

# Effect of Hematopoietic Stem Cells and Platelet-Rich Plasma on the Healing of Experimental Skin Burned Tissues: A Comparative Study in Adult Male Mice

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## Original Article

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## ABSTRACT

**Background:** Unfortunately burns are a common problem, leading to scarring or death. Platelet rich plasma (PRP) is a well known method that could be used as topical application for burn either second or third degree; it harbors many growth factors that can accelerate the healing.

**This Study Aimed:** To analyze the use of PRP in deep second-degree burn, in comparison with the hematopoietic stem cells (CD34+) in mice.

**Materials and Methods:** Seventy male adult mice were divided into four groups (control group, burn only, burn treated with CD34+ cells (injected once intradermal with  $0.3-0.4 \times 10^6$  /kg and burn treated with PRP (1 ml injected once intradermal) at the edge of burn after 24hs from its induction. All burned groups were exposed to thermal burn. On day 9 and 19, the animals were sacrificed and skin biopsies were taken for H&E and Masson's trichrome staining. Assessment of angiogenesis and quantification study for matrix metalloproteinase 13 (MMP13) was done.

**Results:** Wounds treated with PRP revealed fast wound closure in comparison with burned only group. Significant decrease in collagen fibers and increase in MMP13 deposition, as well as angiogenic markers (Angio1 and 2 and VEGF) expression was observed. However CD34+ cells treated group showed highly significant improvement in previously mentioned markers. Histopathological changes of burned skin improved in all treated groups after 19 days particularly in CD34 + cells treated group.

**Conclusion:** These results indicated that CD34+ cells treatment could exert beneficial effects on healing process more than that of PRP in second degree burns.

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**Key Words:** Angiogenesis, collagen, hematopoietic stem cell, MMP13, platelet-rich plasma.

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## INTRODUCTION

The skin wound repair is a complex pathological sequence and is formed of several phases' inflammation, epithelium repair and granulation tissue formation. The remodeling of matrix and wound contractions are the final steps. It is controlled by different cytokines and growth factors. A predominantly collagenous acellular scar is mostly the final result of healed skin. Scar might form up to 70% of the basal tensile force of healthy skin associated with effective new extracellular matrix (ECM) remodeling<sup>[1]</sup>. New collagen formation is mainly originating from dermal

Fibroblasts during burn healing; moreover they produce several matrix metalloproteinases (MMPs) that could help in remodeling the stroma<sup>[2]</sup>. MMPs are zinc-dependent endopeptidases, can cleave all ECM molecules and many non-matrix substrates<sup>[2,3]</sup>. The skin MMPs are secreted by endothelial cells, fibroblasts, keratinocytes and inflammatory cells such as macrophages, lymphocytes, and monocytes, several factors are controlling their formation and function. MMPs are rapidly expressed and activated during wound healing. MMP formation can be enhanced by a lot of signals, as: cytokines

(EGF, HGF, FGF, VEGF, PDGF, TNF- $\alpha$ , KGF, TGF- $\beta$ ), hormones<sup>[4]</sup>. MMPs are also activated in several pathological and physiological conditions, as tumor and angiogenesis. They are implicated in stimulation of cell migration, growth factors and proteinases activation, during wound repair<sup>[2,3]</sup>. MMP-13 and MMP-9 exert vital roles in healing of wounds through different mechanisms. MMP-13 has a vital role in the granulation tissue maturation, including myofibroblast modulating function, inflammatory reaction, new vessel formation, and matrix degradation<sup>[5]</sup>. Furthermore, it has important role in migration of keratinocyte, wound contraction and re-epithelization. It affects wound contraction through its effects on the following substrates: Collagen III, II, I, X, IX, IV, and XIV, fibronectin, tenascin, gelatin, serpins, aggrecan, fibrillin and laminin<sup>[6]</sup>. Scar formation and wound contraction during the healing process of deep partial or full-thickness burns treated by the known methods are still unsatisfactory in majority of cases ; therefore, it is essential to discover new therapy that might reduce the harmful effect of burn in humans<sup>[7]</sup>.

Several methods are used for treatment of thermal injuries either concentrated platelets in platelet-rich plasma (PRP) that may provide a higher amount of several bioactive growth factors<sup>[8]</sup>. Recently cellular therapy is new trend especially mesenchymal stem cells (MSCs) due to their characteristics features as prolonged ex vivo proliferation, multilineage potentiality as well as immunomodulatory properties. Stem cell application for regeneration after skin burns may have important clinical significance. On the other hand, umbilical cord blood is a valuable origin of hematopoietic stem cells (HSCs) for clinical cases and is considered the big source of stem cells with naive immunity<sup>[9,10]</sup>. It gives 30% less multipotent stem cells than that obtained from adult bone marrow (10). Moreover cord blood transplanted has the same disease free survival as well as mortality occurred in bone marrow transplants<sup>[11]</sup>. Therefore in cases of skin burns as well as other skin wound healing umbilical cord is considered new hope of “off-the-shelf” cell- skin engineerin<sup>[12]</sup>. It has been found that co-treatment with epithelial and mesenchymal stem cells might give synergistic effects<sup>[13]</sup>. However, questions regarding which type of stem cell is better in burn therapy are still not answered? To determine whether transplantation of human hematopoietic stem cells (CD34+ cells) or platelet rich plasma has been proved to be effective in healing of skin burn?

So far, no studies had been done to determine whether hematopoietic stem cells have distinguishable properties in terms of regenerative, pro-angiogenic or anti-inflammatory activities in cases of skin burn injury?

### **Purpose**

This study had been performed to elucidate the efficiency of the hematopoietic stem cell in pathogenesis of skin burn wounds in animal model in comparison to PRP in this respect. It is required for better understanding

properties of this cell type, as well as determining if these cells are appropriate type to be used in skin burn.

## **METHODS AND MATERIALS**

### ***The preparation of platelet-rich plasma***

The peripheral blood was taken from healthy individuals after written consent. 20 mL was collected into tubes contained citrate-dextrose-acid and at 1000g for 10 minutes. It was centrifuged to get PRP at the surface of the test tube. PRP was further centrifuged to obtain a platelet concentrate (PC) at 1500g for 10 minutes. The final product was 4.5 times platelets more than platelet-poor plasma and the baseline. The PC was kept in sterile tube and added human thrombin (0.2 ml /1mL PC) and calcium gluconate to it immediately before injection to activate platelets. The 20 ml of whole blood yields 2 ml PRP<sup>[8]</sup>.

