RESEARCH ARTICLE

Effects of Topical Pentoxifylline on Induced Thermal Burn in Mice

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ABSTRACT

Objective: To evaluate the effects of topical application of pentoxifylline in the treatment of induced burn in mice and to compare it with silver sulfadiazine.

Methods: Pentoxifylline-hydroxypropyl methylcellulose (HPMC) topical gel formulations were prepared by mixing homogeneously 1g of pentoxifylline with 3g of HPMC gel under continuous stirring, and the final weight was made up to 100 mL. The experimental animals (72 mice) were divided into four groups, each subdivided to three groups according to the three-day interval (7, 14, and 21st day) after thermal injury, each with eight animals. The animals were anesthetized then thermal injury was done by a metal bar. The wound surface area was measured every seven days, and the animals have been sacrificed by ether overdose at these intervals. Then, skin sections are stained with (hematoxylin and eosin) and Masson trichrome for histological scoring after being evaluated by immunohistochemical assay to estimate the expression of fibroblast growth factor and epidermal growth factor.

Results: Pentoxifylline group (Gr4) showed a significant reduction in the burning area ($p<0.05$), PTX and SSD groups have a significant increase in immunohistochemical expression of fibroblast growth factor (FGF), epidermal growth factor (EGF) in comparison with gel base and burn without treatment (BWT) groups, and skin histopathological examination were measured on days 7, 14, and 21of burn injury experiment. Histopathologic evaluations on day 7 showed that neovascularization induced granulation tissue was greater in the pentoxifylline group (PTX) than silver sulfadiazine (SSD), gel base, and BWT groups. On day 21, re-epithelialization scores were showed higher and maximum level in the PTX group than the SSD, gel base and BWT groups. Inflammatory cells scores in BWT, SSD, and gel base groups showed higher than PTX group.

Conclusion: the results suggest that topical use of pentoxifylline showed significant efficacy in wound healing activities than silver sulfadiazine cream. Histopathological evaluation results showed significant improvement in inflammatory response, granulation tissue formation, and re-epithelization, which may be due to the anti-inflammatory, anti-oxidant, and angiogenic activity of pentoxifylline and the appropriate regulation FGF and EGF.

Keywords: Fibroblast growth factor, Pentoxifylline, Silver sulfadiazine, Wound healing.

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INTRODUCTION

A burn is a type of skin injury, or other tissues, caused by heat, cold, electricity, chemicals, friction, or radiation.[1] It is the most severe form of trauma that has affected humanity since time immemorial, and that over the years, the scientific revolution has improved the results in its treatment.[2]

Despite advances in burn care techniques, there is still a tendency for therapeutic failure in patients with sustained burns, especially of a large percentage of total body surface area (TBSA); Right management includes an expert multidisciplinary approach that solves all the issues a burn patient has to face. In addition to that, modification in medical treatment protocols and seeking new mechanisms involved in the pathogenesis of burn may be helpful in the successful treatment of burn patients.[3] Burns is now considered as one of the most devastating forms of trauma that afflict humans because it induces local and systemic damage that seriously alter homeostasis.[4] The local changes in burn wounds are classified by *Jackson* into three zones: The zone of coagulation at the central focus of injury is generally thought to consist of devitalized tissue. The most peripheral zone is termed hyperemia, characterized by vasodilation, inflammatory changes without structural damage. Between these zones, an intermediate region of indeterminate prognosis arises, which is termed the zone of stasis.

The zone of stasis is often best identified in mid to deep dermal burns and represents a region of vascular stasis and ischemia. From a clinical perspective, this region poses some

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of the greatest challenges for the burn team. This tissue has the potential to heal or to progress to a full-thickness lesion.[5]

