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# Immunohistochemical and gene expression analysis of autologous platelet rich fibrin for distal limb wound defects healing in donkeys *(Equus asinus)*.

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**ABSTRACT**

***Objective:*** To evaluate the effect of platelet-rich fibrin (PRF) in the promotion of distal limb wound defects healing in donkeys.
***Design:*** A randomized experimental design
***Animals:*** Twelve clinically healthy male donkeys, weighing, 130–230 kg and aged 4 –5 years were allocated into three groups(4 animals/each) and undergo a 6cm$^2$ (2cm X 3cm) 2 wound defects on the dorsolateral surface of right metacarpal and metatarsal regions for each donkey. Control (group A): the wound defects were left for spontaneous healing. In groups B and C, the wound defects were treated with either one application of PRF (B) or with three consecutive applications of PRF (a week interval) (C). Wound defects healing were evaluated clinically, histologically and immunohistochemically, in addition to gene expression patterns of angiogenic and myofibroblastic genes vascular endothelial growth factor (VEGF-A), collagen type 3 α1 (COL3α1), and fibroblast growth factor 7 (FGF-7) and tissue growth factor β1 (TGFβ1) were performed.
***Results:*** The healing percentage of single and three PRF applications was significantly higher (P <0.05) (84.6%, and 93.7% respectively) than in control one (66.7%). The number of days needed for complete wound healing was considerably shorter in repeated PRF treated wound defects (63.2±2.8) compared with single PRF and untreated wound defects (71.6±3 and 86.3±3, respectively). Semi-quantitative evaluation of histological sections at 15 and 45 days post-operative showed a significant difference (P<0.05) in epithelization, PMNL, fibroblasts, tissue macrophages, neo-angiogenesis and new collagen scores in both PRF groups compared to control one. Qualitative analysis of immunohistochemical views of the wound defects showed a significant immunostaining difference against EGFR, VEGF, and TGFβ stain between both PRF treated groups and control one. Immunohistochemical analysis of cells stained for epidermal growth factor receptor (EGFR), VEGF, and TGFβ at 15 and 45 days after interference was higher in both PRF treated groups compared to control one, but three PRF application showed the highest rates. The relative expression of FGF-7, TGFβ1, VEGF-A, and COL3α1 genes was higher in both PRF groups compared to control one, but the triple PRF group revealed the highest expression.
***Conclusion and clinical relevance:*** Application of PRF could improve the healing of distal limb wound defects in donkeys.

***Keywords:*** Donkeys, Platelet-rich fibrin, Distal limb wound, Second intension healing.

## 1.INTRODUCTION

Achieving proper healing of equine lower leg wounds as well as decreasing allied complications stills considered a significant challenge to veterinarians. Distal limb wounds are frequently prone to many complications because of lack of blood supply [1], bony prominence, and lack of supportive soft tissue [2], high rate of motion over joints, and the tendency for infection[3]. Ideal wound healing necessitates an increase in contraction rate, acceleration of

epithelialization, and avoidance of exuberant granulation tissue [3].

Platelet-rich fibrin(PRF)contains fibrin mesh plus extra leukocytes with a great concentration of growth factors (GFs) that substitute for platelet-rich plasma (PRP) as an effective therapy for tissue repair[4]. PRF membrane exhibits a gradual steady-state release of platelet growth factors for as long as seven days. In a veterinary aspect, the influence of PRF on the healing of cutaneous wound had been investigated in dogs[5]on tendon repair in sheep[6],on chronic open wounds in bucks[7] and distal limb wounds in donkeys[8]The present study aimed to evaluate the effect of single and three application of PRF on distal limb wound defects in donkeys clinically, histologically and immunohistochemically, in addition to the gene expression patterns of angiogenic and myofibroblast genes (VEGF-A), collagen type 3 α1 (COL3α1), and fibroblast growth factor 7 (FGF-7)and (TGFβ1) were performed.

## 2. MATERIALS AND METHODS

### *2.1. Animals*

Twelve male healthy donkeysof4-5 years old,weighted130 - 230 kg, free of scars or blemishes on the metacarpal and metatarsal regions, were subjected to the experimental work. These animals were clinically examined for signs of discomfort or lameness. Two weeks before the experiment, donkeys were housed in a separate hygienic condition, fed on a balanced ration, and administered anthelmintic drug (Equiveen Paste, (Ivermectin Paste at 0.2 mg/kg BW, Adwia Company, Egypt).

### *2.2. Preparation of PRF*

Twenty milliliters of venous blood was collected from each operated donkey in vacutainer tubes (without anticoagulant) and immediately centrifuged at 3000 rpm for 10 minutes; three distinct layers were formed, the middle layer (PRF clot) was collected, prepared then applied[4]

