Apremilast Topical Effect in Induced Hypertrophic Scar of Rabbits

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Abstract

The aim of this study was to assess the effect of Apremilast in experimentally induced hypertrophic scar in the rabbits. A total of 40 healthy male New Zealand white rabbits between 6 and 12 months of age were divided randomly into 4 groups (n = 10). Group I include apparently healthy control rabbits. Group II include induced hypertrophic scar control and received no treatment only base gel. Group III rabbits with induced hypertrophic scar treated with triamiconlone acetonide (TAC) 0.1% as a standard medication. Group IV rabbits with induced hypertrophic scars were treated with Apremilast 5%. On the first day, 4 surgical incisions was done using an 8-mm biopsy punch on the ventral surface of the rabbit ear down to cartilage. Triamcinolone acetonide (TAC) and Apremilast are given to the developed scars topically on day 31. The results include an examination on skin histopathology, the level of transformation growth factor beta-1 (TGF-β1), and collagen III in skin tissue. In the treatments, the inflammatory scores, scar index and immunological scores of TGFβ1 and collagen III were highly significantly decreased compared to the hypertrophic induced scar group (P≤ 0.001). Also There was significant reduction in fibroblast count compared to the group of induced hypertrophic scars (P < 0.05). Apremilast was efficacious in the treatment of hypertrophic scar because of their capacity to reduce inflammations and fibroblast counts and scar index of the hypertrophic scar, In addition to reducing immunological scores and were almost comparable to that of topical TAC.

Keywords: Hypertrophic scar, Apremilast, SEI, TGF-β1

1. Introduction
The human body responds to an injury by starting the wound healing process and the formation of scars. Scars are useful neo-formation tissues, but they lack the same characteristics and functions as the physiological tissue they replace (1).

Wound healing consists of a series of processes that are initiated by intracellular and intercellular biochemical pathways and work together to preserve tissue integrity and homeostasis. Among the cellular components involved are the coagulation cascade and the inflammatory pathways. Fibroblasts, keratinocytes, and endothelial cells, as well as immune cells including neutrophils, monocytes, macrophages, lymphocytes, and dendritic cells, have been implicated (2). Wound healing mechanisms that, in their optimal state, result in the formation of a generally indeterminate, flat, and thinly lined normotrophic scar. However, if an over wound healing occur, abnormal scars may form (3). This happens when There is a persistent inflammatory process, a prolonged proliferation phase, and reduced remodeling ,when there is an imbalance between biosynthesis and degradation, driven by apoptosis and ECM deterioration (4).

Hypertrophic scar (HSc) is a form of atypical scars. Atypical scar forms include excessive collagen deposition, resulting in elevated scar tissue texture. Following an initiating injury, the wound healing process begins as it would with normal scarring, but the buildup of repair matrix takes longer, with growing morphologic and biochemical abnormalities (3). HSc are a prevalent issue following burn injuries and other skin damage, with 67 percent of Caucasians and 74 percent of Chinese individuals experiencing them after severe burns. HSc causes physical and psychological issues in survivors because to its reddish color, elevated appearance, and poor flexibility. Targeted therapies can be used early on to avoid severe scarring and produce positive functional and aesthetic results (5).

Another aspect to consider is Poor diet; diabetes, obesity, and prior radiation exposure all contribute to tissue that does not recover properly. Medications can also contribute to impaired tissue repair (6). Hypertrophic scars are common after damage to the deep dermis (7).

Hypertrophic scars are hard, red or pink-colored elevations of the skin that are usually pruritic and only extend to the edges of the original lesion. These scars appear 4 to 8 weeks after the accident, then develop rapidly for up to 6 months before gradually receding over a few years .Histologically, Flattened epidermis, replacement of the papillary and reticular dermis by scar tissue, which is largely formed of fine, well-organized type III collagen orientated parallel to the epidermis, are the most typical findings in HSc (8).
HSc has no hair follicles, sebaceous glands, or sweat glands, and the typically undulating rete ridges between the epidermis and dermis are straightened. The proliferation and migration of fibroblasts, myofibroblasts, endothelial cells, and immune cells causes the hypocellular dermal layer to become hypercellular. As the number of cells grows, so does the amount of extracellular components deposited, resulting in an unequal generation of ECM elements. And the existence of many vertically aligned blood vessels (6).