The systemic pathophysiologic changes following thermal injuries affect multiple organs and body systems, leading to clinical manifestations including shock, intestinal alterations, respiratory and renal failure, immunosuppression, and others. Major thermal injury is associated with extreme hypermetabolism and catabolism being the principal metabolic manifestations after successful resuscitation from the shock phase of the burn injury.[6] Numerous growth factors, including epidermal growth factor (EGF), transforming growth factor-beta (TGF-β), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), and platelets derived growth PDGF, are involved in wound healing, and their expression and/or the expression of their receptors is altered during wound healing.[7] VEGFs and their receptors, especially vascular endothelial growth factor receptor-2 (VEGFR-2) (Kinase insert domain receptor (KDR), may promote early events in angiogenesis, particularly endothelial cell migration and proliferation. Fibroblast growth factors, especially FGF2 (basic fibroblast growth factor, bFGF) and its receptor bFGFR, are preferentially involved in angiogenesis and modulate cell growth and differentiation.[8] Pentoxifylline (PTX) is a known agent whose pharmacological properties are variable. Dimethylxanthine derivative increases the level of cyclic adenosine monophosphate (cAMP) in the blood vessels' smooth muscle and is categorized as a vasodilator.[9] Pentoxifylline inhibits the synthesis of inflammatory mediators, decreases cytokine release, and suppresses leukocyte function, which controls the inflammation in wound healing.[10] Pentoxifylline reduces the levels of pro-inflammatory cytokines (IL-1b, IL-6, and TNF-a).[11] Pentoxifylline can reduce oxidative stress via its effect on oxygen-free radicals production, improving wound healing.[12]

MATERIALS AND METHODS

A. Experimental Animals

The animals were housed in a metal cage (one animal per cage). Before beginning the study, animals were left for at least 48 hours to adapt to controlled temperature conditions in the animal room, allowing free access to water and food ad libitum. Seventy-two adult albino female mice, each weighing nearly (20–25g), were used in this study and divided into four groups. Each group was divided into three sub-groups representing three-day intervals: (day 7,14, and 21) after burn induction. Group 1 Induced burn group (burned with no treatment) and Group 2 Induced burn group treated with Standard drug (Silver sulfadiazine 1%) applied topically twice daily. Group 3 Induced burn group treated with gel base (hydroxypropyl methyl cellulose-HPMC) applied topically twice daily. Group 4 Induced burn group treated with pentoxifylline applied topically twice daily.

B. Thermal Injury

A method described by Wu *et al.,* 2012 was used with modification.[13] In a water bath, the cylindrical metal bar (diameter 1.6 cm; height, cm; total weight 65 g) was heated and kept in equilibrium for 15 minutes with the presence of a thermometer in water. Deep second-degree burn wound was induced on the back of the animal under general anesthesia (ketamine 90 mg/kg; Xylazine 10 mg/kg) by applying a heated bar to 95 c with a bar weight pressure on mice shaved back for approximately 10 seconds. For each animal the same metal bar was used to achieve standardized burn-depth.

C. Preparation of Gel Base

Hydroxypropyl methylcellulose (2 g) was dispersed gradually into the preheated water (90°C). The entire HPMC solution was stirred with a magnetic stirrer until it produced a homogeneous appearance. The solution was then left in the refrigerator overnight until a transparent gel was formed.

D. Preparation of Pentoxifylline Gel (1%)

Pentoxifylline-HPMC topical gel formulations were prepared by mixing 1 g of pentoxifylline with HPMC gel under continuous stirring until all pentoxifylline powder dissolved homogeneously, and the final weight was made up to 100 mL.[14]

E. Dissection and Skin Tissue

The mice were anesthetized using a piece of cotton soaked in ether and placed in a closed container for a few minutes to be anesthetized by inhalation. Then, a blade incision for the skin of the burned area was performed after the mice were transformed into the work table and the burn wound was dissected. Biopsies were taken each week (on post-burn days 7, 14, 21). Biopsies included the wound bed as well as the healthy skin of the wound margins.

F. Wound Size Measurement (Burning Area)

The measurements were taken on days 1, 7, 14, and 21 every seven days and were camera shot (12 megapixels). On the first day, the wound area was considered 100 percent, and on subsequent days the wound areas were correlated with the area of the wound on the initial days. To measure the rate of wound healing, the size of the lesions was determined by keeping the test animal in a good position, and the wound margin was drawn on a transparent plastic sheet using a fine-tipped pen.[15]

Immunohistochemistry

A. Sample Preparation

Paraffin-embedded tissue blocks of study and control groups were collected. New sections were made from each of the paraffin-embedded blocks as follows:

- Five µm thick sections were made on ordinary slides to be subjected to hematoxylin and eosin stain as previously described. This was conducted to confirm the diagnosis and wound grade.
- Five µm thick sections were made on positively charged slides.

Tests were performed on the prepared slides for immunohistochemistry to detect epidermal growth factor and fibroblast growth factor.