### *2.3. Experimental study*

On the day of surgery, a catheter (14gauges -2 inch) was aseptically placed into the jugular vein of the operated donkeys. Metacarpal and metatarsal regions were prepared aseptically for surgery. The operated donkeys received a single intramuscular dose of penicillin-based antibiotic (6 mg/kg bodyweight procaine penicillin with 4.5 mg/kg bodyweight benzathine penicillin equivalent to 1 ml per 25 kg bodyweight) (Norocillin LA, Norbrook Co.UK). TIVA was obtained by intravenous injection (IV) of 0.05mg/kg acepromazine maleate 1.5% (Castran, Interchemie Co., Holland). Fifteen minutes later, 2% xylazine HCL (1mg/kg) was injected IV (Xylaject-Adwia Company., Egypt). Five minutes later,propofol 1% was injected IV as a bolus dose of 2mg/kgthen continued by an infusion rate of 0.2 mg/kgbwt/min for maintenance of anesthesia (Pofol, Eimc Co., Dongkook, Korea).A tourniquet was done to the region overhead carpal or hock joint. A sterile metal rectangular template (3×2cm) was used for the creation of full-thickness wounds defects on the dorsolateral surface of metacarpi and metatarsi regions. Wound defects were created on two limbs of each donkey (two wounds on each limb, upper and lower wounds with minimum 4-8 cm apart). Donkeys were allocated into three groups. Group (A): the defects were left spontaneous healing, and without applications of PRF, groups (B&C) were subjected to single PRF and triple PRF application, respectively (4 animals, with16 wounds defects per group). The freshly prepared PRF membrane was fenestrated before its application on the treated wounds .Then, the tourniquet was removed, and the wounds of two limbs were bandaged after the application of non-adherent dressing and changed every two days. In group (C), PRF had reapplied again at days 7 and 14 post-operative.

### *2.4. Wound healing assessment*

#### *2.4.1. Clinical wound healing evaluation*

Wound size, epithelialization, and granulation tissue development were evaluated for all wound defects via digital photographs directly taken after wound defects at day zero until complete epithelialization of the wound defects using tape measures and digital caliper[9],

#### *2.4.2. Histopathological evaluation*

Tissue samples were obtained on 15 and 45 days after interference. Histopathological processing was performed according to the standard technique. Samples were stained with Hematoxylin & Eosin (H&E) and Masson Trichrome stain (MTs) [10]

#### *2.4.3. Immunohistochemical analysis*

Paraffin-embedded tissue was cut (4 µm thick) and mounted on saline coated glass slides. The sections were deparaffinized in xylol and dehydrated through a graded ethanol series. Antigen retrieval was performed by autoclaving at 120˚C for 10 min at pH 6.0. Endogenous peroxidase activity was blocked with 3% $H_2O_2$ for 10 min. Tissue sections were then incubated primary antibodies against TGFβ, FGF, EGFR (ready to use, Bio Genex). They were incubated at room temperature for one h, followed by washing three times with phosphate buffer saline. The tissue slides were incubated anti-rabbit secondary antibodies at room temperature for 30 min and visualized by incubation with the three diaminobenzidinetetrahydrochloride liquid system (Dako) at room temperature for 5 min. The sections were subsequently counterstained with hematoxylin.

#### *2.4.4. Gene expression analysis*

Tissue samples were homogenized and lysed using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). RNA concentrations and purities were measured using an Implen spectrophotometer (Implen, Westlake Village, CA, USA). For each sample, cDNA was synthesized from 1 $\mu$g total RNA using a Sensi Fast cDNA synthesis kit (Bioline, Taunton, MA, USA.). The newly formed complementary DNA (cDNA)was mixed with the master mix (TaKaRa, Otsu, Japan) and suitable target primers to explore the response of the tissue to the induced wound: (COL3α1), (VEGF-A), (FGF-7), and (TGFβ1). Reactions

will be performed on a Pikoreal system (Thermo Fischer Scientific, Waltham, MA, USA). At each time point, gene expression of the removed tissue will be compared to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a housekeeping gene.

Results were adjusted to normal, according to the level of GAPDH. Three replicates from every biological sample were used, and results will be expressed as the mean and standard error.

*2.5. Statistical analysis*

Statistical analyses were performed using a statistical software program (Graphpad Prism, version 5.1). The Homogeneity of groups was evaluated by the Kruskal walis test to study the effect of various interventions and time. A triple measure ANOVA was performed. The results were presented considered the effect of the time, time*treated interaction. When there is a significant effect, a one-way ANOVA was performed at each time point. Results were considered significant when $P<0.05$. Data were presented as the mean ± standard deviation (SD.)

## 3. Results

*3.1. Clinical findings*

The intervals needed for complete healing of the wound defects were significantly shorter in both triple, and single PRF treated wound defects (63.2±2.8 & 71.6±3.8, respectively) compared with untreated one (86.3±3.1).Rates of wound defects contraction, epithelization, and healing were significantly higher in both treated PRF groups compared with the control group, but three PRF application revealed the highest rates (Figure 1)(Table3).

*3.2. Histopathological Findings*

Semi-quantitative evaluation of histological sections at 15 and 45 days after interference showed a significant difference ($P<0.05$) in epithelization, PMNL, fibroblasts, tissue macrophages, neo-angiogenesis and new collagen scores in both PRF treated groups compared to control one. Also, there was a significant difference in three PRF application groups compared to a single one (Figure 2) (Table4).Masson trichrome stain (MTS) threw the light on collagen development which demonstrated at 15 days after interference minimal fine scattered bluish stained fibrils of collagen without collagen in most of the granulation tissue in control wound defects (Figure 3A), fine bluish stained fibrils of collagen forming strands running throughout granulation tissue in single PRF treated wound defects (Figure 3B) and bluish stained collagen forming bundles haphazardly arranged all over the granulation tissue in triple PRF treated wound defects (Figure 3C). At 45 days, MTS revealed less bluish stained collagen fibrils forming strands in control wound defects (Figure 3D), bluish collagen bundles haphazardly condensed around blood vessels, and perpendicular to parallelly arranged bundles in single PRF treated wound defects (Figure 3E) and bluish collagen bundles arranged in normal wavy bundles forming parallel fibrocollagenous tissue in the dermis in triple PRF treated wound defects (Figure 3F).

