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Topical Isoxsuprine in experimentally induced hypertrophic scar in rabbits

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ABSTRACT

Hypertrophic scars are pathological scars which result from exaggerated skin proliferation following a wound and injury. Although many theories have been implicated for keloidogenesis, the precise patho-physiological cause is still masked. Different treatment strategies have been tried in their management, but there is no satisfactory option for treating hypertrophic scar currently; moreover the standard steroid therapy is associated with numerous local side effects, and there is a need for researches in new treatment options. The aim of this study is to evaluate the role of topical isoxsuprine in experimentally induced hypertrophic scar in rabbits. In the current experimental study, 40 healthy male albino rabbits between 12 and 14 months of age were studied. These rabbits were categorized into five groups: healthy animal group (n = 8), hypertrophic scar without treatment (n = 8), hypertrophic scar treated with triamcinolone acetonide gel (n = 8), and hypertrophic scar treated with isoxsuprine gel (n = 8). Histological assessment of skin biopsy, including the conventional hematoxylin and eosin stain, and immunohistochemistry for transforming the growth factor beta 1 level (TGF-β1) and collagen 3 alpha1 (COLIIIαI) in skin tissue was done. The immunohistochemical score of TGF-β and collagen III was highest in group 2 (hypertrophic scar without treatment), followed by group 3 (hypertrophic scar treated with triamcinolone acetonide gel) and group 4 (hypertrophic scar treated with isoxsuprine gel) – no significant difference between them since $p >$ 0.05, and then by group 1 (healthy control group). Regarding histopathological scores of inflammation, the scar height, and scar index, the scores were highest in was highest in group 2 (hypertrophic scar without treatment), followed by group 3 (hypertrophic scar treated with triamcinolone acetonide gel) and group 4 (hypertrophic scar treated with isoxsuprine gel) – no significant difference between them since $p >$ 0.05,

with the exception of index of scar, and then by group 1 (healthy control group). It was concluded that isouxoprine in a topical formulation greatly reduced inflammation and scar formation in deep wounds in a manner comparable to that seen with triamcinolone.

Keywords: *hypertrophic scar; isoxsuprine; rabbit*

INTRODUCTION

During the process of wound healing, one of the unwanted complications is the development of hypertrophic scars.[1] Hypertrophic scar is characterized by excessive connective tissue formation within the confines of the original wound itself; however, connective tissue deposition may exceed the original wound site, and as such it is called a keloid.[2] A number of risk factors are associated with keloid and hypertrophic scars.[3,4] One of these risk factors is the presence of skin tension at the side of the wound. The areas of skin tension such as the chest, the back, and the upper arms are more prone to hypertrophic scar and keloid formation, and the areas of minimal skin tension such as the eyelids are less prone to hypertrophic scars and keloid formation.[5–8] Some systemic factors such as systemic hypertension have been to be associated with increased incidence of hypertrophic scar development.[9] Systemic inflammation is another recognized risk factor associated with both keloid and hypertrophic scar.[10] Repeated trauma has also been blamed as a risk factor for hypertrophic scar development. One such example is the use of earrings; the frequent episodes of putting on and taking off earrings are associated with repeated minor trauma, and the likelihood of scar development.[11] Second and third degree burns are well recognized risk factors of keloid and hypertrophic scar development, particularly if the period of healing lasts longer.[12] Another well-known risk factor is the presence of infection.[13] Genetic factors such as single nucleotide polymorphism have been shown to be associated with increased risk of hypertrophic scar formation.[14] The incidence rate of hypertrophic scar is higher in pregnant women and adolescents; in addition, it is more commonly seen in African Americans than in Caucasians.[15,16]

From a clinical perspective, there is a distinction between hypertrophic scar and keloid; however, from pathologic point of view they share more or less the same pathogenic mechanism. In the usual process of wound healing, inflammation, angiogenesis, fibroblast proliferation, and tissue remodeling works in an orchestrated manner, and pathologic scar formation occurs when there is disruption of one of these steps – in particular tissue remodeling.[17]

Isoxsuprine is a drug with the ability of direct relaxation of uterine and vascular smooth muscle fibers, stimulation of beta aderonceptors, production of positive chronotropic and inotropic effects, and dilatation of blood vessels and in particular those supplying skeletal muscles. There are three principal mechanisms that induce the pharmacodynamics of this drug. The first is the stimulation of beta aderonceptors, the second is the inhibition of α-adrenoceptors, and the third one is the direct papaverine-like Spasmolytics of smooth muscles.[18] The observation that beta blockers induce the formation of skin pathology through the enhancement of angiogenesis,[19,20] we suggest that the use of bet agonist, such as isoxsuprine, may counter act this mechanism, resulting in reduction of scar size and resultant disfigurements.