B. Evaluation of Immunohistochemistry Results

Proper and accurate application of kit instructions led to the appearance of a brown precipitate in positive cells on

tissue sections. Quantification of fibroblast growth factor and epidermal growth factor protein expression was evaluated under light microscopy at X40. The counting of positive cells was performed at X40.

Quantification of IHC was performed according to the following semiquantitative scores based on the percentage of positively stained cells as following :0, no staining; 1, ≤ 25%; 2, 26–50%; 3, 51–75 %; and 4, 76–100%.[16]

Assessment of Histopathological Changes of Skin Sections

Histopathological changes of skin tissue of each mouse were evaluated and scored for each group on the 7th, 14th, and 21st days as follows:

- The inflammatory response, characterized by the presence of polymorphonuclear leukocytes (PNM)
- Granulation tissue, characterized by the presence of fibroblasts, myofibroblasts, and neovascularization
- Re-epithelialization, characterized by migration and proliferation of the epidermal cells

The score was made for all parameters evaluated: zero = absent, 1 = mild presence, 2 = moderate presence and 3 = strong presence.

RESULTS

Effect of Different Treatments on Burning Area Contraction

On day 1, the mean and standard deviation of the burning area in animal groups, (G1) is the control group without treatment, (G2) is the group treated with Silver Sulfadiazine, (G3) is the group treated with Gel base, (G4) is the group treated with Pentoxifylline (Figure 1), were 160.5736.47, 179.2038.32, 196.2938.12 and 150.2146.21, respectively (Table 1). The results showed that no significant differences ($P > 0.05$) among all experiment groups.

Also, on day 7, there was a decrease in burn area contraction with no significant difference in all groups. The mean and standard deviation of the burning area in animal groups, (G1) is the control group without treatment, (G2) is the group treated with Silver Sulfadiazine, (G3) is the group treated with Gel base, (G4) is the group treated with pentoxifylline, were 177.3133.20, 185.1339.28, 168.9827.52 and 15260.37, respectively (Table 2).

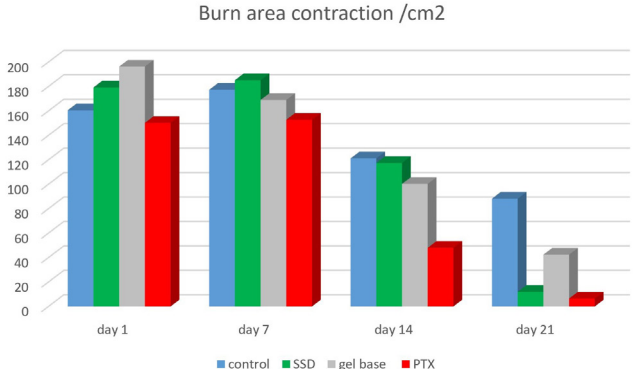

Figure 1: Burn area contraction/cm^2 of study groups on days of the experiment. SSD= Silver sulfadiazine, PTX= Pentoxifylline

In day 14, the mean and standard deviation of the burning area in animal groups, (G1) is the control group without treatment, (G2) is the group treated with Silver Sulfadiazine, (G3) is the group treated with Gel base, (G4) is the group treated with pentoxifylline, were 121.1418.57, 117.3950.04, 100.2820.54, and 47.9725.84, respectively (Table 3). There was a significant statistical association, P-value 0.05, between types of treatment used and wound size.

In day 21, the mean and standard deviation of the burning area in animal groups, (G1) is the control group without treatment, (G2) is the group treated with Silver Sulfadiazine, (G3) is the group treated with Gel base, (G4) is the group treated with Pentoxifylline, were 88.2149.03, 11.988.59, 42.3821.45 and 6.452.09, respectively (Table 4). There was a significant statistical association, p-value 0.05, between the type of treatment used and wound size, all groups showed a decrease in burn area contraction. However, distinctive decline in PTX

Table 1: Mean comparison of wound size on day one. Mann-Whitney test used, p-value 0.05 considered significant, SSD= Silver sulfadiazine, PTX= Pentoxifylline

Group	*Mean*	*Standard deviation*	*p-value*
Group 1 - Control	160.57	36.47	–
Group 2 - SSD	179.20	38.32	0.51
Group 3 - Gel base	196.29	38.12	0.18
Group 4 - PTX	150.21	46.21	0.58