### ***CD34+ cells preparation***

28 ml umbilical cord blood was obtained from Women's Health Hospital, Assiut University during delivery after signature of written consent. It was done according to Giarratana *et al.*, (2005): Centrifugation of the whole blood density gradient e.g. Ficoll-Paque™, Lymphoprep™ was used to separate peripheral blood mononuclear cells, thus harvesting cells from the blood plasma. Washing of cells once with buffer was done. Up to 10<sup>8</sup> cells were suspended in a volume of 300  $\mu$ L of buffer. Magnetic labeling was done for separation using 2 mL conjugated CD34 Micro Beads to mouse monoclonal antibody anti-human CD34 (mouse IgG1 isotype) + 2 mL Blocking Reagent FcR, human by the following protocol<sup>[14]</sup>:

Magnetic labeling: we determined cell number and centrifuged cell suspension at 300.g for 10 minutes and aspirated all supernatant then suspended the pellet of cells in buffer 300  $\mu$ L for up to 10<sup>8</sup> cells and added FcR 100  $\mu$ L blocking reagent for up to 10<sup>8</sup> total cells. Then CD34 MicroBeads 100  $\mu$ L was put on 10<sup>8</sup> total cells. Good Mixing and incubation in the refrigerator (2–8 °C) for 30 minutes was done. Conjugated CD34 fluorochrome antibody was added to recognize other epitope than QBEND/10 (e.g. clone AC136: # 130-081-002 CD34-PE) or conjugated CD45 fluorochrome antibody (e.g. # 130-080-202 CD45-FITC) and incubated in the dark for 5 minutes in the refrigerator (2–8 °C) then the cells washed by adding buffer 5–10 mL for up to 10<sup>8</sup> cells and centrifuged at 300g for 10 minutes. The supernatant was aspirated before suspension of the cells (up to 10<sup>8</sup> cells) in 500  $\mu$ L of buffer. Then magnetic separation was done in the magnetic field by putting column of a fit MiniMACS™ separator. The column was removed from the separator to put it on a collecting tube then the buffer was pipetted onto the column to get out the magnetically labeled cells. We used second MS or LS column to elevate the purity of CD34+ cells.

### ***Hematopoietic progenitor cell purity evaluation***

After isolation of CD34+ cells they were stained with

CD34-PE (# 130-081-002), debris and dead cells from the analysis were excluded according to propidium iodide fluorescence and scatter signals. The Haematopoietic cell population was identified on the basis of its morphology<sup>[15]</sup>.

### **Flow cytometric analysis**

CD34 Cells identification was made by flow cytometry as mentioned by the Cellular Therapy International Society. Monoclonal PE-conjugated antibodies for CD34 and FITC (BD Pharmingen, San Diego, CA, USA) were incubated with the sorted hematopoietic cells at room temperature for 30 min. To stain the cells as control we used isotype control IgG. Phosphate – buffered saline (PBS) was used to wash the 4 % formaldehyde fixed cells and analyzed on a FAC scan flow cytometer (San Jose, CA, USA, Becton-Dickinson). Viability of cells and counting were made by using haemocytometer and trypan blue exclusion test.

### **Experimental design**

#### **Ethics Statement**

All the animal procedures were done according to the standards set forth in the guidelines for the use and care of animals in the experiments by the Committee for the Purpose of Control and Supervision of Experiments on Animals and the National Institutes of Health. This study was approved from Ethical Committee of the College of Medicine, Assiut University.

#### **Thermal burn induction**

The animals were injected intraperitoneally by 0.4 mg/kg mebacaine. The dorsal furs were removed and the animals were put in a plastic mold to expose 10% of the total body surface area (TBSA). 90°C water was put in the exposed surface for 35 seconds. Sever full thickness burn was formed completely after 24 hours. After induction of the burn, all mice were divided and kept in special cages at 22°C with good environment. They were allowed to free access to standard chow and water.

Animals: A total number of 70 male mice at 8–10 weeks of age weighing 50 g were used. We divided the animals into four groups as follow:

Group I (G1): control group included 10 mice, received no treatment.

Group II (GII): Burned only animals 20 mice, after induction of thermal burn they were divided equally into two subgroups (10 mice each).

GII a: they were left for 9 days then sacrificed.

GII b: they were left for 19 days then sacrificed.

Group III (GIII): CD 34+ cells treated group, included 20 mice. They were injected once intradermal with  $0.3\text{--}0.4 \times 10^6$  /kg CD34+ cells in 1ml PBS (20.000 cells /Mice) at the edge of the burn wound 24 hours after burn induction<sup>[16]</sup>.

#### **Then they were divided equally into two subgroups (10 mice each)**

G III a: they were left for 9 days then sacrificed.

GIII b: they were left for 19 days then sacrificed.

Group IV (GIV): PRP treated group included 20 mice and they were injected once intradermally with 1 ml PRP at the edge of burn wound 24 hours after burn induction<sup>[17]</sup>. Then they were divided equally into two subgroups (10 mice each)

G IV a: they were left for 9 days then sacrificed.

GIVb: they were left for 19 days then sacrificed.

#### **Wound healing observation**

We observed the healing of scalded skin in mice from all experimental groups at 1, 4, 9, 11, 13 and 19 after the thermal injuries. The wound area was measured using a ruler and we calculated the percentage of healing in each group in comparison to the baseline (first day of measurement).

#### **Histological studies**

After 24 hours from the end of experiment the animals were anesthetized by intraperitoneal injection of Mebacaine (0.4 mg/kg), and skin samples were taken from all groups. Each sample was divided into three pieces; one was fixed with 10% formalin solution for further histological examination and the other two pieces were stored in -80oC freezer for (qRT-PCR) and western blotting.

Immersion fixation for 12 hours was done and the biopsies were routinely processed by paraffin embedding for H&E. and Masson's trichrome

#### **Western Blotting technique for quantification of MMP13**

SDS–PAGE electrophoresis was done by total acrylamide 10% under non-reduced or reduced conditions. Coomassie Brilliant Blue R- 250 stained proteins for western blot analysis. Electrophoresis by 10% SDS–PAGE and electroblotting onto PVDF membranes was made for samples<sup>[18]</sup>. Skimmed Milk 5% blocked non-specific binding of IgG. Diluted primary antibodies were incubated with the membranes for 1 h. The primary polyclonal antibody of anti-Mouse MMP-13 at 1: 100 was used. Washing the membranes thoroughly then peroxidase-conjugated goat anti-rabbit IgG at a 1:1000 dilution was added and incubated at room temperature for 1 h. Protein bands was determined by the ECL + western blotting detection system (GEL DOC EZ, Biorad).

Assessment of stem cell survival and propagation after transplantation: using HLADQB1-0201 gene to identify human stem cells.

#### **Assessment of angiogenesis**

Relative mRNA expression of genes was analyzed



using qRT-PCR for Ang-1, Ang-2, VEGF as well as GAPDH (House keeping gene). PCR primers were used to reveal gene expression in wound samples:

HLA-DQB-1-0201: Forward primer GTGCGTCTGTGAGCAGAAG

Reverse primer GCAAGGT CGT GCGGAGCT (Size 205 b.p.)

Ang-1: Forward primer TTCCAGCTTGGCTGGATGT

Reverse primer TCTGTGTAACCGTTCAGCGT (Size 150 b.p.)

Angio-2: Forward primer GAGAGCTCAGGACGCAAGTT

Reverse primer GTTCTGGACCTGGTACTGCC (Size 173 b.p.)

VEGF: Forward primer CAGACTATTCAGCGGACTCACC

Reverse primer CCTCAAACCGTTGGCACGA (Size 152 b.p.)

GAPDH (House keeping gene) GAPDH TaqMan® Gene Expression Assays GAPDH TaqMan®. The assay probe spanned an exon junction. This assay detected gDNA, Gene Transcripts: Entrez Gene ID 14433 Gene Name glyceraldehyde-3-phosphate dehydrogenase Chromosome Location Chr.6: 125161338-125166511 on Build GRCh38. Mouse GAPDH. F306 AGGCCGGTGCTGAGTATGTC

R1280 TTTGAGGGTGCAGCGAACTT

### ***RNA isolation and reverse transcription***

Tissues were washed twice after grinding in PBS (warm), and then they separated with trypsin 0.5%. They were washed in PBS (cold), and Trizol (Invitrogen, Carlsbad, CA) was used to isolate RNA passed on the protocol of the manufacture.

