) (Table 3and Fig. 3).

Table 4: The histopathology findings of skin biopsies of control and treatment groups

Time (day)	Control group	Treatment group
3	Area of hemorrhage and polymorph nuclear cells infiltration mainly neutrophils	Area of hemorrhage extensive polymorph nuclear cells infiltration with fibrin network deposition
7	Neutrophils infiltration and fibrine net work formation (Fig. 4)	Granulation tissue replaced most of the incision area with congested blood vessels, neutrophils and mononuclear cells infiltration (Fig. 5)
14	Immature granulation tissues with mononuclear cells infiltration (Fig. 6)	Matures granulation tissue in the incisional area with mononuclear cells infiltration (Fig. 7)
21	Thick epidermal layer over the granulation tissue with short rete ridge (Fig. 8)	Normal epidermal area covers mature granulation tissue (Fig. 9)
28	Presence of epidermis layer cover the mature granulation tissue (Fig. 10)	Melanin pigment in the basal layer of epidermis cover the granulation tissue (Fig. 11). In another section, reformation of hair follicles and sebaceous glands (orang arrow) in the incisional site (Fig. 12)

**Histopathologic findings:** The pathognomonic histopathology features of skin biopsies harvested from both groups, reflect the acceleration and improvement in wound healing subjected to PRP treatment with enhancing of total wound healing at the end of the study, in comparison to untreated wounds, which are shown in Table 4.

## DISCUSSION

During a follow-up for 28 days, we did not record any secondary complications of the wounds in both groups, this may ascribe to highly strike antiseptic and appropriated post-operative cares. This findings were in accordance with Azari *et al.* (2008) in goat. In contrast Carter *et al.* (2003), observed infections in two wounds out of 32 when used PRP for treatment of cutaneous wounds in horse. These differences in the results of the studies may be related to the number of animals used in each study. The vital parameters were elevated in the first week post-wounding in both groups but with no significantly differences  $p>0.05$ . These increments in parameters level may be attributed to acute inflammation evoked from wounding. Then the values retained gradually to its normal levels. These findings were in agreement with previous study (Crovetti *et al.*, 2004).

The morphometric evaluation of wounds in the present study, showed that the progress of epithelialization was faster in wound treated with PRP compared with control wounds during four week study. It has been demonstrated that PRP prompted cutaneous wound repair via differentiation into multiple skin cell types including; keratinocytes, endothelial cells, pericytes and monocytes (Anitua *et al.*, 2006). Wu *et al.* (2007), suggested that PRP engrafted in cutaneous wound accelerate re-epithelialization, through their ability to stimulate the keratinocytes to differentiate into various epithelial cell types such as; skin epithelial cells, after systemic administration *in vivo*.

Re-epithelialization of wounds begins within hours after injury. Epidermal cells from skin appendages such as hair follicles quickly remove clotted blood and damaged stroma from the wound space (Kirsner and

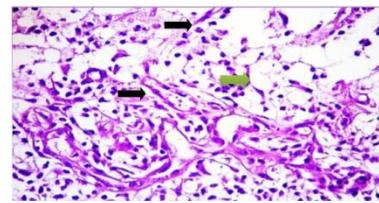


Fig. 4: Histopathologic section of wound related to control group, day 7<sup>th</sup> post-wounding, show's neutrophils infiltration (green arrow) and fibrine net work formation (black arrows) (H&E; X40)

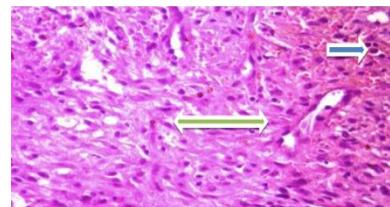


Fig. 5: Histopathologic section of wound related to treatment group on day 7<sup>th</sup> post-wounding, show's granulation tissue replaced most of the incisional area (green arrow), with congested blood vessels, neutrophils and mononuclear cells infiltration (blue arrow) (H&E; X40)