Table 2: Mean and standard deviation of wound size for each group on Day Seven. Mann-Whitney test used, p ≤ 0.05 considered significant

Group	*Mean*	*Standard deviation*	*p-value*
Group 1 - Control	177.31	33.20	–
Group 2 - SSD	185.13	39.28	0.79
Group 3 - Gel base	168.98	27.52	0.69
Group 4 - PTX	152.78	60.37	0.69

Table 3: Mean and standard deviation of wound size for each group on day fourteen. Mann-Whitney test used, p ≤ 0.05 considered significant

Group	*Mean*	*Standard deviation*	*p-value*
Group 1 - Control	121.15	18.57	–
Group 2 - SSD	117.39	50.04	0.89
Group 3 - Gel base	100.28	20.54	0.13
Group 4 - PTX	47.97	25.84	0.002

Table 4: Mean and standard deviation of wound size for each group on day twenty-one. Mann-Whitney test used, p ≤ 0.05 considered significant

Group	*Mean*	*Standard deviation*	*p-value*
Group 1 - Control	88.21	49.03	–
Group 2 - SSD	11.98	8.59	0.003
Group 3 - Gel base	42.38	21.45	0.06
Group 4 - PTX	6.45	2.09	0.002

treated groups more than control, SSD and gel base groups (PTX and SSD groups) showed highly significant differences compared to control and gel base groups.

Immunohistochemistry

Fibroblast Growth Factor

The mean and standard deviation of FGF in animal groups, (G1) is the control group without treatment, (G2) is the group treated with Silver Sulfadiazine, (G3) is the group treated with Gel base, (G4) is the group treated with Pentoxifylline, were 1.630.91, 3.380.51, 1.250.70, and 3.750.46 respectively. A significant statistical association, $p < 0.05$, was between the type of treatment used and FGF.

Immunohistochemical expression scores of FGF resulted in highly significant ($p = 0.0001$) increased with silver sulfadiazine and pentoxifylline treatment (3.38 ± 0.51) and (3.75 ± 0.46), respectively compared with (1.63 ± 0.91) in the induced thermal injury control group (Figure 2).

Epidermal Growth Factor

The mean and standard deviation of EGF in animal groups, (G1) is the control group without treatment, (G2) is the group treated with Silver Sulfadiazine, (G3) is the group treated with Gel base, (G4) is the group treated with Pentoxifylline, were 1.750.70, 2.750.46, 1.751.03 and 3.360.51 respectively. There was a significant statistical association, P-value 0.05, between the type of treatment used and EGF.

Immunohistochemical expression scores of FGF resulted in highly significant (P=0.0001) increased with pentoxifylline treatment (3.36 ± 0.51) compared with (1.75 ± 0.7) in the induced thermal injury control group (Figure 3).

Histomorphological Finding

Inflammation

At day seven, the mean and standard deviation of inflammation degree in animal groups, (G1) is the control group without treatment, (G2) is the group treated with Silver Sulfadiazine, (G3) is the group treated with Gel base, (G4) is the group treated with Pentoxifylline, were 3.000.00, 2.500.53, 3.000.00 and 2.000.00, respectively. On day fourteen were 2.750.46, 2.630.51, 3.000.00, and 1.00 0.00, respectively. On day twenty-one were 2.130.64, 1.380.51, 2.000.00, and 1.50 0.67, respectively.

Histopathological score reflective of inflammation was highly significant ($p \leq 0.001$) decreased with the pentoxifylline treated group compared with other treated and non-treated groups.

Granulation Tissue

At day seven, the mean and standard deviation of granulation tissue production in animal groups, (G1) is the control group without treatment, (G2) is the group treated with Silver Sulfadiazine, (G3) is the group treated with Gel base, (G4) is the group treated with Pentoxifylline, were 0.000.00, 2.251.03, 1.000.00 and 3.000.00, respectively. On day fourteen were 2.001.30, 2.251.03, 1.000.00 and 0.000.00, respectively. At day twenty-one were 0.88 ± 0.35, 0.00 ± 0.00, 0.38 ± 0.51 and 0.000.00, respectively.