Aspects of wound healing are regulated by transforming growth factor-beta. Scarring can result from changes in TGF expression or signaling. When compared to controls from site-matched normal skin, hypertrophic fibroblasts cells generate substantially more TGF-β, resulting in overexpression of pro-fibrotic factors. TGF-β1 mRNA expression is roughly fivefold higher in HSc than in normal skin, and this increase was also found in fibroblasts cells isolated from HSc compared to normal fibroblasts (9).

Apremilast is a phosphodiesterase 4 inhibitor (PDEI4) that acts intracellularly by increasing intracellular cAMP levels by inhibiting the breakdown of cyclic adenosine 3’, 5’-monophosphate (cAMP). This inhibition decreases pro-inflammatory mediator expression while increasing anti-inflammatory mediator expression (10). PDEI4 is implicated in a number of inflammatory processes that result in cutaneous problems. As a result, effective medications in these pathways might have a therapeutic function in dermatology (11).

Immune and inflammatory dysregulation are the root causes of the majority of dermatologic disorders. Many dermatologic disorders can thus be treated using medicines that have immunomodulatory and anti-inflammatory properties. Corticosteroids are one of the most helpful medicines, but their long-term adverse effects limit their use (12). These drugs have comparable effects as corticosteroids, but with fewer side effects. As a result, they can either minimize the need for corticosteroids or improve their efficacy (11).

Apremilast had a wide therapeutic index and had noticeable impacts on innate and cellular immunity, particularly the release of inflammatory mediators. In 2014, Apremilast (brand name Otezla®) was approved for the treatment of people with active psoriatic arthritis and moderate-to-severe plaque psoriasis (13). Apremilast is now available in tablet form in doses of 10, 20, and 30 mg for oral use (14). However, this method of administration has significant drawbacks in terms of side effects, first-pass metabolism, and is not suited for individuals who have difficulty swallowing. Topical treatment that targets a particular inflammatory mediator on the skin, in particular, offers a local pharmacological action with fewer adverse effects. Because it permits medicines to be directed
to the afflicted areas within the skin, this method provides a simple and painless option in the treatment of dermatological disorders (15).

Finally Apremilast has been shown to improve the appearance and clinical results of inflammatory diseases. In general, Apremilast is a breakthrough and a prize in the field of PDE4 inhibitor discovery (16).

2. Materials and Methods

2.1. Animals and experimental conditions

This animal research is cross-sectional study was subjected to the control and the supervision of the protocol that was reviewed by the Institutional Review Board (IRB) in Al-Nahrain University/College of Medicine following the approval of the scientific committee of Pharmacology Department in the College of Medicine/Al-Nahrain University. New Zealand White rabbits were housed under controlled environmental conditions (20±2°C, 14:10h light:dark cycle) and allowed ad libitum access to food and water. A total of 40 healthy male New Zealand white rabbits between 6 and 12 months of age were randomly divided into 4 groups (n=10). Punch biopsy was employed to induce hypertrophic scar in rabbits’ ear. Group I include apparently healthy control rabbits. Group II include induced hypertrophic scar control and received no treatment only base gel. Group III rabbits with induced hypertrophic scar treated with triamiconlone acetonide (TAC) 0.1% as a standard medication. Group IV rabbits with induced hypertrophic scars were treated with Apremilast 5%. Hypertrophic scar model was described by Caliskan, Gamsizkan (17). The animals were anasthetized by an injection of 0.25 ml of ketamine (15 mg/kg b.w.): xylazine (10 mg/kg b.w.) mixture into the marginal ear vein (18). On the first day, surgical wounds were created using an 8-mm biopsy punch. On the ventral surface of one ear, four incisions were carefully made down to cartilage. The removal of the perichondrial layer delayed epithelization, and wounds were bandaged with sterile gauze for 1 day after hemostasis was established with manual pressure. The final scars were produced on day 30 and treated for 21 days.