*3.3. Immunohistochemical analysis*

Qualitative analysis of immunohistochemical views of the wound defects showed a significant immunostaining difference against EGFR, VEGF, and TGFβ stain between both groups and control one (Figures4,5&6). The average count of cells immunostained for EGFR, VEGF, and TGFβat 15 and45 days post-operative showed a significant increase in both PRF groups compared to control one. In addition, there was a significant increase in triple PRF group compared to a single PRF one (Table5).

*3.4. Gene expression analysis*

The relative expression of FGF-7, TGFβ1, VEGF-A, and COL3α1 genes was higher in both PRF groups compared to control one, but the triple PRF group revealed the highest expression and statistical analysis revealed these expressions (Table6).

**Table: 1.** Explanation of used scale in the semi-quantitative evaluation of histological sections.

| No | Epithelization | PMNL | Tissue macrophages | Fibroblasts | Neo-angiogenesis | New collagen |
|---|---|---|---|---|---|---|
| 0 | Thickness of cut edges | Minimum | Absent | Absent | Absent | Absent |
| 1 | Migration of epithelial cells | Mild | Mild | Mild | Mild | Mild |
| 2 | Bridging of the incision | Moderate | Moderate | Moderate | Moderate | Moderate |
| 3 | Complete regeneration | Marked | Marked | Marked | Marked | Marked |

The histological structures and processes (epithelization, polymorphonuclear leucocyte (PMNL), tissue macrophages, fibroblasts, new collagen and neoangiogenesis) were semi-quantitatively evaluated in coded slides according to the following scale: 0, 1, 2, and 3.

**Table 2.** List of primer used in gene expression analysis**.**

| Gene | Primer sequence | Accession number |
|---|---|---|
| GAPDH | F: GGAGTAAACGGATTTGGCC<br>R:CATGGGTGGAATCATACTGAAA | XM_014834961 |
| TGF-β1 | F:TAATTCCTGGCGCTACCTCA<br>R:CATGAGGAGCAGGAAGGGT | HM569606 |
| FGF-7 | F: GACAGTGGCAGTTGGAATTGT<br>R: CAACAAACATTTCTCCTCCACTG | NM_001163883 |
| VEGF | F: TCATTTCTCCAGGGTTTACCCT<br>R:ATTTGGGGGAGTAGAAGAGCAA | XM_014837457 |
| COL3 α1 | F: TTCCTGGGAGAAATGGTGACC<br>R:GGAGAATAGTTCTGACCACCAGT | XM_014852914 |

**Table 3.** Mean values and SD of wound size ($cm^2$), wound contraction (%), Epithelization (%) and Wound healing (%) in donkeys in control and both PRF treatment groups.

| Criteria | Groups | Time post treatment (week) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1st | 3rd | 5th | 7th | 9th | 10th |
| Wound size ($cm^2$) | A | 7.2±0.3$^{a}$ | 5.5±0.08$^{a}$ | 4.4±0.3$^{a}$ | 3.7±0.2$^{a}$ | 3.2±0.3$^{a}$ | 2.8±0.2$^{a}$ |
| | B | 6.5±0.08$^{b}$ | 4.7±0.1$^{b}$ | 2.9±0.8$^{c}$ | 2.0±0.1$^{d}$ | 1.5±0.2$^{b}$ | 1.2±0.13$^{c}$ |
| | C | 6.4±0.1$^{b}$ | 4.3±0.12$^{d}$ | 2.3±0.1$^{d}$ | 1.5±0.13$^{e}$ | 1.0±0.13$^{d}$ | 0.7±0.1$^{d}$ |
| Wound contraction (%) | A | -20.7±4.6$^{b}$ | 8.7±1.8$^{d}$ | 27.3±4.8$^{d}$ | 39±2.8$^{e}$ | 47.5±5.1$^{d}$ | 53.3±3.5$^{e}$ |
| | B | -7.6±1.5$^{a}$ | 21±2.2$^{b}$ | 51.6±2.8$^{b}$ | 67.7±2$^{c}$ | 74.3±2.6$^{b}$ | 80.1±2.0$^{c}$ |
| | C | -6.9±2.0$^{a}$ | 28.7±2$^{a}$ | 62.5±2$^{a}$ | 74.5±2.4$^{b}$ | 84±1.5$^{a}$ | 89.2±1.9$^{b}$ |
| Epithelization (%) | A | 0.0±0.0 | 9.6±1$^{d}$ | 32.7±1.8$^{d}$ | 45.7±1.9$^{d}$ | 50.8±1.9$^{d}$ | 54.7±1.8$^{c}$ |
| | B | 0.0±0.0 | 27.6±1.8$^{b}$ | 56.7±1.8$^{b}$ | 73.7±1.7$^{b}$ | 84.7±1.7$^{b}$ | 89.7±1.7$^{b}$ |
| | C | 0.0±0.0 | 34.7±1.6$^{a}$ | 68.8±1.6$^{a}$ | 81.6±1.8$^{a}$ | 89.7±1.8$^{a}$ | 94.7±1.9$^{a}$ |
| Wound healing (%) | A | -20.7±4.6$^{b}$ | 8.8±1.6$^{d}$ | 24.6±1.8$^{d}$ | 41.7±1.6$^{d}$ | 56.5±1.8$^{d}$ | 66.7±1.7$^{d}$ |
| | B | -7.6±1.5$^{a}$ | 30.6±1.7$^{b}$ | 43.7±1.8$^{b}$ | 64.6±1.8$^{b}$ | 79.6±1.8$^{b}$ | 84.6±7.6$^{b}$ |
| | C | -6.9±2.0$^{a}$ | 36.7±1.7$^{a}$ | 51.6±1.7$^{a}$ | 73.6±1.8$^{a}$ | 88.6±1.8$^{a}$ | 93.7±5.0 $^{a}$ |