MATERIAL AND METHODS

In the current experimental study, 56 healthy male albino rabbits in the age range of 12 to 14 months, which were obtained from the local markets, were studied. These animals were kept in the animal house at the Faculty of Medicine/Al-Nahrain University, Baghdad. They were allowed 48 hours

to acclimatize to the animal room conditions of controlled temperature (28–30 ºC). They were allowed free access to food and water. The study was approved by the institutional ethical approval committee pertaining to Faculty of Medicine/ Al-Nahrain University.

Animal grouping

Forty rabbits were divided into seven groups with eight rabbits in each group: Healthy animal group (group 1, $n = 8$); hypertrophic scar was induced and the animals left without treatment (group 2, $n = 8$), hypertrophic scar was induced, and then the animals treated with triamcinolone acetonide gel (standard drug) 0.1% twice daily for 28 days (group 3, $n = 8$); and hypertrophic scar was induced scar then the animals treated with isoxsuprine gel 3 % twice daily for 28 days (group 4, $n = 8$).

Induction of hypertrophic scar in rabbits

The induction of hypertrophic scar was based on the method described by Cliskan *et al.* in 2016.[21] To induce anesthesia in the experimental animals, Ketamine at a dose of 45 mg/kg and xylazine in a dose of 5 mg/kg were given intramuscularly. An 8 mm biopsy punch was performed after making a surgical wound. Wounds, four in number, were created on the ventral surface of an ear in a depth till the cartilage. Delaying was done by removing of the perichondrial layer. Hemostasis was carried out by manual pressure, and then a sterile gauze was used to cover the wounds for a period of one day. Waiting for the scars to harvest, each rabbit was housed alone. Scars were obtained on day 30.

Preparation of gel formulations

A similar technique was used to prepare gel formulations of chemicals, and a magnetic stirrer was used on need. Solution (A) was prepared by dissolving a weighted concentration of isoxsuprine in a 10 ml of ethanol alcohol. Solution (B) was prepared by weighing approximately 3 g of the gelling agent (hydroxy propyl methyl cellulose) and adding it to 75 g of distilled water, followed by stirring it to dissolve the solution. These solutions, A and B, were thoroughly mixed and the final weight was 100 g. The samples were allowed to equilibrate at room temperature for at least 24 hours.[22]

Variables included in the current study

The variables included in the current study were the histopathological study of skin sections and the immunohistochemical expression of transforming growth factor beta 1 level (TGF-β1) and collagen 3 alpha 1 (COLIIIαI) in skin tissue.

Tissue harvesting and evaluation of scars

After induction of anesthesia, a punch biopsy of 11 mm with 3 mm adjacent skin margin was carried out on the two best samples from each animal.[23] The tissue biopsies were then fixed with 10% buffered formalin, and then processed to produce paraffin blocks. Sections were made from these blocks on glass slides, and stained with hematoxylin eosin and immunohistochemistry. Hematoxylin and eosin stained slides were then examined by a well-trained pathologist to determine the degree of inflammation, height and index scar and size of it. Immunohistochemical sections were placed on positive charged slides and were immune stained with an antibody against collagen III and transforming growth factor (TGF-β1) marker.[24] The average score was then calculated and compared among groups.[25]

Assessment of histopathological changes of skin sections

Tissue sections were assessed as follows: measuring the SEI index "the ratio between the highest vertical height of scar area between perichondrium and skin surface to the highest vertical height of normal area around the scar between perichondrium and skin surface". The measurement was performed by a calibrated eyepiece reticule by a blinded examiner.[26] The degree of inflammation, fibroblast counts, and wound size were evaluated in a semi-quantitative manner. The degree of inflammation was evaluated

according to the following scores: 0 = none, 1 = mild, 2 = moderate, and 3 = severe. Fibroblast count was evaluated according to the following scores: 0 = absence of fibroblasts; 1 = few fibroblasts, 2 = presence of disorganized fibroblasts, and 3 = presence of fibroblasts parallel to the wound surface. The size of the wound was evaluated according to the following scores: 0 = closed wound, 1 = small, 2 = medium, and 3 = large.[27]