2.2. Preparation of gel formulations

Gel formulations of chemicals was prepared as following: first, to prepare base gel 3 gram of gelling agent hydroxy propyl methyl cellulose (HPMC) was weighted and added to 75 ml of warm distilled water (70 °C) then stirred with magnetic stirrer for 2 hours to obtain homogeneous gel (solution A). Second, 0.1gram of triamcinolone acetonide was weighted then dissolved in 10 ml of absolute ethanol alcohol to prepare (solution B) as standard drug. Third, a solution prepared from
(20ml of polyethylene glycol 400 and 10 ml of propylene glycol after mix and heated to 50 °C) then allow 5g of weighted Apremilast to dissolved in it using magnetic stirrer for 10 minutes until complete solubility is achieved to make solution B. Solution A and B were mixed thoroughly and the final weight was made up to 100 ml to prepare TAC gel 0.1% and Apremilast gel 5% respectively (19). The prepared gels were given twice daily for 21 days topically.

2.3. Sample collection and preparation

After the animals were anesthetized at the end of the study (51 days), each animal tissue sample was collected using 11 mm bunch biopsy with more 3 mm margin of adjacent skin. Each tissue sample was stored in 10% buffered formaldehyde solution prepared in sections then send for histological and immunohistochemical analysis. The inflammatory degree, fibroblast count, and index of scar were all determined using the hematoxylin–eosin (H & E) technique. The remaining sections were placed on positively charged slides and immunostained with antibodies against collagen III and the TGF-β1 marker. The average (mean ± SD) will be calculated for each group (20).

2.4. Assessment of histopathological changes

The scar elevation index (SEI) is calculated by dividing the maximum vertical height of the scar section between the perichondrium and the skin surface by the highest vertical height of the normal area around the scar between the perichondrium and the skin surface. A blindfolded examiner used a calibrated ocular reticule to quantify each wound (17, 21). Inflammation levels and fibroblast numbers were assessed semi-quantitatively. The following scores were used to assess the degree of inflammation: 0 indicates no, 1 indicates mild, 2 indicates moderate, and 3 indicates severe. The number of fibroblasts was measured using the following criteria: 0 indicates that there are no fibroblasts; 1 indicates that there are few fibroblasts; 2 indicate the existence of disorganized fibroblasts; and 3 indicate the presence of fibroblasts parallel to the wound surface.

2.5. Immunohistochemistry IHC Detection of Collagen III, TGFβ1

(I) Anticollagen III antibody: Rabbit polyclonal antibody to collagen III (Code number:SL0549R) (Sunlong biotech). (II) AntiTGFβ1antibody: Rabbit polyclonal antibody to TGFβ1 (code number SL0086R) (Sunlong biotech).

(III) Rabbit specific HRP/DAB (ABC) detection IHC kit(Abcam) (code:Ab64261).

2.6. Evaluation of IHC results
To detect primary antibody-antigen complexes, a biotinylated, cross-adsorbed, and affinity purified secondary anti-rabbit IgG was used. Following reaction with an enhanced detection reagent, application of kit resulted in the appearance of a brown precipitate in positive cells on tissue sections using light microscopy at X20. The degree of the immunohistochemistry response of collagen was determined by rating the signal intensities on a scale of: 0= undetected, 1= low density, 2= medium density, 3=dense, 4=very dense (22). TGF-β1 immunoreactivity was determined and recorded as the score: Absence of immunoreactivity had score 0, Weak immunoreactivity received a score of 1, moderate immunoreactivity received a score of 2, and strong immunoreactivity received a score of 3 (23).

2.7. Statistical analysis
Data were collected and presented using two statistical software programs: the statistical package for social science (SPSS version 23) and Microsoft Office Excel 2016. All results are presented as means ± SD. Comparison of mean values between two groups was carried out using independent t test. P ≤ 0.05 was considered significant and highly significant when p ≤ 0.01 (24).

3. Results

3.1. Visual remark (Healing rate)
In group II (induced hypertrophic scar untreated group) all animals show inflammatory signs from the first day, with partial wound closure starting on the fourth day and excessive fibrosis formation (100% induction) at 30th day (Figures 1).

Figure 1. Stages of tissue healing and hypertrophic scars formation (duration 30 days)
In group III (treated with TAC): shown gradual decrease of inflammatory signs with closure of wound and moderate decrease of thickness after 21 days of treatment healing symptoms were visible immediately after treatment (Figure 2).