A (control), B (single PFR), C (triple PRF)
Time, p<0.0001; Time * treatment, Wilks'Lambada, p<0.0001; All within interactions, Wilks'Lambada, p<0.0001
Means with different superscript letters in the same column are significantly different at p<0.05

**Table 4.** Semi-quantitative evaluation of histological changes / structures during skin wound healing in in donkeys in control and both PRF treatment groups.

| Treatment groups | Day | Epithelization | PMNL | Tissue macrophages | Fibroblasts | Neo-angiogenesis | New collagen |
|---|---|---|---|---|---|---|---|
| A | 15 | 0.3±0.5$^{b}$ | 0.5±0.5$^{c}$ | 0.5±0.5$^{b}$ | 0.8±0.4$^{b}$ | 0.8±0.4$^{c}$ | 0.2±0.4$^{c}$ |
| B | | 1.2±0.8$^{a,b}$ | 1.5±0.5$^{b}$ | 1.3±0.8$^{a,b}$ | 1.7±0.5$^{a,b}$ | 1.5±0.5$^{c}$ | 1±0.0$^{b}$ |
| C | | 1.3±0.5$^{a,b}$ | 1.8±0.4$^{a,b}$ | 1.5±0.5$^{a,b}$ | 1.8±0.4$^{a}$ | 1.7±0.8$^{c}$ | 1.3±0.5$^{b}$ |
| A | 45 | 1.2±0.4$^{d}$ | 0.8±0.4$^{c}$ | 1±0.0$^{b}$ | 1.7±0.5$^{c}$ | 1.5±0.5$^{b}$ | 0.7±0.5$^{b}$ |
| B | | 2±0.0$^{b}$ | 2.3±0.5$^{a,b}$ | 2.3±0.5$^{a}$ | 2.3±0.5$^{a,c}$ | 2.3±0.5$^{a,b}$ | 2.5±0.5$^{a}$ |
| C | | 2.8±0.4$^{a}$ | 2.8±0.4$^{a,b}$ | 2.8±0.4$^{a}$ | 3±0.0$^{a}$ | 2.7±0.5$^{a}$ | 2.8±0.4$^{a}$ |

A (control), B (single PFR), C (triple PRF)
Time, p<0.0001
Time * treatment, Wilks'Lambada, p<0.0001
All within interactions, Wilks'Lambada, p<0.0001
Means with different superscript letters in the same column at the same time point are significantly different at p<0.05

**Table 5.** Average count of cells immunostained for EGFR, VEGF and TGF-β1 in surgically induced wound defects in donkeys in control and both PRF treatment groups.

| Treatment groups | Day | EGFR | VEGF | TGFβ |
|---|---|---|---|---|
| A | 15 | 43.8±1.7$^{e}$ | 120.5±1.9$^{f}$ | 25.5±2.5$^{f}$ |
| B | | 155.2±6.9$^{d}$ | 184.7±4.2$^{d}$ | 75±3.7$^{d}$ |
| C | | 256.2±8$^{b}$ | 267±5.8$^{b}$ | 144.8±5.2$^{b}$ |
| A | 45 | 76.2±3.1$^{e}$ | 151.5±6.3$^{f}$ | 86±3.7$^{e}$ |
| B | | 187.5±2.3$^{d}$ | 221.2±5.2$^{d}$ | 133.3±4.5$^{c}$ |
| C | | 316.2±4.6$^{b}$ | 322.3±9.6$^{b}$ | 174.5±4$^{b}$ |

A (control), B (single PFR), C (3 PRF); Time, p<0.0001; Time * treatment, Wilks'Lambada, p<0.0001; All within interactions, Wilks'Lambada, p<0.0001
Means with different superscript letters in the same column at the same time point are significantly different at p<0.05

**Table 6.** Mean values and standard errors of relative expression of FGF-7, TGF-β1, VEGF- A and COL3 α1, at two points time (15 & 45 day) in in donkeys in control and both PRF treatment groups.