Both HLA-DQB1-0201 and HLA-B2704 genes were used to assess survival and propagation of human HSCs after transplantation<sup>[9]</sup>. Specific unique primers sets for (Angio1&2 and VEGF) were used.

### ***Morphometric and statistical analysis***

Morphometric measurements were done by using computerized image analyzer software system (Leica Qwin 500; Leica, Cambridge, UK) attached to a camera in Leica universal microscope at the Faculty of Medicine, Histology Department, Assiut University. The measurement of the area density of collagen fibers in dermis in Masson's trichrome staining sections was done. It was performed in 10 non-overlapping fields (X100) from each section in all groups.

Mean  $\pm$ SEM were expressed all data. The chi-square test compared nonparametric data. The student t test compared the others. P values less than 0.05 were considered significant.

## **RESULTS**

### ***Characteristics of CD34+ cells***

Hematopoietic progenitor cells (HPC) were isolated from cord blood, characterized and identified by expression of the CD34 surface antigen. They exhibited morphological and phenotypic characters of CD34 +cells (Figure 1A).

Macroscopic Evaluation (clinical assessment) was done on several durations to plot a curve that represents the percentage of reduction in the size of burn ulcer after different durations, it was observed that the most rapid healing occurred in CD34 treated group then PRP treated group, the slowest rate was observed in burn only group (Figure 1B). (Table 1)

### ***Histological Examination***

#### ***G1: The control group***

H&E sections of control group examination revealed that thin skin had two layers epidermis and underlying dermis. The epidermis was stratified keratinized squamous epithelium. The junction between epidermis and dermis was prominent. The dermis contained two connective tissue layers reticular and papillary. The papillary layer was formed of thin loosely arranged fibers but the reticular had thick dense arranged ones. Sebaceous glands and hair follicles appeared in the reticular layer (Figure 2). Masson's trichrome stained sections showed that papillary layer contained fine collagen fibers while reticular layer contained wavy thick bundles (Figure 3).

GIIa: After 9 days of burn induction, wide area of epidermal loss was observed with separation between dermis and epidermis in some areas of burn surface. Inflammatory cells were aggregated in the dermis (Figure 4). Masson's trichrome stained sections observed marked degeneration of dermis with denaturized red collagen (Figure 5).

GIII a: After 9 days of CD34+ cells treatment, there was reduced inflammatory cellular infiltration and appearance of thin regenerated epidermis (Figure 6). Fine collagen fibers in the dermis and some denaturized ones were observed in Masson's trichrome sections (Figure 7).

GIV a: After 9 days of PRP treatment incomplete re-epithelization was observed with reduced inflammatory cells aggregates (Figure 8). In Masson's trichrome fine denaturized collagen fibers in the dermis was frequently seen (Figure 9).

GII b: After 19 days of burn induction there was incomplete regeneration of epidermal surface, appearance of hair follicles in dermis with decrease inflammatory cellular infiltration (Figure 10). Sections of Masson's trichrome revealed some scattered collagen fibers running in different directions (Figure 11).

GIII b: After 19 days of CD34+ cells treatment marked regeneration of the epidermis was observed. Multiple sebaceous glands and hair follicles were noticed in dermis (Figure 12). Masson's trichrome examined sections exhibited obvious deposition of collagen fibers parallel to the epidermis (Figure 13).

GIV b: After 19 days of PRP treatment thin regenerated epidermis was observed with incomplete healing of hair follicles (Figure 14). Masson's trichrome sections revealed prominent deposition of collagen fibers in one direction parallel to the epidermis (Figure 15).

### Morphometric and statistical results

There was a significant decrease in collagen fibers area percentage in sections of Masson's trichrome in all groups as compared to control group. A significant decrease after 9 days from induction of burn in GIIIa as compared to GIIa was observed. However no significant change in GIVa as compared to GIIa. After 19 days a significant increase in GIIIb and GIVb as compared to GIIb was observed. However GIIIb had the higher collagen fibers area percentage among the untreated and treated burn groups (Figure 16).

Western Blotting analysis for MMP13 revealed highly significant increase in deposition after 9 days in PRP treated group and significant reduction in CD 34+ cells treated group in comparison with burn group. After 19 days of treatment significant increase in MMP13 deposition was observed in CD 34 treated group, while PRP treated group revealed significant reduction in deposition in comparison to that of CD34 treated group. The CD34 treated group was considered the highest significant MMP13 deposition after 19 days (Figure 17).

### Assessment of Angiogenesis using qRT-PCR

Significant increase in expression of Angio1 gene especially in CD 34 as well as PRP treated groups after 9 days of treatment in comparison with burn only group. After 19 days of treatment the reduced expression of Angio1 gen in CD 34 as well as PRP treated groups but it was still significantly upregulated specially in PRP treated group in comparison with burn only group (Figure 18A and Table 2).

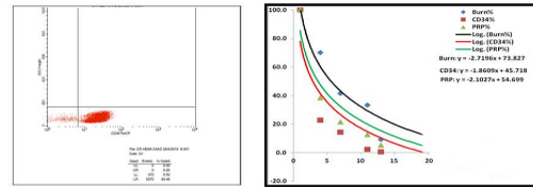
A significant decrease in expression of Angio2 gene especially in PRP as well as CD34+ cells treated groups after 9 days of treatment in comparison with burn only. After 19 days of treatment a significant increase in the expression of Angio2 gene in CD 34 treated group was observed, however PRP treated group was downregulated in comparison with burn only group (Figure 18B and Table 2).

A significant decrease in expression of VEGF gene especially in CD 34 treated groups, however significant upregulation was observed in PRP treated group after 9 days of treatment in comparison to burn only. After 19 days of treatment highly significant increased expression of VEGF gene in CD 34, however PRP treated group was downregulated in comparison to burn only (Figure 18 C and Table 2).

### Tracking the Stem cells using qRT-PCR

Expression of HLA gene in CD34+ cells treated groups after 9 days of treatment in comparison with control, indicating the presence of viable human derived cells, after 19 days of treatment increased expression of HLA gene in CD 34 in comparison with control, indicating the presence of multiplying viable human derived cells (Data not shown).

Fig 1 : Flow cytometry characterization



A) Hematopoietic progenitor cells (HPC), identified by expression of the CD34 surface antigen, show morphological and phenotypic heterogeneity in cord blood.

B) The most rapid healing occurred in Cd34 treated group (Red curve) then PRP treated group (green curve), the slowest rate was observed in burn only group (black curve)

Fig. 1: (A & B): (A) Cord blood hematopoietic progenitor cells (HPC), were identified by positive CD34 surface antigen, show morphological and phenotypic characters of CD34 +cells by flow cytometry. (B) clinical assessment reveal rapid progressive reduction in the size of burn ulcer in CD34 treated group more than that in PRP, while the slowest is observed in burn only group that reflect that the most rapid healing occurred in CD34 treated group then PRP treated group, the slowest rate is observed in burn only group.