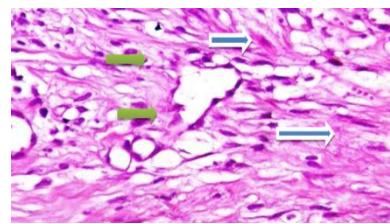


Fig. 6: Histopathologic section of wound related to control group, 14 days post-wounding, show's immature granulation tissues (blue arrows) with mononuclear cells infiltration (green arrows) (H&E; X40)

Eaglstein, 1993). The expression of integrin receptors on epidermal cells allows them to interact with a variety of Extracellular-Matrix (ECM) proteins (e.g., fibronectin and vitronectin) that are interspersed with stromal type I collagen at the margin of the wound and interwoven with the fibrin clot in the wound space. As re-epithelialization ensues, basement-membrane

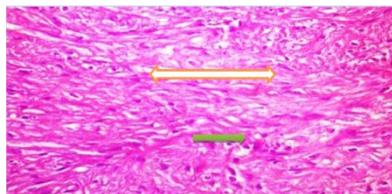


Fig. 7: Histopathologic section of wound related to treatment group, 14 day post-wounding, show's, mature granulation tissue in the incisional area (white arrow), with mononuclear cells infiltration (green arrow) (H&E; X40)

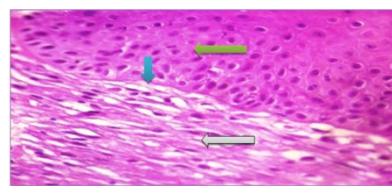


Fig. 8: Histopathologic section of wound related to control group, 21days post-wounding, show's thick epidermal layer (green arrow) over the granulation tissue (grey arrow) with short rete ridge (blue arrow) (H&E; X40)

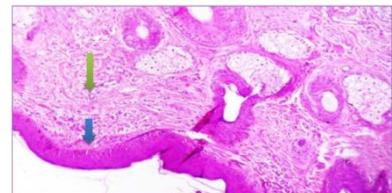


Fig. 9: Histopathologic section of wound related to treatment group, 21days post-wounding, show's normal epidermal area (blue arrow) covers mature granulation tissue (green arrow). (H&E; X20)

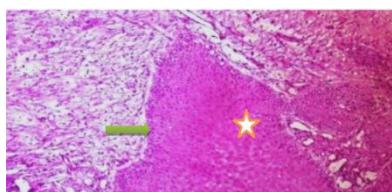


Fig. 10: Histopathologic section of wound related to control group, 28 days post-wounding, show's the presence of epidermis layer (star) cover the mature granulation tissue (green arrow). (H&E; X40)

proteins reappear in a very ordered sequence from the margin of the wound inward. Epidermal cells revert to their normal phenotype, once again firmly attaching to the reestablished basement membrane and underlying dermis (Leitner *et al.*, 2006).

The results of current study showed that wound contraction percent was greater in treatment wound compared to the control wound. Swaim *et al.* (2001), indicated that in second-intention wound healing,

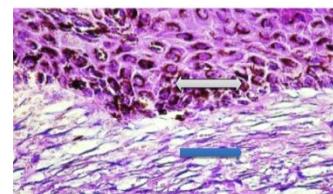


Fig. 11: Histopathologic section of wound related to treatment group, 28 days post-wounding, show's melanin pigment in the basal layer of epidermis (grey arrow) cover the granulation tissue (blue arrow) (H&E; X40)

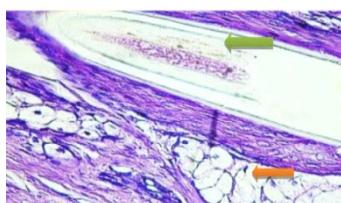


Fig. 12: Histopathologic section of wound related to treatment group, 35 day post-wounding, show's the reformation of hair follicles (green arrow) and sebaceous glands (orange arrow) in the incisional site (H&E; X40)

closure is achieved by contraction and epithelialization. A greater contribution of wound contraction accelerates healing because contraction occurs faster than epithelialization. Wound contraction is defined as the centripetal movement of the original wound margins. This process occurs due to contraction of myofibroblasts in granulation tissue. Myofibroblasts are essential to wound contraction and healing. They differentiate from fibroblast and are characterized by presence of stress fibers containing  $\alpha$ - actin isoform that is expressed in smooth muscle. It has been confirmed that PRP expressed Transforming Growth Factor (TGF) which activate fibroblasts and differentiate into myofibroblast and increase the number of these cells to promote wound contraction.