| Groups | Day | COL3 α1 | | FGF-7 | | TGF-β1 | | VEGF- A | |
|---|---|---|---|---|---|---|---|---|---|
| A | 15 | 1 | 0.058$^{d}$ | 1 | 0.04$^{g}$ | 1 | 0.08$^{e}$ | 1 | 0.03$^{f}$ |
| B | | 3.2 | 0.04$^{c}$ | 6.05 | 0.24$^{de}$ | 3.72 | 0.15$^{cd}$ | 4.12 | 0.13$^{de}$ |
| C | | 5.5 | 0.2$^{b}$ | 7.65 | 0.11$^{cd}$ | 6.35 | 0.13$^{b}$ | 6.57 | 0.12$^{bc}$ |
| A | 45 | 1 | 0.115$^{d}$ | 1 | 0.2$^{g}$ | 1 | 0.05$^{e}$ | 1 | 0.12$^{f}$ |
| B | | 6.06 | 0.8$^{b}$ | 9.6 | 0.33$^{b}$ | 6.3 | 0.14$^{b}$ | 8.81 | 0.14$^{b}$ |
| C | | 11.6 | 0.04$^{a}$ | 13.6 | 0.14$^{a}$ | 11.2 | 0.15$^{a}$ | 11.79 | 0.12$^{a}$ |
| Row factor | | 0.0001 | | 0.0001 | | 0.0001 | | 0.0001 | |
| Column factor | | 0.0001 | | 0.0001 | | 0.0001 | | 0.0001 | |
| interaction | | 0.0001 | | 0.001 | | 0.0001 | | 0.0001 | |

(control), B (single PFR), C (triple PRF)
eans with different superscript letters in the same column at the same point time are significantly different at p<0.0001


**Figure 1.** Gross appearance of the of progress cutaneous wound defects healing in distal limbs of donkeys which showed that at third week after interference, regular granulation tissue but slightly above skin level in the control group (A), detection of epithelization in single PRF group (B), healthy granulation tissue and marked degree of epithelization in repeated PRF group (C). At seven$^{th}$ week, progress of the epithelization process in control group (D), nearly complete epithelization of single PRF wound defects (E) and obviously & completely progress in repeated PRF treated wound defects (F).

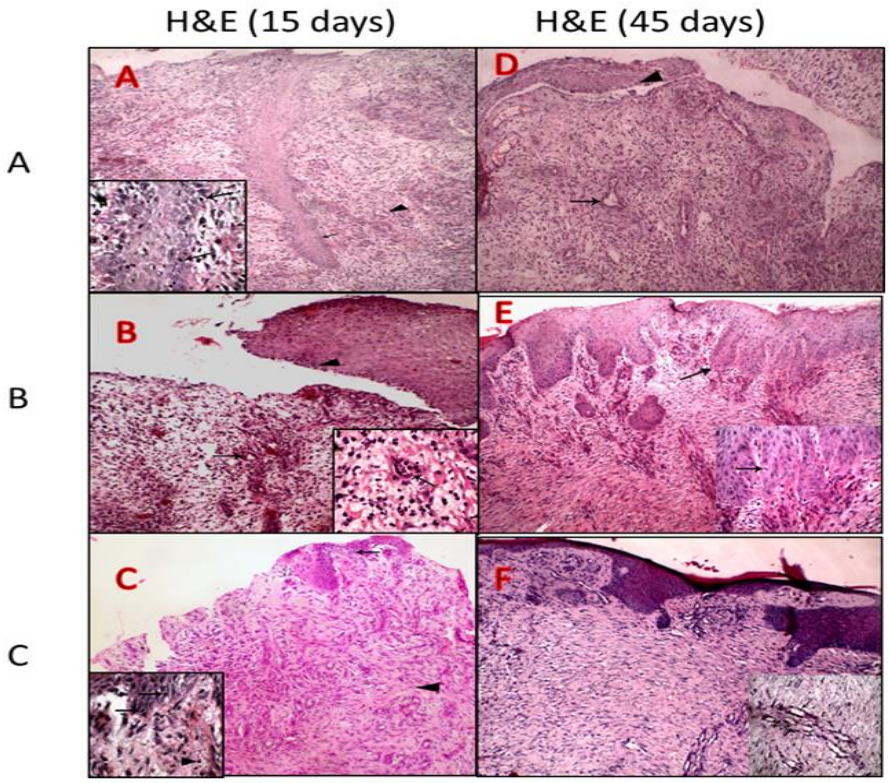


**Figure 2.** Histological findings in the control group (A, D), single PRF group (B, E) and repeated PRF group (C, F) at 15 &45 days showed (A) immature blood vessel (arrowhead), with frequent mitosis of the epithelium (arrows);(B) immature blood capillaries (inset) thickness and migration of epithelium (arrowhead);(C) fibro-collagenous proliferation (arrowhead), reepithelization with active mitosis in basal cell layer(inset). (D) some mature blood vessels (arrow), mitosis with the migration of epithelium on the surface of the wound (arrowhead) (E) papillated rete ridges, and hyperplasia of the epidermis (inset),(F) reepithelization with complete layers of epidermis, mature blood capillaries (inset).