Figure 2. Treatment with TAC (GIII). A. application of topical TAC gel on induced model  B. after 21 days of treatment

In group IV (treated with Apremilast): rapid wound healing signals, remarkable decrease of inflammatory signs occurred after starting treatment with closure of wound and reduction in scar thickness as in figure 3.

Figure 3. Treatment with Apremilast (GIV). A. application of topical Apremilast gel on induced model  B. after 21 days of treatment

3.2. Comparison among induced non-treated group with other studied groups

In comparison between healthy controls and induced non-treated hypertrophic scar of rabbits there was a highly significant increase in inflammation, fibroblast count, SEI, collagen III, and TGF-β in comparison with control group, P<0.001 (Table 1). While Comparison between non-treated induced hypertrophic scars group with TAC 0.1% and Apremilast treated group, a significant reduction was
noticed among TAC group and Apremilast group according to the inflammation, fibroblast count, SEI, collagen III, and TGF-β in comparison with induced non treated group, P<0.05 as in table 2 & 3 respectively.

Table 1. Comparison between control group and induced non-treated group

<table>
<thead>
<tr>
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<th>Control group</th>
<th>Induced non-treated</th>
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<tr>
<td></td>
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<td>Mean</td>
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<tr>
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<tr>
<td>Fibroblast count</td>
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<tr>
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<td>3</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>0.8</td>
<td>0.422</td>
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Table 2. Comparison between non-treated induced group and TAC treated group

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<th>TAC mean</th>
<th>SD</th>
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<tr>
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<tr>
<td>Collagen type III</td>
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<td>0.316</td>
<td>&lt;0.001</td>
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<tr>
<td>TGFβ1</td>
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<td>0</td>
<td>1.1</td>
<td>0.316</td>
<td>&lt;0.001</td>
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Table 3. Comparison between non-treated induced group and Apremilast treated group

<table>
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Figure 4. Histopathological Comparison between study groups. A. maximum vertical height of the scar section between the perichondrium and the skin surface (x4). B. Inflammation rates and fibroblast presentation (x20). I. Healthy control group. II. Induced non treated group. III. TAC treated group. IV. Apremilast treated group.

In figure 4; healthy control tissue shown no inflammation & no fibroblast cell (IB), induced group show moderate inflammation, presence of disorganized fibroblast cell & few organized fibroblast cell (IIB), TAC group shown mild inflammation and few disorganized fibroblast cell (IIIB), Apremilast group shown no inflammation and few fibroblast cell (IVB). (Yellow arrow point on inflammatory cell, red arrow point on disorganized fibroblast cell and black arrow point on organized fibroblast).

3.3. Comparison between TAC treated group and Apremilast group

Showed that Apremilast treated group was significantly reduced SEI levels in comparison with TAC treated group, P<0.05, as in table 4 and figures 5 and 6.
Figure 5. Immunohistochemical (TGF-β1 & collagen III) expression in ECM (x20).
A. TGF-β1 (brown colour mainly in epidermis) B. collagen III (brown colour mainly in dermis).
IA. Control group shown absence immunoreactivity of TGF-β1. IB. Control group shown low density of collagen III. IIA. Induce non treated shown strong immunoreactivity of TGF-β1. IIB. Induce hypertrophic scar shown dense of collagen III. IIIA. TAC group show a weak immunoreactivity of TGF-β1. IIIB. TAC group shown low density of collagen III. IVA. Apremilast group shown weak immunoreactivity of TGF-β1. IVB. Apremilast group shown medium density of collagen III.

Figure 6. Comparison between induce non treated, TAC and Apremilast treated groups
Table 4. Comparison between TAC treated group and Apremilast treated group

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<td>TGFβ1</td>
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<td>0.316</td>
<td>0.7</td>
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According to the correlation between different variables in the study (inflammations, fibroblast count, SEI, collagen type III, and TGF-β); there was a positive significant correlation between each other among the studied sample, P<0.001 (Table 5).