EVALUATION OF IMMUNOHISTOCHEMICAL RESULTS

A biotinylated, cross-adsorbed, and affinity purified secondary anti-mouse IgG was used to detect primary antibody-antigen complexes adhered to a glass microscope slide; following a reaction with an enhanced detection reagent, proper and accurate application of kit instructions led to the appearance of a brown precipitate in positive cells on tissue sections. Quantification of collagen protein expression was evaluated under light microscopy at X40. The counting of positive cells was performed at X40.

The extent of the immunohistochemical reaction of ECM proteins, such as collagen was measured by ranking the signal intensities according to the following scale: – (absent), + (mild), ++ (moderate), +++ (marked).[28] Stained slides were examined to identify immunoreactivity for TGF-β1. The scoring system was done, and the score recorded was the average intensity of the expression: The absence of immunoreactivity had a score 0, weak immunoreactivity had score 1, moderate immunoreactivity had score 2, and strong immunoreactivity had a score 3.[24]

Statistical analysis

Using two statistical software programs, the statistical package for social science (SPSS version 22) and Microsoft Office Excel 2013, the data was collected, summarized, analyzed, and presented. All the obtained results are presented as means ± SD. Comparison of mean values between two groups was carried out using Mann Whitney U test and unpaired t test. On the other hand, data for multiple comparisons were performed by Kruskal Wallis test, Post hoc Turkey test and one-way ANOVA. $P \leq 0.05$ was considered significant and highly significant when $p \leq 0.01$.[29]

RESULTS

Healing rate

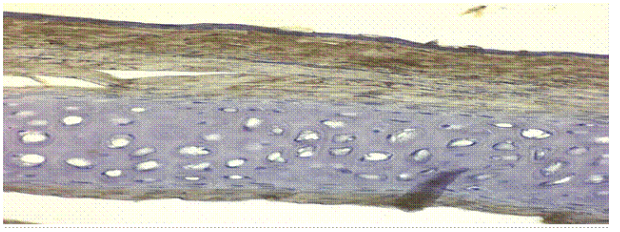
The appearance of untreated induced hypertrophic scar: normal healing process involves three overlapping phases, inflammation (0–3 days), cellular proliferation (3–12 days), and remodeling (3–6 months). So in this group, the inflammatory signs are seen from the first day in all animals with partial wound closure, starting from the fourth day, and excessive formation of fibrosis (100% induction) on the 30th day. In group 3 (induced hypertrophic scar in rabbits treated with triamcinolone acetonide), the healing signs were very clear, starting after the treatment with fading of inflammatory sign. Finally, there was complete wound closure and decreased thickness of scar after 28 days of treatment. In Group four (induced hypertrophic scar in rabbits treated with isoxsuprine gel): remarkable decrease of inflammatory signs occurred after starting the treatment with a closure of wound and no sign of thickness after 28 days of treatment.

Immunohistochemistry

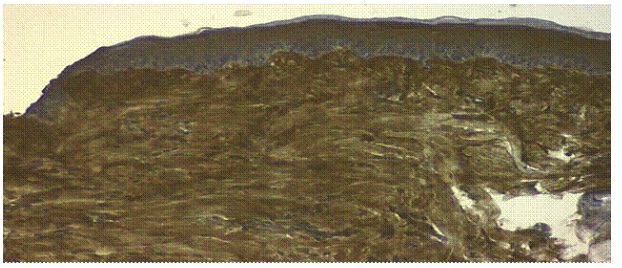
The immunohistochemical score of transforming growth factor (TGF-β1) and collagen III are shown in Table 1, in which comparison of mean score among study groups was carried out. The score was highest in group 2, followed by group 3 and group 4, (no significant difference was noted between them since $p > 0.05$), and then by group 1 (healthy control group). The results are shown in Figures 1, 2 and 3.