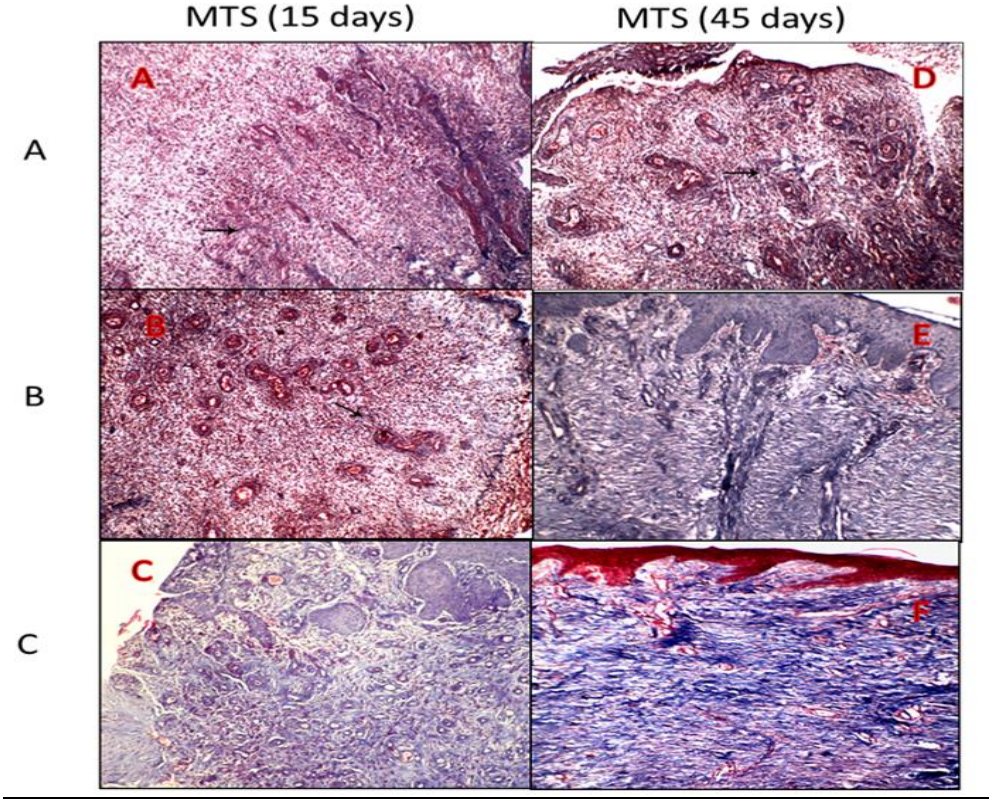


**Figure3.** Masson trichrome stain of the control group(A, D), single PRF group (B, E) and repeated PRF group (C, F) at 15 & 45 days showed; (A) minimal fine scattered bluish stained collagen fibrils (arrow),(B) fine bluish stained strands fibrils of collagen (arrow),(C) haphazard bundles bluish stained collagen. (D) less bluish stained collagen fibrils forming strands principally at perivascular (arrow) (E) bluish collagen bundles haphazardly condensed around blood vessels and perpendicular to parallelly arranged bundles (F) bluish collagen bundles arranged in normal wavy bundles forming parallel fibrocollagenous tissue in the dermis.

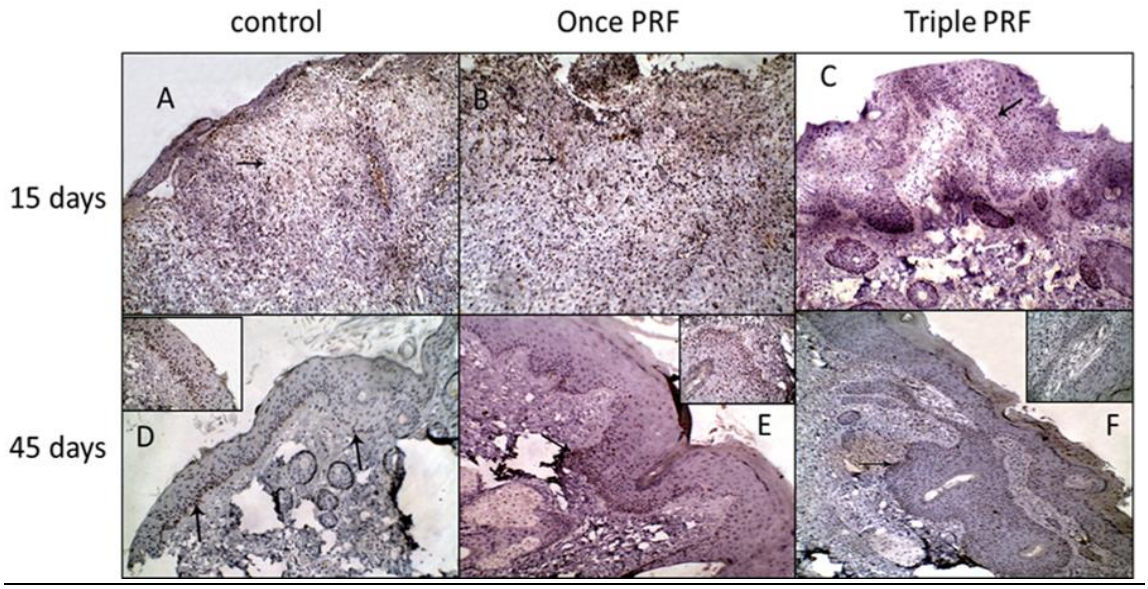


**Figure 4.**Immunohistochemical views in control (A, D), once PRF (B, E) and triple PRF(C, F) treated wound defects at 15 & 45 days against EGFR showing a strong positive brown immunostaining for epithelial cells in triple PRF group compared to once PRF and control groups.