On day seven there was a significant increase in granulation tissue production in PTX and SSD groups compared with

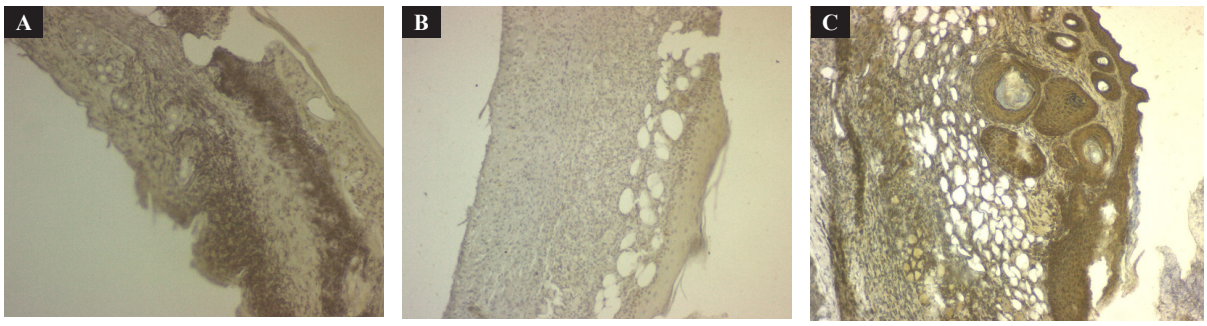

Figure 2: Immunohistochemical expression of FGF in ECM of treatment groups: (A) Induced burn shown moderate-intensity FGF in SSD treated group (X20); (B) Induced burn shown mild-intensity FGF in gel base treated group (X10); (C) Induced burn shown high-intensity FGF in PTX treated group (X10)

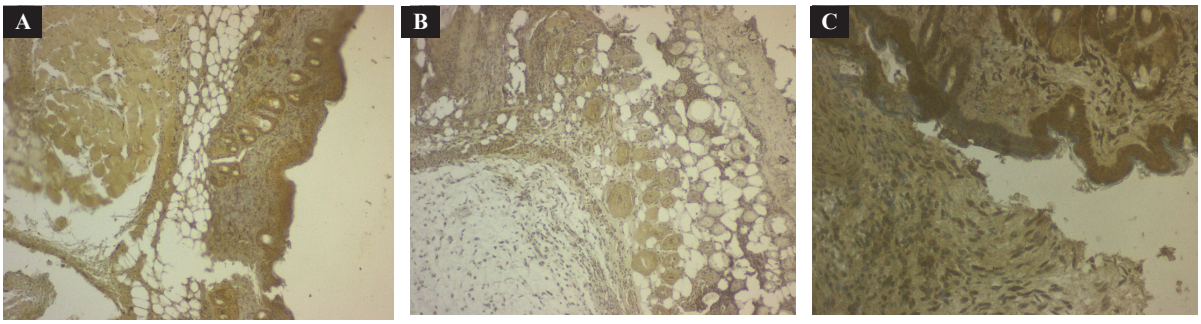

Figure 3: Immunohistochemical expression of EGF in ECM of treatment groups: (A) Induced burn shown moderate-intensity EGF in SSD treated group (X10); (B) Induced burn shown mild-intensity EGF in gel base treated group (X10); (C) Induced burn shown high-intensity EGF in PTX treated group (X20)

other groups (p-value < 0.05). While at day fourteen, there was a distinctive elevation in granulation tissue production with significant differences (p <0.05) in the control group compared with other groups, and granulation was absent in the PTX-treated group. There was a significant decrease in granulation tissue production in the control and gel base groups on day twenty-one. Granulation tissue was absent in PTX, and SSD-treated groups.

Re-epithelialization

At day seven, the mean and standard deviation of Re-epithelization degree in animal groups, (G1) is the control group without treatment, (G2) is the group treated with Silver Sulfadiazine, (G3) is the group treated with Gel base, (G4) is the group treated with Pentoxifylline, were 0.000.00, 1.000.00, 0.00 0.00 and 0.75 0.46, respectively. On day fourteen were 0.500.53, 1.25 0.46, 0.000.00 and 3.000.00, respectively. On day twenty-one were 1.75 0.70, 3.00 0.00, 3.00 0.00, and 2.75 0.46, respectively.

The histopathological score was extremely significantly increased with PTX, and SSD treated groups compared with control and gel base groups. (Figure 4).