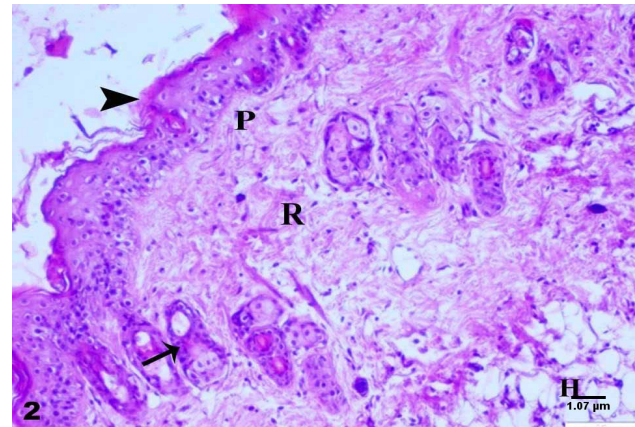


Fig. 2: A photomicrograph of section of mice thin skin of control group showing epidermal layers of keratinized stratified squamous epithelium with thin horny layer (arrowhead). The dermis is consisted of superficial thin papillary (P) and deep thick reticular (R) layers. Cross sections of hair follicles and sebaceous gland (arrow) were seen. Note the underlying hypodermis (H). H&E (scale bare 1.07).

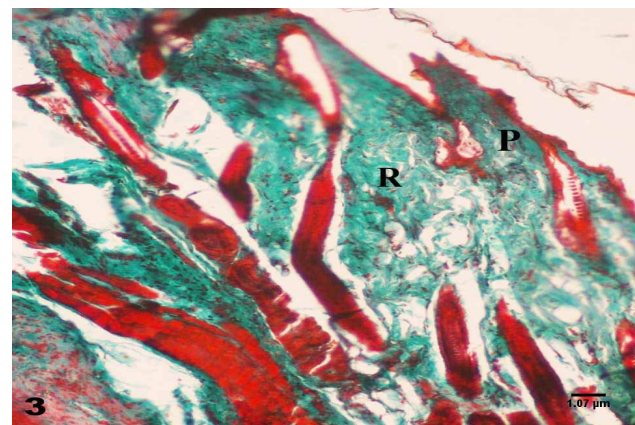
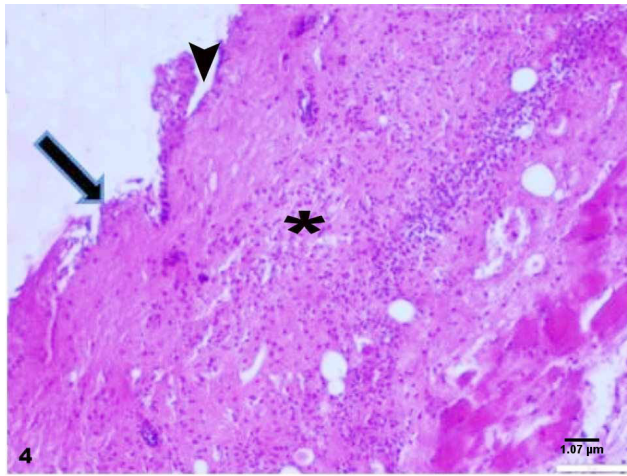
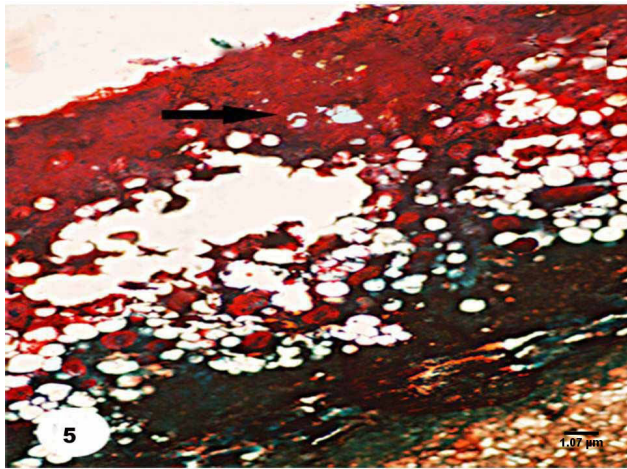


Fig. 3: A photomicrograph of section of mice thin skin of control group showing loosely arranged thin collagen fibers in papillary (P) and thick wavy bundles in reticular (R) layers. Masson's trichrome (scale bare 1.07).

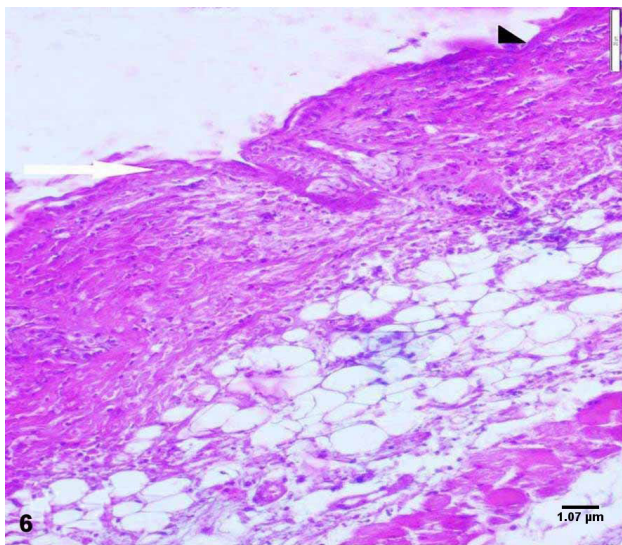




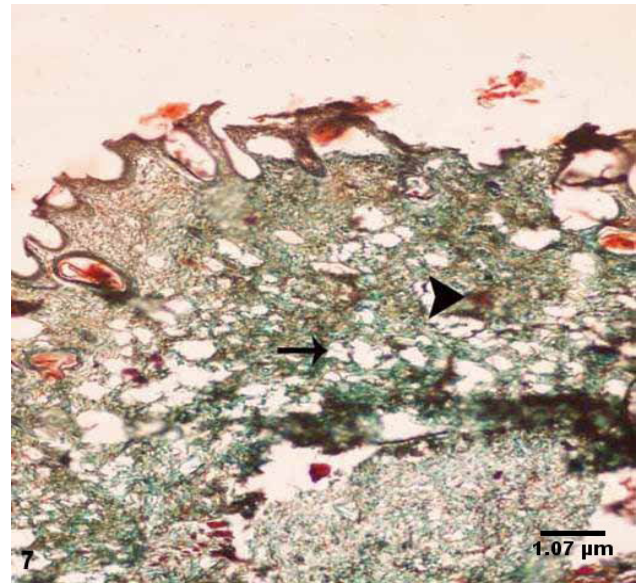
**Fig. 4:** A photomicrograph of burn group after 9 days showing wide area of epidermal loss (arrow) and separation between dermis and epidermis in some areas of burn surface (arrowhead). Note the presence of inflammatory cells in the degenerated dermis (\*). H&E (scale bare 1.07)