Table 5. Correlation between the study variables

<table>
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<tr>
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<th>Collagen III</th>
<th>TGF-β</th>
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<td>.704**</td>
<td>.758**</td>
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<tr>
<td></td>
<td>P .000</td>
<td>.000</td>
<td>.000</td>
<td>.000</td>
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<tr>
<td>Fibroblast Count</td>
<td>r 1</td>
<td>.758**</td>
<td>.561**</td>
<td>.503**</td>
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<tr>
<td></td>
<td>P .000</td>
<td>.000</td>
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</tr>
<tr>
<td>SEI</td>
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<td>.639**</td>
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4. Discussion

Hypertrophic scar (HSc) is a pathological symptom resulting from excessive deposition of collagen and dermal fibrosis in a connective tissue due to skin dermal injury (25). Any medications that modulate the abnormalities in the period of wound healing could be of benefit in the therapeutic process of scars. Studies and drug manufacturing confirmed that it takes more than 10 years to discover and develop a new drug into the markets, at an average cost exceeding 2.6 billion US dollars. As a result, new therapeutic uses for the existing medications may be discovered through drug repurposing is cost
effective and promising (26). Different therapeutic modalities, such as corticosteroids, topical silicone gel sheeting, surgical excision, laser therapy, cryotherapy and radiotherapy, have been used to treat HSc; however, none of these modes of treatments has proven completely effective. That’s why, the molecular mechanism underlying scar formation still needs to be studied and clarified, and successful treatment scar remains a challenge. As the pathogeneses of HSc have not been elucidated, and current therapeutic approaches have limited effectiveness (27).

The HSc model of the ear of rabbits was successful as there were significant differences between induced non treated group and control healthy group in response to inflammation, fibroblast count, SEI, collagen type III, and TGF-β1, the results is high significant increase (p ≤ 0.001), and it is consistent with the finding of Zhang, Liu (28). Many animal models of HSc, such as the white pig model and mice model, have been utilized to discover the mechanism of scarring and test novel treatments. The inability of these models to mimic the natural process of HSc formation was their primary limitation. Humans are the only ones who get abnormal scars, such as HSc. The fibromuscular layer under the dermis of laboratory animals is thought to represent the major pathogenic difference between animals and humans (29). In animal models, healing from injuries and lesions is therefore dependent on wound contraction rather than re-epithelialization. The rabbit ear model of HSc is well-established and frequently utilized in experiments and researchs (30). Many evidences indicate that scar formation show a marked preference for the locations on the body that are frequently subjected to tension like the anterior chest (31). Also, fibroblasts from deeper layers of skin had lower collagenase levels but higher amounts of TGF-β1, collagen, and α-smooth muscle actin. These fibroblasts are thought to be similar to HSc fibroblasts and may play a key role in scar formation and development. Taken together, these facts help to explain why HSc occurs more frequently after deep dermal injuries in some body regions than after superficial lesions (32, 33).

Treatment group by the TAC have high significant reduction in fibroblast count and SEI as compared with group of induced HSc (p ≤ 0.01). Extremely high significant reduction was observed in TAC group (p ≤ 0.001) in inflammation, TGF-β1 and collagen III in comparison to induced HSc group. Those findings reported also by Pessoa, Melhado (34) and Carroll, Hanasono (35). TAC is an effective and the most commonly used treatment for HSc, The steroid group was designed because any prospective therapy should outpace the current criterion of steroid as standard therapy with fewer side effects on. TAC significantly suppressed cell proliferation and TGF-β1 expression, leading to a significant induction of matrix metalloproteinase-2 and a down-regulation of production of type III collagen. The lower effect of TAC on apoptosis might be an explanation for the higher recurrence rates in hypertrophic scars treated with TAC alone (36).
There is a considerable reduction in collagen III in TAC, which is consistent with (37). In addition to a drop in TGF-β1 after treatment with triamcinolone acetonide, one possible mechanism for collagen drop in the extracellular matrix is the influence on plasma protease inhibitors, allowing collagenase to breakdown collagen. Regarding SEI, TAC was shown a significant decrease in the SEI measurements that agrees with Caliskan, Gamsizkan (17), and disagreeing with Saulis, Mogford (21) that observed an insignificant decrease in SEI after 4 weeks’ treatment with onion extract and TAC in rabbits ear model.