DISCUSSION

Burning Area Contraction

The wound area of each animal was measured on days 1, 7, 14, and 21 post-burn in all groups. Since the wound contraction is

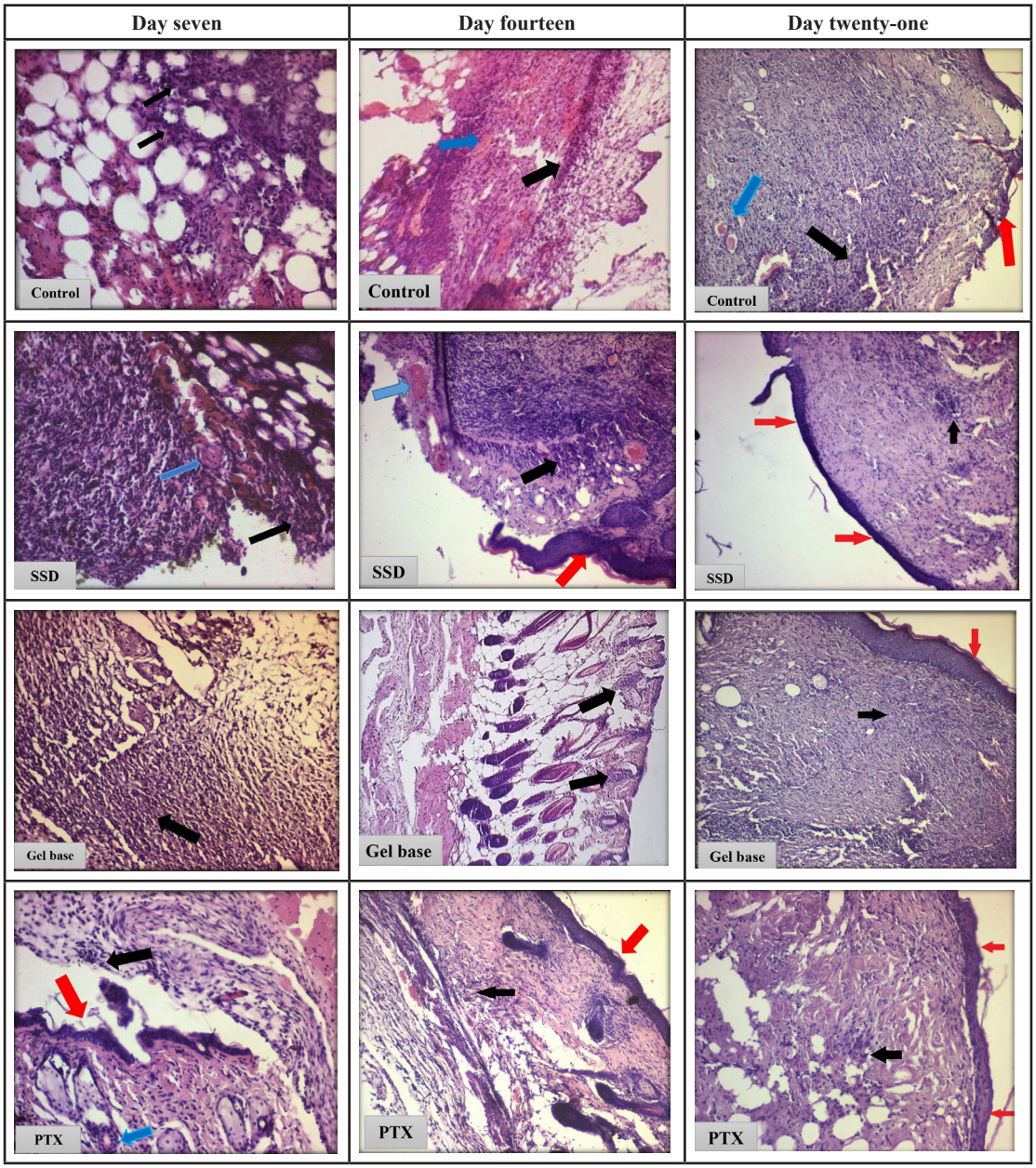

Figure 4: Light microscopic section of mouse skin histopathological study for all groups showing: inflammatory cells (black arrow), granulation tissue (blue arrow), and re-epithelization (red arrow).

mediated by specialized myofibroblasts found in the granulated tissue.[18] So, the increase in wound contraction in the PTX-treated group might result from fibroblasts' enhanced activity.