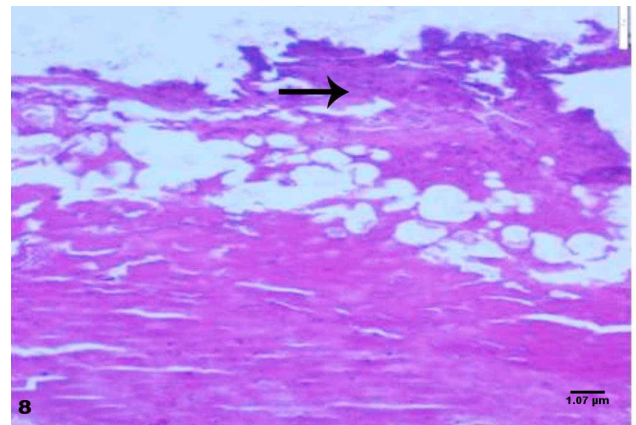
**Fig. 5:** A photomicrograph of burn group after 9 days showing denaturalized red collagen (arrow). Masson trichrome stain (scale bare 1.07)



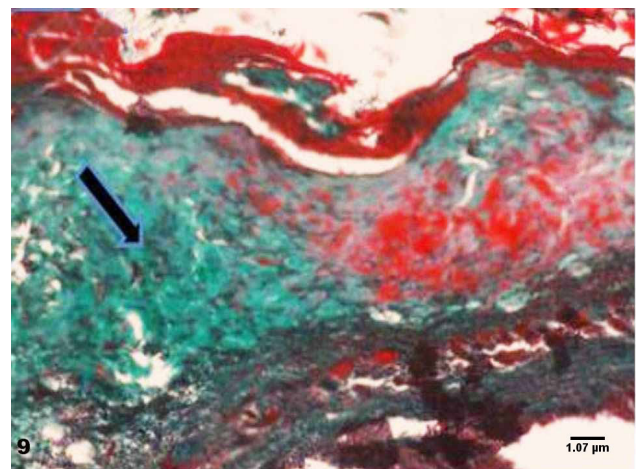
**Fig. 6:** A photomicrograph of CD34+ cells treated group (9 days) showing appearance of thin epidermis (arrow) in multiple areas with sites of defected epithelium (arrow head) H&E (scale bare 1.07).



**Fig. 7:** A photomicrograph of CD34+ cells treated group (9 days) showing fine collagen fibers are observed in the dermis (arrow) while some of them appear denatured (arrowhead). Masson's trichrome stain (scale bare 1.07).

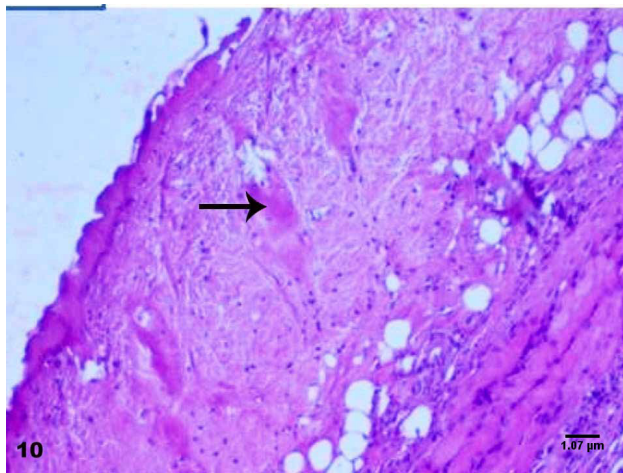


**Fig. 8:** A photomicrograph of PRP treated group (9 days) showing A) Incomplete re-epithelization (arrow) but no inflammatory cells observed in the dermis. H&E (scale bare 1.07).

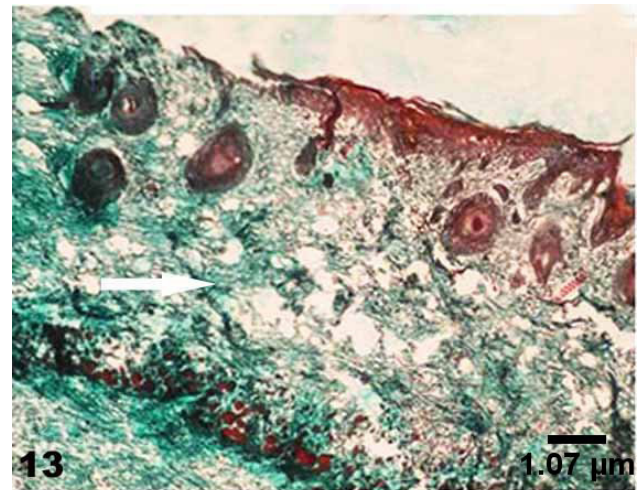


**Fig. 9:** A photomicrograph of PRP treated group (9 days) showing Fine collagen fibers are observed in the dermis (arrow). Masson's trichrome stain (scale bare 1.07).

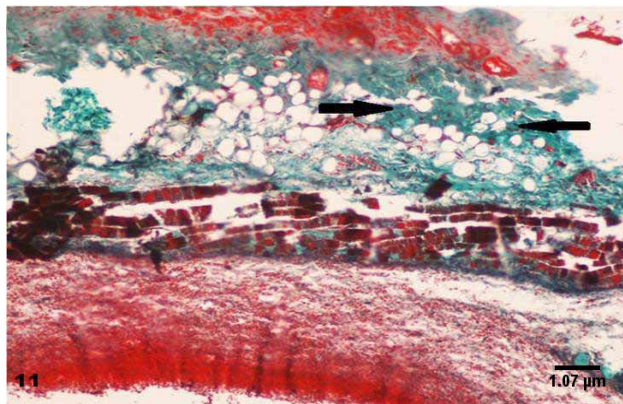




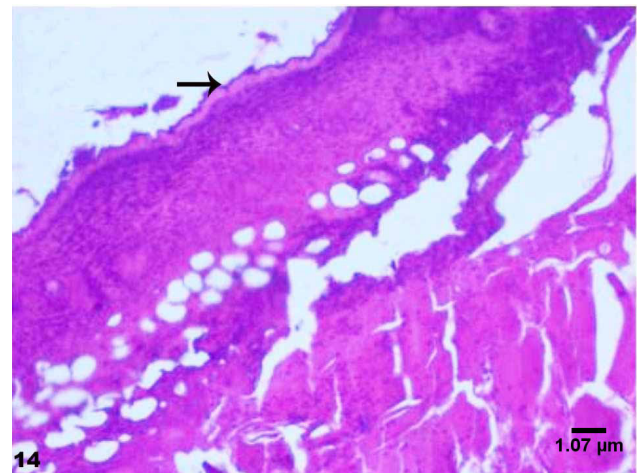
**Fig. 10:** A photomicrograph of burn group (19 days) showing incomplete regeneration of epidermal surface with appearance of hair follicles in dermis (arrow) with observed decrease in the inflammatory cells. H&E (scale bare 1.07).