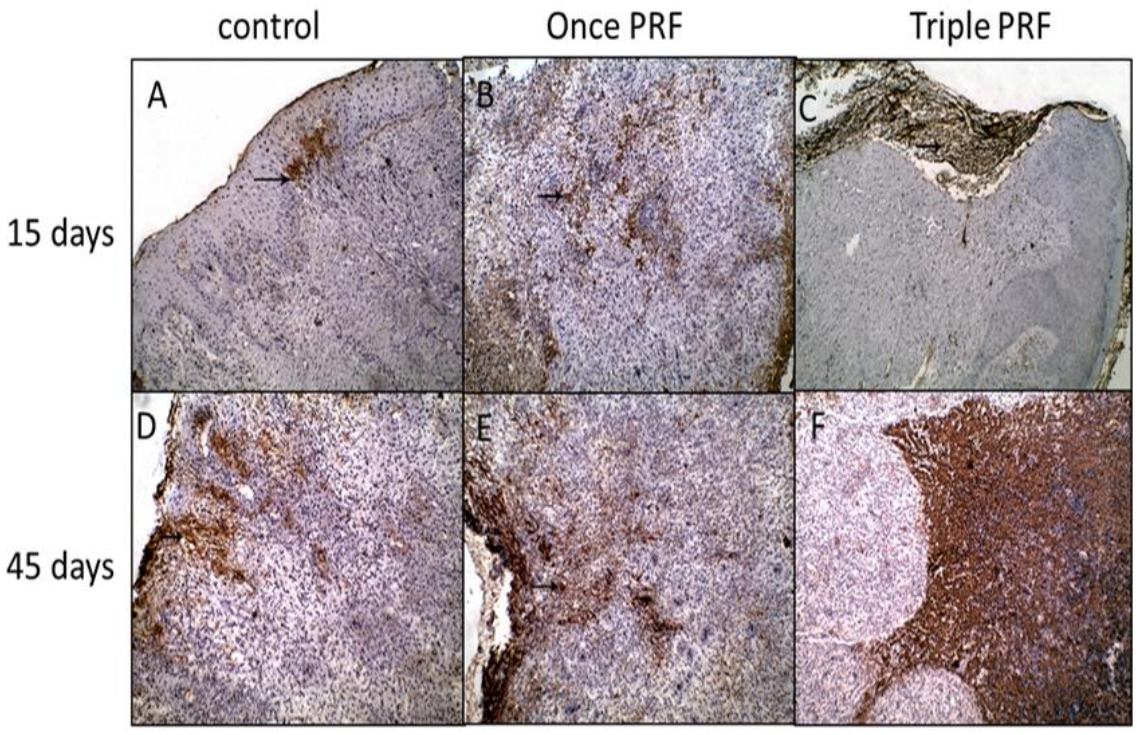


**Figure5.**Immunohistochemical views in control (A, D), once PRF (B, E) and triple PRF(C, F) treated wound defects at 15 & 45 days against VEGF showing a strong positive brown immunostaining for newly formed angioblasts in triple PRF group compared to once PRF and control groups.

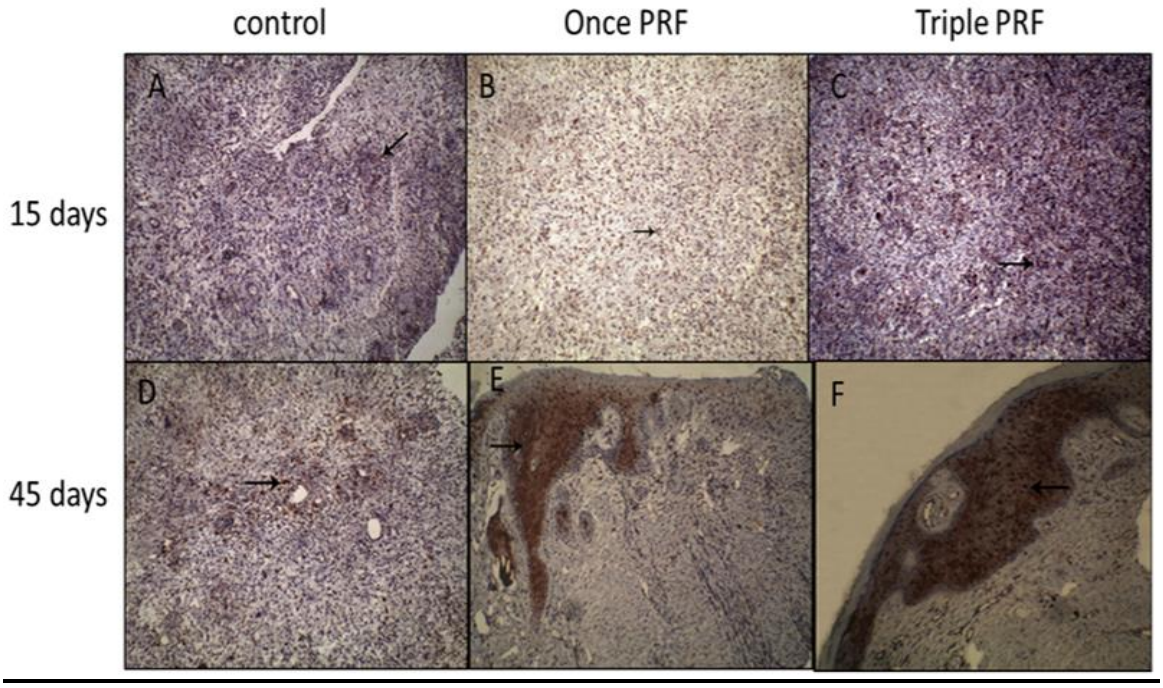


**Figure 6.**Immunohistochemical views in control (A, D), once PRF (B, E) and triple PRF(C, F) treated wound defects at 15 & 45 days against TGFβ stain showing a strong positive brown immunostaining for fibroblasts in triple PRF group compared to once PRF and control groups.