Histological findings

A biopsy was taken from animals as explained in chapter two. Inflammation was assessed by an



(A)



(B)

FIGURE 1. Cytoplasmic immunohistochemical expression of TGF-β1 in dermis(×20) (A) Normal tissue showing low intensity of TGF-β1; (B) Induced hypertrophic scar shows high intensity of TGF-β1

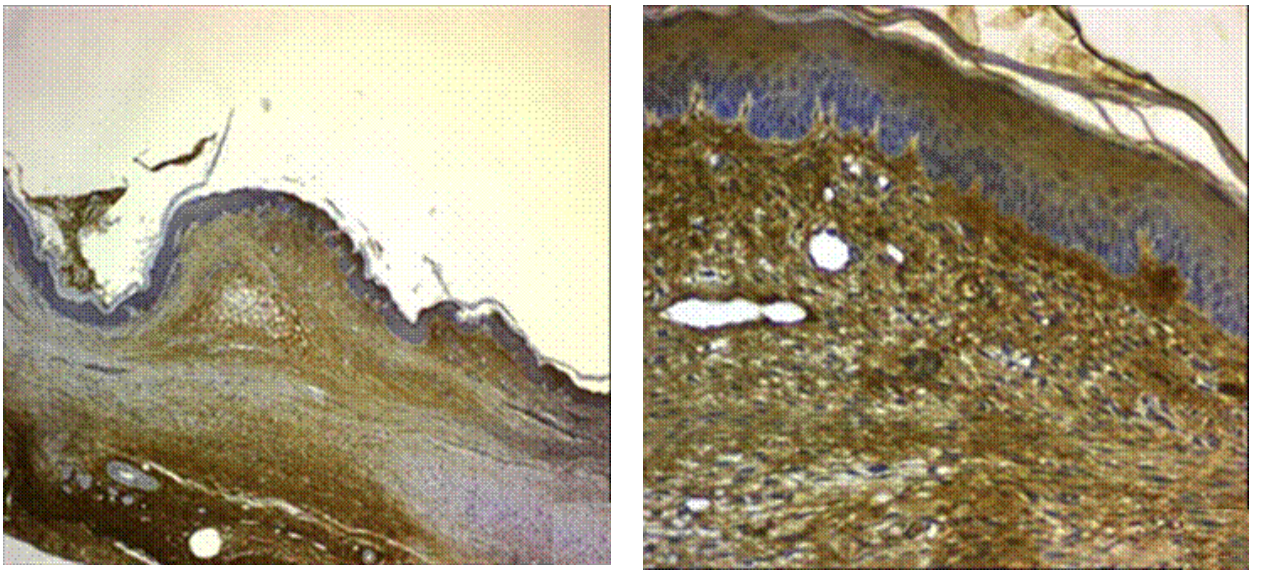
expert pathologist and graded as mild, moderate, and severe. Mild inflammation was given a score of 1, moderate inflammation was given a score of 2, and severe inflammation was a given a score of 3. All results are shown in the Table 2 and Figures 4 and 5. The scores are shown in Table 2. The score was the highest in group 2 (hypertrophic scar) followed by 3 and 4 (no significant difference between them since $p > 0.05$ with the exception of index of scar), and then by group 1 (healthy control group).