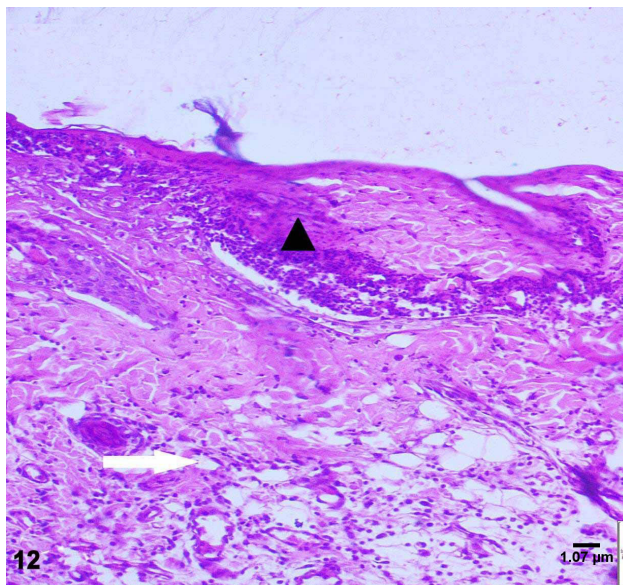
**Fig. 13:** A photomicrograph of CD34+ cells treated group (19 days) showing prominent deposition of collagen fibers is observed in one direction parallel to the epidermis (arrow). Masson's trichrome stain (scale bare 1.07).



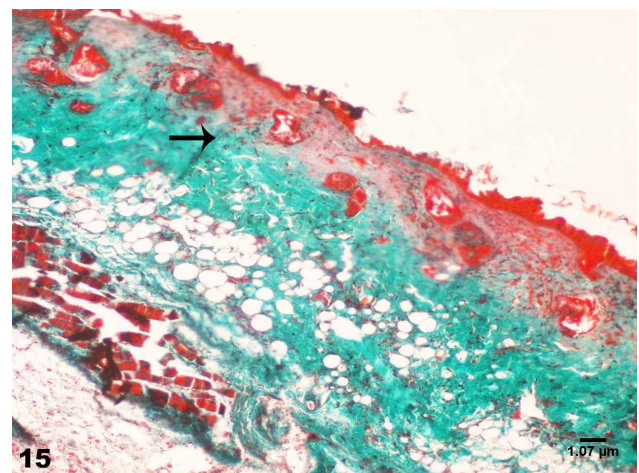
**Fig. 11:** A photomicrographs of burn group (19 days) showing some scattered collagen fibers (arrow) were observed in different directions. Masson's trichrome stain (scale bare 1.07).



**Fig. 14:** A photomicrograph of PRP treated group (19 days) showing thin regenerated epidermis is observed (arrow) and incomplete healing of hair follicles. H&E (scale bare 1.07).



**Fig. 12:** A photomicrograph of CD34 +cells treated group (19 days) showing regeneration of the epidermis (arrowhead). Multiple hair follicles and sebaceous glands are seen (arrow). H&E (scale bare 1.07).



**Fig. 15:** A photomicrograph of PRP treated group (19 days) showing prominent deposition of collagen fibers is observed in one direction parallel to the epidermis (arrow). Masson's trichrome stain (scale bare 1.07).

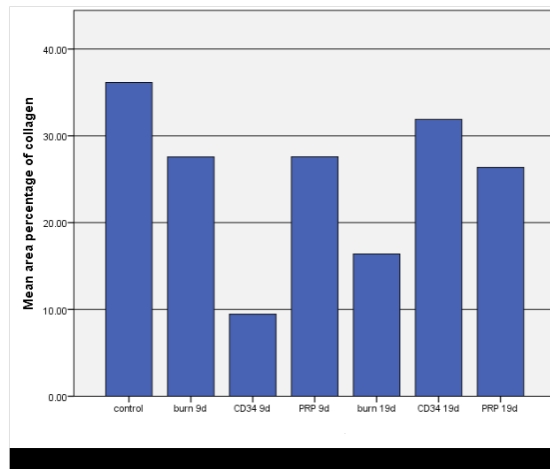


Fig. 16: Quantification of collagen fibers area percentage in Masson's trichrome stained sections of different groups. Graphics show the mean.

Fig 17

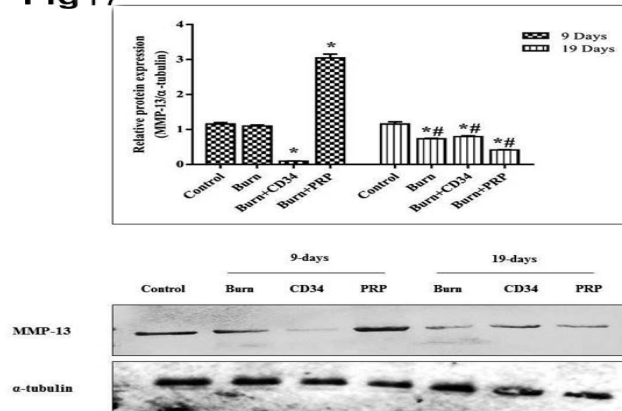


Fig. 17: Western Blotting analysis of MMP13: (A) Western blot analysis revealed that differential expression of MMPs after stem cells and PRP. Alpha tubulin was the loading control for western blot analysis. (B) Quantitative assessment of protein in gel using Image J to quantify protein bands on a PAGE gel revealed significant increase in MMP13 expression in PRP and decrease in CD34 treated group after 9 days in comparison to that burn only, however a significant increase was observed in CD34, as well as PRP treated groups after 19 days treated group \* Significant as compared to 9 days burn only group, # Significant as compared to 19 days burn only group. Graphics show the mean.

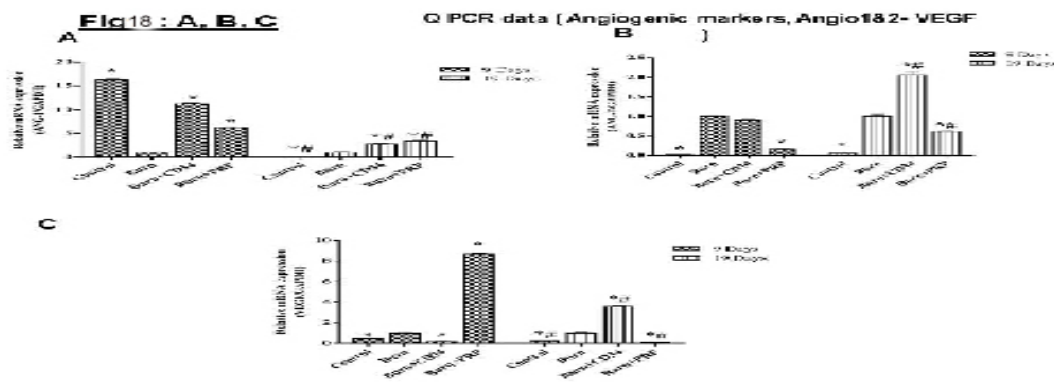


Fig. 18 (A&B&C): A) Angio1 gene: A significant increase in expression of specially in CD 34 as well as PRP treated groups after 9 days of treatment in comparison with burn only, After 19 days of treatment the reduced expression of Angio1 gen in CD 34 as well as PRP treated groups but it is still significantly upregulated especially in PRP treated group in comparison with burn only. B) Angio2 gene: A significant decrease in expression of especially in PRP as well as CD 34 treated groups after 9 days of treatment in comparison to burn only, After 19 days of treatment a significant increased expression of Angio2 gene in CD 34, however PRP treated group is downregulated in comparison with burn only. C) VEGF gene: A significant decrease in expression of especially in CD 34 treated groups, however a significant upregulation is observed in PRP treated group after 9 days of treatment in comparison to burn only, After 19 days of treatment highly significant increased expression of VEGF gene in CD34, however PRP treated group is down regulated in comparison with burn only.