Wound contraction involves a complex and superbly orchestrated interaction of cells, ECM and cytokines. During the second week of healing, fibroblasts assume a myofibroblast phenotype characterized by large bundles of actin-containing microfilaments disposed along the cytoplasmic face of the plasma membrane of the cells and by cell-cell and cell-matrix linkages (Masur *et al.*, 1996). The appearance of the myofibroblasts corresponds to the commencement of connective-tissue compaction and the contraction of the wound. The contraction probably requires stimulation by TGF and Platelet-Derived Growth Factor (PDGF) attachment of fibroblasts to the collagen matrix through integrin receptors and cross-links between individual bundles of collagen. Collagen remodeling during the transition from granulation tissue to scar is dependent on continued synthesis and

catabolism of collagen at a low rate. The degradation of collagen in the wound is controlled by several proteolytic enzymes termed matrix metalloproteinase, which are secreted by macrophages (Liu *et al.*, 2002).

Histopathological sections, reveals hemorrhage and inflammatory cells during the first seven days in addition to the formation of granulation tissue. These observations were recorded by Azari *et al.* (2008), in goats in which the new stroma, often called granulation tissue, begins to invade the wound space approximately four days after injury. Numerous new capillaries endow the new stroma with its granular appearance. Macrophages, fibroblasts and blood vessels move into the wound space at the same time. The macrophages provide a continuing source of growth factors necessary to stimulate fibroplasia and angiogenesis; the fibroblasts produce the new ECM necessary to support cell in growth; and blood vessels carry oxygen and nutrients necessary to sustain cell metabolism. Werner and Grose (2003), indicated that growth factors, especially PDGF and TGF in concert with the ECM molecules, presumably stimulate fibroblasts of the tissue around the wound to proliferate and produce collagen fibers which resemble a bridge connecting the ends of the wound in addition, the fibroblasts are responsible for the synthesis, deposition and remodeling of the extracellular matrix. The formation of new blood vessels is necessary to sustain the newly formed granulation tissue. Angiogenesis is a complex process that relies on extracellular matrix in the wound bed as well as migration and mitogenic stimulation of endothelial cells (Wu *et al.*, 2007).

In the present study, the re-epithelialization of epidermis in PRP treatment group started at the second week post-wounding while it appeared in fourth week in control group and this continuation was supported by a study of Knighton *et al.* (1990). On another hand, Wynn (2007), stated that Re-epithelialization of the wound can be conceptually viewed as the result of three overlapping keratinocyte functions: migration, proliferation and differentiation. The sequence of events by which keratinocytes accomplish the task of re-epithelialization is generally believed to begin with dissolution of cell-cell and cell-substratum contacts. This is followed by the polarization and initiation of migration in basal and a subset of supra-basilar keratinocytes over the provisional wound matrix.

Well differentiated keratinocytes of epidermis with scar formation in the dermis were observed in treatment group at day 28. While, in randomized prospective study by Dyson (1997), showed that scar formation at day 35<sup>th</sup> post-wounding and indicated that the epidermis regenerated progressively from the surrounding wound margins. The neoepidermis showed a complete spectrum of changes. Near the wound margin, the differentiation of the neoepidermis and regeneration of the dermo-epidermal junction were more advanced than toward the wound center, where the proliferative index was significantly increased. Also Bennett and Schultz, (1993), referred that the wound becomes covered with

epithelial tissue within two weeks, minimal collagen will be deposited and no scar will form. Generally, if a wound takes longer than three to four weeks to become covered, a scar will form.

Based on the results of the present study, transplantation of PRP that were isolated from caprine blood had positives effect on second intention cutaneous wound healing in goats. In conclusion, this study demonstrates the beneficial effect of PRP in cutaneous wound healing via fast epithelialization and effective wound contraction.

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