This study found a significant improvement in the Apremilast treated group in comparison with non-treated induced group, there is highly significant reduction according to the inflammation, SEI, collagen III, and TGF-β and significant reduction in fibroblast count. Apremilast show better anti-inflammatory effect than TAC, Anti-inflammatory potential of Apremilast was confirmed for its ability to decrease the production of IL-6 and IL-8 in many in vitro models. This effect was confirmed histologically by reduction in infiltration of inflammatory cells, and immunologically by decreasing inflammatory cytokines IL-8, IL-17A and TNF-α, which suggests that this formulation could be used as an attractive topical treatment for skin inflammation (38). On the other hand, In vivo, Apremilast significantly reduced epidermal thickness and proliferation, decreased the general histopathological appearance of psoriasis form features and reduced expression of TNF-α, human leukocyte antigen-DR in addition to intercellular adhesion molecule-1 in the lesion of that skin (39).

When compared to healthy volunteers and patients without HSc, the numbers of IL-4+ T cells (Th2), as well as levels of IL-10 and TGF-β, were considerably greater in blood of patients with HSc in the early stages following burn injuries. Additional Th subsets, called Th17, Th22, Th9, and T follicular helper (Tfh) have been recognized recently (40). Although the function of these subsets in HSc is unknown, both Th17 and Th22 are essential producers of IL-22, which plays a key role in tissue healing. IL-22 promotes cell survival, epithelial cell proliferation, and barrier function. As a result, IL-22 plays an essential role in developing extracellular pathogen resistance. Delay in epithelialization and bacterial colonization, as previously noted, are key predictors of severe scarring. As a result, IL-22 has the potential to lower the risk of HSc by encouraging fast epithelialization and reducing bacterial colonization. Apremilast is PDE4I member, this group of drugs has been shown to influence both innate and adaptive responses in a growing body of studies. In macrophages, neutrophils, monocytes, and dendritic cells, inhibiting PDE4 exhibited regulatory actions (41). Furthermore, PDE4I inhibited T cell receptor (TCR)-induced activation of T cells, resulting in decreased cytokine and chemokine release from T helper-1 (Th1), Th2, and Th17 cells, whereas PDE4 inhibition may have little effect on the
phenotype and function of B cells (41). In addition, increased cAMP levels in keratinocytes and epithelial cells may suppress inflammatory responses and control cell development and barrier activities. These facts were implicated also in vivo by Huff and Gottwald (42), they used Apremilast on inflammation in a non-FDA-approved fashion, to treat 50 years old lady with vitiligo. After six weeks of Apremilast treatment, the patient was starting to re-pigment. Three months after initiating treatment, 60 mg intramuscular TAC was added with apremilast 30 mg twice daily was maintained. Giving that many years of using steroids alone had yielded no improvement.

This significant improvement in apremilast treated group in spite of its poor water solubility prove the success of the prepared formula to release the drug, and that agree with Sarango-Granda, Silva-Abreu (38) who prepare apremilast as microemulsion and demonstrated its capacity to release the incorporated drug following a first-order kinetic model while also guaranteeing a local anti-inflammatory effect with reduced systemic adverse effects due to the high drug retention in the skin.

Apremilast treated group showed a superior reduction in SEI and better TGF-β1 in comparison to TAC, and that can be explained as apremilast is PDE4 inhibitors, PDE4I inhibit several profibrotic activities of fibroblasts and these effects are more pronounced in the presence of TGF-β1. The inhibitory effect of the PDE4I is mediated by potentiating endogenous PGE2 signaling, which in turn acts, at least in part, by stimulating cAMP and protein kinase-A. In addition to inhibiting the breakdown of cAMP, PDE4 inhibitors potentiate TGF-β1-induced prostaglandin E2 production. By augmenting an endogenous feedback control mechanism, PDE4 inhibitors have the potential, as therapeutic agents, to limit TGF-β1-driven fibrosis. Also according to Thangapazham, Sharma (43), TGF-β1 induces angiogenesis, therefore a reduction in TGF-β1 in all groups was associated with wound closure in the generated hypertrophic scar.

The current study reported a positive significant correlation between the inflammatory response parameters among the studied samples. This suggests that the inflammatory response of scar tissue is correlated with each other whatever the therapeutic technique utilized (44).

In Conclusion, Apremilast was effect in reducing scar and is comparable to that of triamcinolone acetonide.

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References