**Table 1:** Represent the different duration of clinical assessments ,measurement is done using ruler , and the calculated percentage of healing in each group in comparison to the baseline (first day of measurement).

Days	Burn%	CD34%	PRP%
1	100.0	100.0	100.0
4	70.2	22.8	38.5
9	41.7	14.3	21.5
11	33.4	2.2	12.6
13	9.3	0.6	5.2
19	3.8	0.4	2.8

**Table 2:** Effect of CD34 and PRP on angiogenetic markers during two different time (9 and 19 days) of burns induced in experimental animals

Gene	ANG-1		ANG-2		VEGF	
	9d	19d	9d	19d	9d	19d
Control	16.23±0.28*	0.152±0.009 <sup>#</sup>	0.02±0.0009*	0.05±0.001*	0.43±0.010*	0.25±0.006 <sup>#</sup>
Burn	1±0.02	1±0.05	1±0.020	1±0.050	1±0.020	1±0.042
Burn+CD34	11.24±0.19*	2.755±0.095 <sup>#</sup>	0.89±0.030*	2.06±0.096 <sup>#</sup>	0.17±0.010*	3.61±0.134 <sup>#</sup>
Burn+PRP	6.15±0.099*	3.349±0.100 <sup>#</sup>	0.15±0.010*	0.61±0.009 <sup>#</sup>	8.65±0.110*	0.12±0.009 <sup>#</sup>

ANG-1, angiotensin-1; ANG-2, angiotensin-2; VEGF, vascular endothelial growth factor; PRP, plasma riches platelets

Data are expressed as mean ± SEM (n = 4-6).

\*Significantly different from burn group at  $p < 0.05$ .

<sup>#</sup>Significantly different from 9 days' burn at  $p < 0.05$ .

## DISCUSSION

Hematopoietic stem cells are considered a good source for renewal of damaged tissues. These cells have characters of self-renewal and multipotent differentiation. They have many advantages like: easy availability, inexpensiveness, short dual time and wide ability of sorting, low immunogenicity<sup>[20,21]</sup>. Umbilical cord blood might be considered a source of hematopoietic and non-hematopoietic stem cells that exert a potential regenerative role in severe burns wound in animal models<sup>[22,23]</sup>. Hematopoietic cells are able to give myeloid, erythroid and lymphoid cells as well as non hematopoietic cells as mesenchymal progenitor, epithelia and endothelial cells<sup>[24]</sup>.

Several authors reported the benefits of hematopoietic stem/progenitor cells (CD34+ cells) in restoring hematopoiesis function in clinical transplantation. It might be due to its inherent ability to effective homing to the bone marrow engraft and niche, and it is essential for their engraftment and repopulation<sup>[25]</sup>.

The thermal burn in this study led to epidermal loss with destruction of the vascular network in the underlying connective tissue. The significant increase in expression of angiogenic markers (Angio1 and VEGF) observed early after 9 days in PRP and CD+34 treated groups. Angio1 contributes to stable new vessels as well as vascular homeostasis and inhibits VEGF-induced expression of inflammatory cell adhesion molecules. This might explain the angiogenic potential of PRP in the present study that responsible for its healing effects as angio1 was upregulated until 19 days while angio2 was downregulated. The angiogenic potential was manifested

early, meanwhile limited or incomplete reepithelization of burned skin occurred. Angiogenic effect was exerted by platelets derived growth factors, and clotting factors that secreted from platelets in the first hour after clotting<sup>[26,27]</sup>. Re-epithelization of burned skin might be resulting from plenty content of TGF-β in PRP which increases proliferation of suprabasal cell and regeneration of the epidermis<sup>[28]</sup>. However, PDGF, bFGF and VEGF triggered the proliferative effect of dermal vessel<sup>[29]</sup>. These proliferative effects might be explained by endothelial cells sensitivity to bFGF and VEGF<sup>[30]</sup>.

The observed complete re-epithelization, reduced inflammatory cells infiltrate and significant increase in angiogenic markers expression (Angio 2 and VEGF) after hematopoietic stem cell (CD34+ cells) treatment especially after 19 days might be resulting from its new vascularizing effect. It was reported that CD34+ cells in the preserved vessels could increase the blood flow of the differentiated progenitor endothelial cells and restore the vascular network<sup>[31]</sup>. High concentration of Angio2 enhances endothelial cells survival and migration from the blood to the connective tissue. This is facilitated by their long cytoplasmic processes and released number of proteolytic enzymes that degrade the matrix and form small tunnels for their movement<sup>[32]</sup>. They proliferate to build cellular cords, which form luminal tubes, thus giving a new capillary. Newly formed capillaries unite to form vascular network and blood flow is retained<sup>[33]</sup>. Moreover it was found that the upregulation of angio2 in association with VEGF induced vascular proliferation, while in VEGF absence, it induced endothelial cell death<sup>[34]</sup>. Similar observations were reported after mesenchymal stem cells and pluripotent

- induced stem cells they improve wound healing by enhancement of angiogenesis<sup>[35]</sup>. In accordance to this the mobilized hematopoietic and endothelial progenitor cells was migrated to the wound causing the inflammatory reaction, self destruction and new vessels formation<sup>[36]</sup>. Similarly, vessel proliferation as well as dermal fibroblast was reported in wound after topical administration of bone marrow cells, in laboratory animals<sup>[36,37]</sup>.

Collagen deposition assessment in the present study revealed increase in collagen in PRP treated animals, more than that detected in CD34+ve cells treated animals especially after 19 days of treatment. This might reflect their potent stimulatory and proliferative effect on fibroblasts. These effects might be resulting from the sensitivity of fibroblasts to growth factors specially bFGF, PDGFa, PDGFb, IGF, and EGF<sup>[38]</sup>. Chemotaxis of fibroblasts could be noticed after topical application of epidermal growth factor causing regeneration of epidermis and increasing the power of wound tenseness<sup>[39]</sup>. Furthermore, it has been shown that PRP and /or CD34+ cells derived transforming growth factor -  $\beta$ 1 modulates cell sorting, multiplication, chemotaxis, and formation of many extracellular matrix proteins. The stimulatory effect of TGF- $\beta$  on fibroblasts might help in collagen fast maturation as well as, its new synthesis in addition to fibronectin, and glycosaminoglycan synthesis during wound healing. Moreover, smooth muscle cell migration and proliferation might be a consequence of endothelial cell growth factor release<sup>[28]</sup>.