DISCUSSION

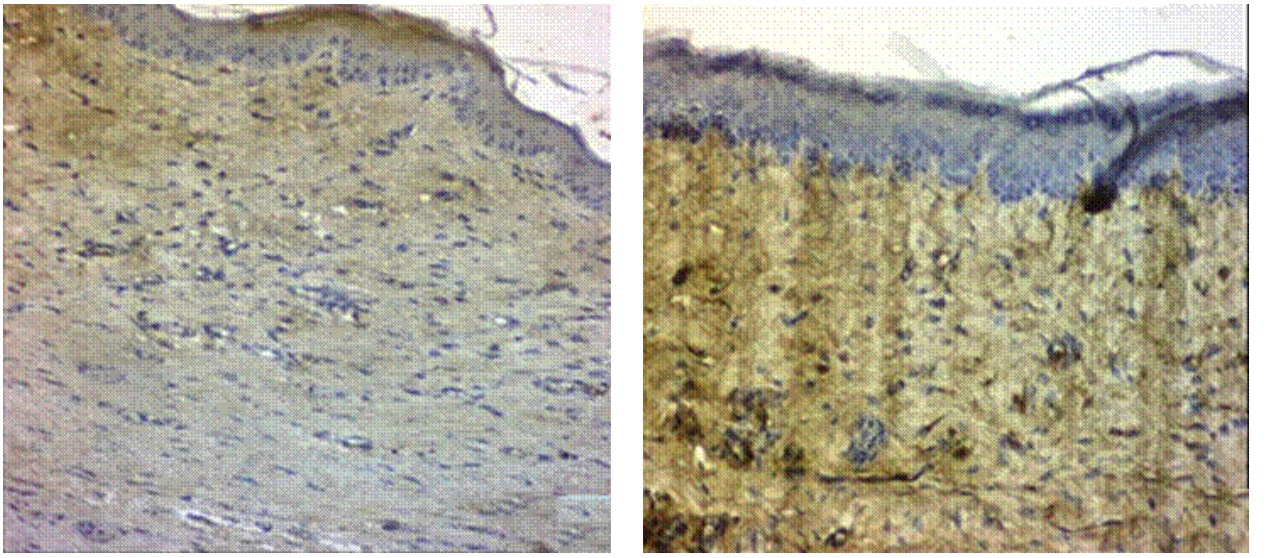
One of the important health issues seen daily by general surgeons and plastic surgeons is the improper healing of wounds. One such example is the development of hypertrophic scar, and in extreme conditions the deposition of connective tissue outside the original wound leading to what is known as a keloid.[30] A number of risk factors have been reviewed in the introduction section of this article associated with hypertrophic scar and keloid development. One such risk factor is burn which is commonly seen in our community.[3,4] In addition to this, scar in women or in young adults on exposed areas of the body may lead to disfigurement and associated psychological upset.[15] Therefore, the development of a pharmacological approach that can reduce or inhibit hypertrophic scar or keloid development appears to be mandatory. Moreover, there are several challenges in the treatment of hypertrophic scar, including longer healing time, lack of specific remedies, side-effects of drugs, and lack of early intervention may decrease the optimal response of treatment.[31]

In this study, based on previous observation that beta blockers are associated with angiogenesis[19,20] it is suggested that the use of bet agonist may counter act this phenomenon, and lead to less deposition of granulation tissue, and less scar development in association with process of wound healing. In the current study, isoxoprine was associated with less inflammation than the group with no topical treatment, and inflammatory score that is comparable to the group of topical steroids. Thus, isoxsuprine will lead to a significant reduction in inflammatory responses associated with wound healing, and eventually less scar formation, as scar amount is a function of the severity and amount of inflammation and tissue destruction.[17] On the other hand, we observed less amount of scar and scar index in association with use of isoxsuprine in comparison with the group with no topical treatment, and the reduction in the amount of scar was comparable to the group in which topical steroid was used. Thus, isoxsuprine is effective in a way similar to topical steroids in this regard.

The process of scar formation is a function of the amount of granulation tissue in case of wound healing, and granulation tissue is a combination of new blood vessel formation (angiogenesis), fibroblast proliferation, and laying down collagen.[32] Therefore, we believe that isoxsuprine by inhibiting



(A) (B)



(A) (B)

FIGURE 2. Cytoplasmic immunohistochemical expression of TGF-β1 in treatment groups. (A) and (B). Hypertrophic scar showing mild intensity TGF-β1 in TAC treated group (×20); (C) and (D) hypertrophic scar showing mild intensity TGF-β1in isoxsuprine treated group (×20)