The present study shows a significant decrease in the measurement of burning area in the SSD treated group than induced burn animals without treatment (control), but this reduction is still less than PTX-treated group, and this is following both Muller *et al.*, 2003 and Hosseinimehr *et al.*, 2010 who also found that SSD cream causes a delay in the healing time of wounds.[19,20]

Immuno-histochemistry

Immunohistochemistry expression of FGF-2 was increased significantly after 7 days of treatment ($p \leq 0.05$) in induced burned with topical silver sulfadiazine (SSD) treated group, and a more significant increase in topical pentoxifylline (PTX) treated group in comparison with both control and gel base-treated induced burn groups.

A study by Rosen *et al.*, 2015 demonstrated that the healing rates are slower in full-thickness wounds treated with SSD cream than those treated with a control aqueous cream.[21] Similarly, using a mouse burn model, SSD applied topically to burns significantly delays wound closure compared with untreated controls. Alterations of the cytokine milieu resulting in decreased recruitment of macrophages are some of the contributing factors to the delayed closure observed, and this can explain the effect of sliver sulfadiazine on inhibition of FGF-2

Since FGF is a mitogenic and angiogenic factor and has a role in granulation tissue formation, the histopathologic findings demonstrating the increased number of blood vessels following PTX treatment were consistent with the increased expression of FGF.

Immuno-histochemistry expression of EGF was significantly increased after 7 days of treatment ($p \leq 0.05$) in the PTX group compared to the control and gel base groups on day 7.

The earliest and highest levels of EGF protein expression may be involved in the mechanism of PTX improving the re-epithelialization wound healing of burns. This is indirectly supported by the EGF enhanced proliferation and migration of keratinocytes, fibroblasts, and endothelial cells in PTX treated wounds. Moreover, this agrees with Simonetti *et al.*, 2017 and Qing, 2017 who found that the increased level of expression of EGFR accelerates wound re-epithelialization, facilitates angiogenesis, increases epithelial proliferation and migration, and induces the inflammatory reaction and contraction of wounds.[22,23]

Histomorphological Finding

This study revealed that PTX significantly attenuated inflammation compared with other treated and non-treated groups on post-burn days 7, 14, and 21. PTX is also known for its anti-inflammatory effect, and this agreement with Deree *et al.*, 2008 that found that PTX suppresses inflammatory mediator synthesis, diminishes cytokine release, and inhibits leukocyte activity, also agree with Bhat *et al.*, 2001 that stated that PTX can reduce oxidative stress through its action on the development of oxygen-free radicals.[24] Hence PTX can improve wound healing with its anti-inflammatory and anti-oxidant properties.

The formation of well-vascularized granulation tissue in the wound bed is essential for wound healing. Granulation tissue provides a substratum for epidermal cells to migrate and cover the wound.[25]

In the present study, the histopathologic scoring showed improvement in granulation tissue production in PTX-treated group at the 7th day of injury which is consistent with the increase in the expression of epidermal growth factor. It contributes to building collagenous tissue and accelerating the generation of wound granulation and epithelial tissue. Further, it accelerates the wound healing process by binding to EGF receptor on epidermal cell and fibroblast cell surface membranes. This agrees with Zhang *et al.*, 2019 that EGF increases granulation tissue production, neutrophil numbers, collagen uniformity, and the extracellular matrix when used to treat diabetic foot ulcers,[26] noticeable microscopic re-epithelialization was observed in PTX and SSD groups on day 7. On day 21 of the study, results with light microscopy showed that the re-epithelialization process in animals treated with PTX was better than in those that received SSD.

Re-epithelialization was complete in four mice of the PTX group showing that PTX has substantial effects on re-epithelialization. Higher rate of re-epithelialization and a lower rate of tissue necrosis in the PTX group are thought to be due to the anti-oxidant, anti-inflammatory, and anti-edema, effects of PTX

CONCLUSIONS

Topical pentoxifylline gel (1%) administered once daily for 21 days provided a significant improvement in burn wounds due to anti-inflammatory and anti-oxidant activity, raising angiogenic activity by enhancing RBC deformability, erythrocyte flexibility facilitating easier vascularization, enhancing tissue oxygenation that increases blood flow in the vessels that proved to be highly beneficial in treatment of skin injury in mice model in compare with topical sliver sulfadiazine cream 1%.

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