The observed inflammatory cells infiltrate in PRP as well as CD34+v cells at 19th day after initial reduction of these cells might reflect the late chemotactic effects especially in CD34 treated animals, these cells might exert immunomodulatory role and antimicrobial action in wound. PRP and /or CD34+v cells gained vascular endothelial growth factor might attract neutrophils and monocytes<sup>[28]</sup>. Similar observation was reported due to paracrine effects MSCs treatment in burned skin that promoting efficient wound healing, through interaction between stem cells and inflammatory cells, thus initial as well as late rise in the inflammatory reaction at the site of wound might be responsible for antimicrobial effect and activation of the healing process. Moreover CD34+ cells induced stimulation of proliferation of resident stem cells might be considered one of the regeneration potentials of these cells<sup>[40]</sup>. Nevertheless other reported a reduced, inflammatory phase might help in reduction bacterial infections and scar formation in treated wounds<sup>[28]</sup>

Significant higher tissue MMP13 expression was observed in the PRP-treated group that was an essential marker in the healing of wound. It has been found that collagenase-3 (MMP-13) exert a potent effect in cleaving collagen fibers and other components of ECM together with transforming growth factor- $\beta$ <sup>[41-44]</sup>. In spite of limited physiological expression pattern of MMP-13 in human adult skin, therefore it is not secreted during usual healing of wound<sup>[45]</sup>. However, its expression by fibroblasts led to minimal scare formation during healing as in adult wounds

in gingiva and skin wounds in fetus<sup>[46,47]</sup>. These findings suggested that MMP-13 has a role in potent collagenous granulation tissue remodeling and therefore efficient repair without scare formation. Furthermore, MMP-13 might be implicated in the pathogenesis of chronic ulcers and was secreted by fibroblasts<sup>[45]</sup>. It was proved that PRP increased the speed of extracellular matrix repair and formation during the wound healing first half<sup>[48]</sup>.

The delayed significant increase in MMP13 expression after CD34+ cells especially after 19 days of treatment might be involved in the potential regenerative, anti-inflammatory as well as angiogenic effect of hematopoietic cells. These assumptions were raised from the well known proteolytic activities of these gelatinase on collagen degradation that help in migration and homing of transplanted cells. Thus the concomitant increase in MMP13 as well as VEGF in CD34+ cells especially after 19 days might help a lot in optimizing the angiogenic effect of VEGF. The synthesis of new blood vessels is a complicated procedure and balanced by the equilibrium between antiangiogenic and angiogenic factors such as VEGF<sup>[49]</sup>. It had been found that VEGF angiogenic activity is obstructed by forming a complex with CTGF42. MMP-13 could emit VEGF through selective degradation of CTGF42 in this complex<sup>[50]</sup>. The persistence of significant upregulation of VEGF in CD34 group up to 19 days might exert a good impact on regenerative potential due to its associated MMP13 expression in comparison to that observed early in PRP treated animals. Similarly It *et al* (2007) reported that choosy CTGF breaking down of the complex CTGF/VEGF might activate VEGF and induce angiogenesis in the tumor xenografts with MMP-7 as well as MMP-13<sup>[51,42]</sup>. Moreover, MMP-13 is also expressed by endothelial cells might be implicated in ECM degradation and so directly controls angiogenesis<sup>[52]</sup>. Thus, all these data raised the suggestion that MMP-13 is participating in contraction of the wound by fibroblast amplification and myofibroblast sorting by enhancement the delayed TGF- $\beta$ 1. Therefore significant late macroscopic wound closure and histological re- epithelialization that reported in MMP-13 KO mice might confirm their importance in cutaneous wound healing<sup>[53]</sup>. The observed concomitant increase in MMP13 expression with angiogenesis could confirm its role in activating of angiogenic markers as well as the beneficial degradation effect on matrix and proliferating keratinocyte migration. One of the important crucial roles played by MMPs in all steps of healing is the modification of the matrix, that permitting the cell moving and tissue restyling. Breaking down of the hemidesmosomes might help keratinocyte aboard during wound healing. Therefore keratinocytes move in one of two pathways: either through the transnational wound matrix or in junction with dermis under the matrix<sup>[54,55]</sup>. The significant increase in collagen deposition in association with MMP13 after CD34+ cells especially after 19 days of treatment might help the removal of damaged or denaturated collagen. Moreover, similar observation was reported after co cultures of fibroblasts with bone marrow derived CD34+ cells, the later were pre-incubated with G-CSF might induce activation of



proMMP-2 and thus activating MMP13<sup>[56]</sup>. RT-PCR results showed that HLAHLADQB1- 0201 genes, exhibited exactly in human cells. They were observed in the tissues in CD 34 treated groups after 9 days as well as 19 days of treatment in comparison to control (Data not shown) thus meaning the existence of living human derived cells<sup>[22]</sup>.

## CONCLUSION

This study suggested that the capability of CD34 stem cells to change the tissue environment through expression of proangiogenic factors. Secretion of collagen as well as MMP13 might contribute more to their multipotential sorting to fix the tissue. Hematopoietic stem cells were found to have a coherent and good style of wound treatment through induction of fast cellular amplification, granulation tissue and collagenous fiber synthesis as well as early full wound healing.

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## CONFLICTS OF INTEREST

There are no conflicts of interest

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## الملخص العربي

## تأثير الخلايا الجذعية المكونة للدم والبلازما الغنية بالصفائح الدموية علي التئام النسيج الجلدي المحترق تجريبيا : دراسة مقارنة في ذكور الفئران البالغة

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**المقدمة:** تعد الحروق من الأسباب الشائعة لحدوث الندبات و الوفاة. ومن الطرق المعروفة لعلاج الحروق من الدرجة الثانية او الثالثة العلاج بالبلازما الغنية بالصفائح الدموية كعلاج موضعي بما تحتويه من العديد من عوامل النمو التي تستطيع الاسراع من التئام الحرق.

**تهدف هذه الدراسة:** أجريت هذه الدراسة لتحليل استخدام البلازما الغنية بالصفائح الدموية في الحرق من الدرجة الثانية ومقارنته بتأثير الخلايا الجذعية المكونة للدم الموجبة 34 في الفئران.

**الطرق ومواد البحث:** أجريت هذه الدراسة على سبعين من الفئران البالغة مقسمة الى أربع مجموعات المجموعة الضابطة ومجموعة الحرق فقط ومجموعة الحرق مع الخلايا الجذعية المكونة للدم الموجبة 34 (-0.3 0.4 × 10 6 /كجم حقنت في داخل الادمه) ومجموعة الحرق مع البلازما الغنية بالصفائح الدموية (1مل حقنت في داخل الادمه) وتم الحقن علي حافة الحرق بعد 24 ساعة من احداثه. كل المجموعات المحترقة تعرضت الي الحرق الحراري. تم ذبح الفئران في اليوم التاسع والتاسع عشر وأخذت عينات من الجلد لصباغتها بالهيماتوكسيلين و الايوسين و كذلك صبغة الماسون . تقييم تكوين الأوعية الدموية ودراسة كمية لانزيم ميتالوبروتيناز المادة الخلالية 13.

**النتائج:** أظهرت النتائج أن الحروق التي عولجت بالبلازما الغنية بالصفائح الدموية اغلاق سريع للجرح بالمقارنة بالحرق فقط ونقص في الياف الكولاجين وزيادة ترسيب في انزيم ميتالوبروتيناز المادة الخلالية 13 ومع زيادة التعبير عن علامات تكوين الاوعية الدموية 1 و 2 وعامل النمو لخلايا المبطنة للاوعية الدموية. وعلي الرغم من ذلك المجموعة المعالجة بالخلايا الموجبة 34 قد اظهرت تحسن ملحوظ في العلامات المذكورة سابقا. كما تحسنت التغيرات النسيجية المرضية للحرق في كل المجموعات بعد 19 يوم وخاصة مجموعة الخلايا الموجبة 34.



**الاستنتاج:** نستخلص من هذه النتائج أن العلاج بالخلايا الموجبة 34 ذو تأثير أكثر في التئام الجروح من البلازما الغنية بالصفائح الدموية في الحرق الحراري من الدرجة الثانية.