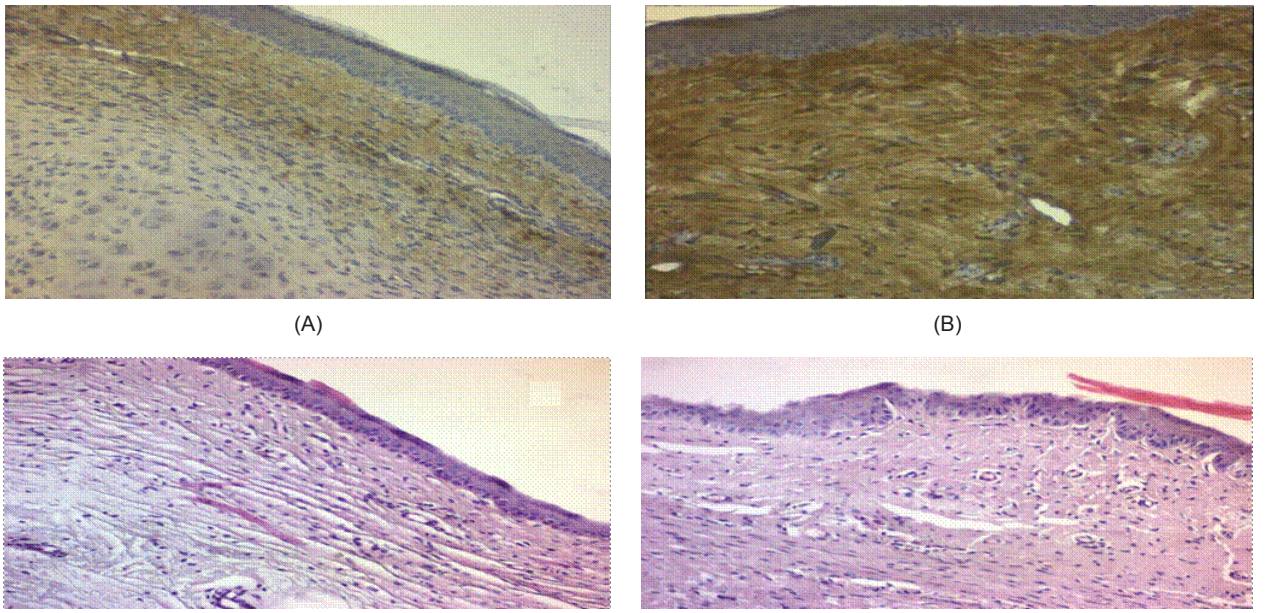
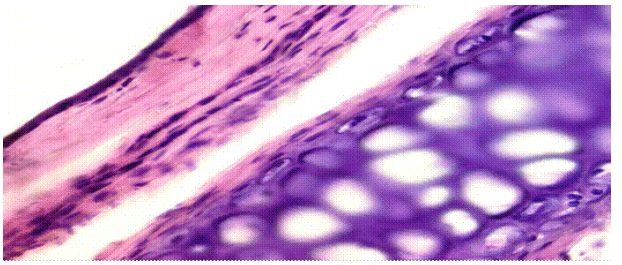


FIGURE 3. Cytoplasmic immunohistochemical expression of collagen III in Dermis (×20). (A) normal tissue showing low intensity of collagen III. (B) Induced hypertrophic scar showing high intensity of collagen III. (C) hypertrophic scar of 0.1% triamcinolone group. (D) Isoxsuprine treated group.

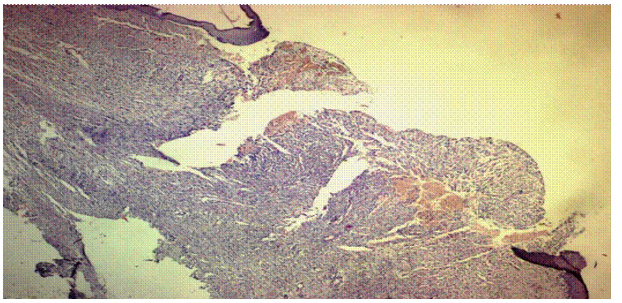
TABLE 1. Comparison of Immunohistochemical Score Mean of Transforming Growth Factor (TGF-β1) and Collagen III among Study Groups

Characteristic	Healthy control group *n* = 10	Hypertrophic scar group *n* = 10	Isoxsuprine group *n* = 10	Triamcinolone group *n* = 10
TGF-β1				
Mean ±SD	1.65 ± 0.30 C	3.96 ± 0.23 A	2.31 ± 0.28 B	2.09 ± 0.25 B
Range	1.3–2.1	3.6–4.4	1.9–2.7	1.8–2.6
Collagen III				
Mean ±SD	1.12 ± 0.24 C	3.88 ± 0.39 A	1.89 ± 0.12 B	1.79 ± 0.15 B
Range	0.9–1.7	3–4.5	1.7–2.1	1.5–2

***n**: number of cases; SD: standard deviation; capital letters were used to indicate the level of significance after performing one way ANOVA test and post hoc LSD test so that similar letters indicate no significant difference while different letters indicate significant difference at $p \leq 0.05$ and letter (A) takes the highest mean value followed by (B) then (C) then (D)*



(A)