## 4. Discussion

PRF has been revealed a clinical advantage to PRP because it possesses the benefits of easier, simple preparation, a balanced exit of GFs, and fibrin production to construct a basic framework that protects growth factors from proteolysis. So, growth factors can keep their activity for a relatively long period (5-7 days) and stimulate tissue regeneration effectively [11, 12].

In the current study, promotion of wound healing was produced in a short period after repeated PRF application (63.2±2.8) compared to single treatment (71.6±3.8) and untreated wound defects (86.3±3.1); it could be attributed as previously mentioned[8] to effective wound contraction and proper epithelization induced PRF. PRF membrane was applied directly after its separation to ensure the maximum release of growth factors to the surgical site[13].The produced exudate after squeezing the membrane can be used to hydrate the membrane as it can be an additional source of growth factors[14]. The freshly prepared membrane in this work was fenestrated to introduce uniform slits, to allow drainage of wound exudate; increase moisture and air exchange, and improve the proper application to the wound bed[15].

The surgically created distal limb wound defects were increased in size during the first week post-operatively then decreased subsequently. This enlargement may be attributed to greater mobility and the skin tension forces that retract the skin edges and cause wound expansion. These observations were similar to those noticed in previous reports[1,8]. Bleeding was markedly decreased in PRF treated wound defects compared to untreated ones which could be illuminated on the basis of the quick formation of a fibrin network, which encourages quick hemostasis as mentioned by[16].The infection signs were not observed after the application of autogenic PRF. This may be relayed on the mutable quantity of leukocytes in PRF that have a bactericidal effect[17]. The absence of inflammation signs in treated wound defects indicates the great capability of PRF in weakening the inflammatory cascades in comparison with non-treated wound defects that showed slight edematous swelling. A clear and advanced rate of epithelialization in single and triple PRF groups (27.6% &34.7 %) compared to control one (9.6%) was recorded in third week post-operative. This may be attributed to the capability of platelets to stimulate differentiation of keratinocytes into different epithelial cell varieties including epithelial cells of the skin as described by[18] The contraction rate of all PRF treated wound defects was higher than in untreated one but repeated PRF treated wound defects showed the highest rates. This finding could be relayed on the amount plus action of myofibroblasts that accountable for the centripetal motion of the wound edges[19, 20].

Histopathological investigation in single PRF treated wound defects compared to untreated one displayed obvious proliferative action in epidermal and dermal layers, and these events were more pronounced in repeated PRF applications. The greater epithelialization was attributed to the amount of growth factors provided by PRF for the wound defects for the long interval[5] and the high mitotic actions of proliferative keratinocytes, which were established in both strata basale and spinosum that consequences in the

construction of specific rete ridges by way of interdigitating among the hyperplastic neo-epidermis besides well-structured dermal connective tissue[21]Significant increase in neo-angiogenesis score in triple PRF treated wound defects compared to single treated and untreated wound defects . This agreed with[22, 23].These findings attributed to the amount of VEGFs and its regulation that lead to an increase in angiogenesis [24].

The expression of VEGF immunohistochemistry displayed a significant increase in single PRF treated wound defects in comparison to untreated ones, and this expression was higher in triple PRF treated wound defects [25]. VEGF is a potent angiogenic factor, and that explained the essential role of PRF in the promotion of wound healing process via neo-vascularization. [26, 27]

Similarly, EGFR immunohistochemistry shown a significant increase in single PRF treated wound defects in comparison to untreated wound defects, and this expression was higher in triple PRF treated wound defects. EGF is released by platelets and is chemotactic for fibroblast, and this showed the important role of PRF in wound healing via accelerating the rate of epidermal regeneration and increased wound tensile strength[28] especially its repeated application. These results were similar to those found previously[18]in lower leg wound defects in donkeys following PRP application. Also, the expression of TGFβ immunohistochemistry exhibited a significant increase in the single PRF group when compared to the control one, and this expression was higher in the triple PRF group. This higher expression in treated wound defects revealed the role of PRF in fibroblasts activation for pre-collagen formation that prompts the deposition of collagen and healing wound as agreed with [26]

The TGFβ1and COL3α1 expressions showed significant overexpression in single PRF treated wound defects compared to untreated ones, and this expression was higher in repeated PR applications[8].similarly, VEGF-A, and FGF-7 revealed overexpression in both PRF group compared to untreated one, but this expression was higher in repeated PRF application. This attributed to the long duration of PRF action [8, 29-31]; they recorded a significant increase of VEGF mRNA expression in stromal cell differentiation in rat bone marrow when PRP was used. Also, FGF expression was confirmed in rat periodontal tissues and in lower leg wound defects in donkeys.

### *Conclusion*

In conclusion, 3 topical applications of PRF as an autologous, enhanced healing of cutaneous wound defects in distal limbs of donkeys as documented clinically, histologically, immunohistochemically, and by gene expression analysis.

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### *Conflict of interest statement*

The authors declare that there is no conflict of interest

### *Animal ethics committee permission*

The current research work is permitted to be executed according to standards of animal research committee in the Faculty of Veterinary Medicine, Mansoura University.

### *Author' contributions*

Mohamed Albahrawy and Esam Mosbah *performed the experiment, statistical analysis, research writing*. Adel Zaghlouland Khaled Aboelnasr *revised manuscript; Mohamed Hamed, performed histopathological interpretation; Mohamed Aladl performed gene expression analysis.*

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