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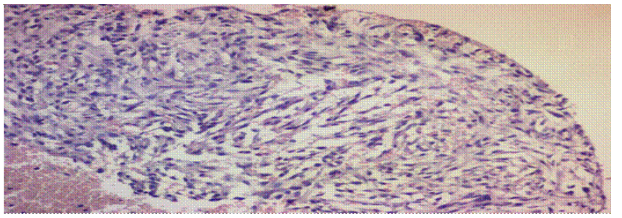
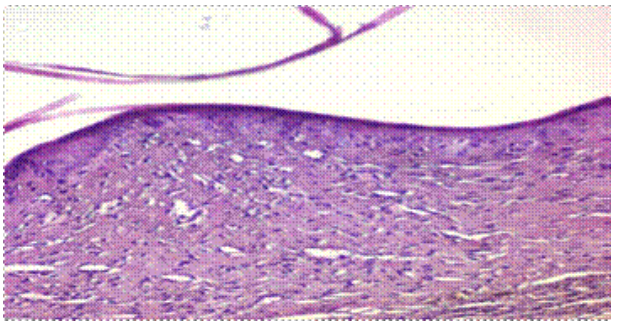


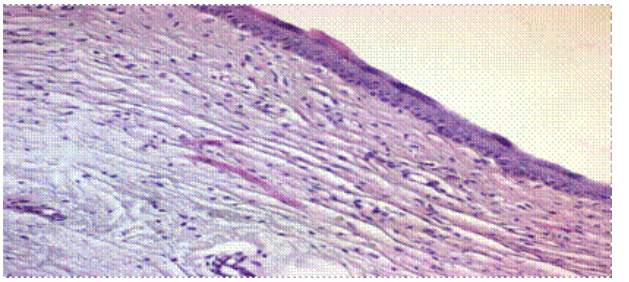
(C)

FIGURE 4. Some of the histologic cross-section of rabbit tissue on POD of 30 (H&E staining). (A) Normal dermal tissue characterized by the absence of inflammatory cell and fibroblast with close wound (×20); (B) Induced hypertrophic scar represent by large open scar (×4); (C) Induced hypertrophic scar tissue represent by severe inflammation and high number of polymorph nuclear cells, also dermis cellularity increases, fibroblasts was sever, and arranged in disorganized manner (×20)

the growth factors that are involved in angiogenesis and fibroblast proliferation and function, namely TGF-β and EGF, leads to significant reduction in eventual scar development. This suggestion was supported by our observation that the immunohistochemical score of both these growth factors was



(A)



(B)

FIGURE 5. Some of the histological sections in treatment groups stained with H and E stain and examined for inflammation (black arrow), fibroblast count, and arrangement (yellow arrow -organized fibroblast), (red arrow - disorganized fibroblast) and scar size (×20). (A) hypertrophic scar of 0.1% triamicinolone gel group. (B) hypertrophic scar of 3% isoxsuprine group

significantly less in a group of isoxsuprine in comparison with the group without topical treatment. Moreover, the score in both the growth factors was comparable to that of the topical steroid group.

Therefore, we can conclude that the use of isoxsuprine leads to reduction in the production of growth factors, TGF-β and EGF, leading to less angiogenesis and less fibroblast proliferation activity in laying down collagen, and eventually less scar formation. Another support in this study to less fibroblast activity is the observation of lower collagen III score in association with isoxsuprine use which comparable to the use of topical steroids. To validate the results of the current study, further experimental work and clinical trials are needed.

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TABLE 2. Histopathological Evaluation and Scores Compared among Study Groups

Characteristic	Healthy control Group *n* = 10	Hypertrophic scar group *n* = 10	Isoxsuprine group *n* = 10	Triamcinolone group *n* = 10
Inflammatory score				
Mean ± SD	0 ± 0 C	3.41 ± 0.35 A	1.33 ± 0.30 B	1.47 ± 0.347 B
Range	0–0	3–4	1–2	1–2
Height of scar				
Mean ± SD	0 ± 0 C	7.96 ± 0.46 A	2.36 ± 0.34 B	2.38 ± 0.37 B
Range	0–0	8–9	2–3	2–3
Index of scar				
Mean ± SD	0 ± 0 D	8.8 ± 0.58 A	2.97 ± 0.21 B	2.27 ±0.27 C
Range	0–0	8–10	3–3	2–3

***n**: number of cases; SD: standard deviation; capital letters were used to indicate the level of significance after performing one way ANOVA test and post hoc LSD test so that similar letters indicate no significant difference while different letters indicate significant difference at $p \leq 0.05$ and letter (A) takes the highest mean value followed by (B) then (C) then (D)*

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