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The Effect of Intralesional Injection of Salbutamol in Experimentally Induced Hypertrophic Scar

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Abstract

Background: Hypertrophic scars are developed as a result of exaggerated dermal tissue proliferation after skin injury .Althoughmany theories regarding generation of hypertrophic scar exist; however the precise pathophysiological process remain unclear. Many various treatment modalities have been implicated in their treatment, however currently there is no satisfactory strategy for treating all keloid lesions; moreover the standard intralesional injection of steroid are associated with a lot of local side effects and there is need for search for other treatment options.

Aim of the study: to evaluate the role of intralesional effect of salbutamol in experimentally induced hypertrophic scar in rabbits.

Materials and methods:

Forty healthy male albino rabbits between 10 and 12 months of age were used in the study. The animals were left for 2 days to acclimatize to the animal house conditions of controlled temperature (28-30ºC), allowed free access to water ad libitum and pellet . Forty rabbits were divided into five groups, ten rabbits pergroup .

Group1. Healthy animal group.

Group2. Hypertrophic scar was induced and the animals left without treatment.

Group3. Hypertrophic scar was induced then the animals treated with intralesional triamcinolone acetonide (standard drug) once weekly for 4weeks.

Group4. Hypertrophic scar was induced scar then the animals treated with intralesional salbutamol 0.2% (investigated drug) once weekly for 4weeks.

The results measures included evaluation of histopathology of dermal sections, transforming growth factor beta 1 level (TGF-β1) , and collagen IIIin skin of rabbit'0s ears .

Results

By comparison with the control group, intralesional administration of salbutamol significantly reduce the scars in ear of the rabbit models , this effect was demonstrated on the basis of histological and biochemical parameters , moreover this therapeutic effect was comparable to standard intralesional steroid injection.

Conclusion

Intralesional salbutamol injection has powerful therapeutic effects on deposition of collagen, fibroblasts hyperplasia , and micro angiogenesis in scars on rabbit ears without demonstration a local side effects.

Keywords: hypertrophic scar, intralesional salbutamol

Introduction

Hypertrophic scar formation is an unwanted complication in the healing process ofwound (1). The keloid scar, is usually used interchangeably with hypertrophic scar , however this is not precise(2). In hypertrophic scar the connective tissue deposited in the original wound while in keloid scar the deposition usually extends to the area beyond of the original wound (3, 4).

The mechanisms of hypertrophic scar formation are complicated ; moreover the process may be determined by multiple factors (1).

From pathophysiological point of view wound healing is divided into three phases inflammatoryphase , proliferative phase , remodeling phase (5) . The scar is formed in the remodeling phase (6)

Immediately after wound, the homeostasis started and the bleeding is controlled by the effect of platelets aggregation at the site of injury (7). The subsequent production of the fibrin clot inhibits the bleeding and provides a framework for the attachment and proliferation of the cells(11) .

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Initiallyduring wounding time ,inflammatory phase begins , where activation of the coagulation cascade that stimulates chemotaxis of neutrophils and then macrophages into the wound (7).
Then inflammatory process progresses (after 2–3 days) into the proliferative phase in whichfibroblasts are attracted into the wound site to synthesize granular tissue (8). This granular tissue is composed of procollagen, proteoglycans,elastin, and hyaluronic acid (9).
This phase becomes a major theme to restoring the barrier function of skin, and the vascular network that lead to generation of granulation tissue (8) .

Re-epithelialization requires two processes of migration and proliferation of keratinocytes and epithelial cells (10)

Re-epithelialization processusually stimulated by a different signals, such as nitric oxide, that synthesized mainly by macrophages(11), cytokines and growth factors, including epidermal growth factor (EGF), and IGF-1 thatsecreted from multiple cell types in the injury (12) .

Restorationof the network of blood vessels is mandatory , since nutrients and oxygen are needed during this phase that involves formation of new blood vessel, also known as 'angiogenesis', that initiated by growth factors, e.g., vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and the serine protease thrombin in the wounds, which activate the endothelial cells of existing vessels (13).
Once the injury is closed, the immature scar can move on to the remodeling phase starts at the end of development of the granulation tissue(8) .During this event mechanical tension and cytokines production (TGF-β) , drive fibroblasts to differentiate into myofibroblasts, that express α-smooth muscle actin which contract the wound. (14) .
Nevertheless myofibroblasts subjected to apoptosis when healing phase is complete (15). At this condition, the produced collagen III is replaced by the collagen I, which has a higher tensile strength but required more time for deposition (8). This remodeling phase can last up to a year depending on the severity of the wound, which over time gradually contracts to regains its integrity however , Some skin components, such as., hair follicles and sweat glands, cannot be recovered after serious injury; and the wound can ever achieve is ∼80% of original tensile strength(16). Hypertrophic scars are common skin manifestations approximately 100 million people suffer from scar-related tissues in the developed world, (17).
The incidence of scarring was different in studies found between 38% and 68% postoperative hypertrophic scars (18) and up to 91% following burn injury, depending on the depth of the wound (19). The pathogenesis of hypertrophic scar involves number of signaling pathways including TGF-β that considered to be the master regulator of fibrosis and its effects on immune modulation , apoptosis , collagen deposition, cell proliferation, differentiation and other processes have been well implicated in hypertrophic scar (20). Moreover there are three subtypes of TGF-β including (TGF-β1, -β2, -β3) that secreted as inactive precursors which require activation before binding to the TGF-β receptors (21).In wound healing the majority of cells express TGF-β in an inactive isoform that promotes the chemotaxis of fibroblasts to the site of injuy (22).

Fibroblasts derived from scars have shown both an alteration in TGF-β signaling and an increased expression of the pro-fibrotic cytokines (23). Moreover ,studies of hypertrophic scarring have indicated increased expression and phosphorylation of the receptor Smads-2 and/or 3 (24, 25) .

Thus, the inhibition of the growth of new blood vessels in the hypertrophic scar might be a promising new strategy for the suppression of scar formation. Salbutamol effectively reduces in the angiogenesis through the inhibition of the production of VEGF and IL-1β, so that it may be considered as an angiogenesis regulator with potential therapeutic uses in different inflammatory diseases (26). The goal of this study was to investigate that hypothesis of Beta 2 receptor activation by intralesional salbutamol administration may be successful in the therapeutic modulation of hypertrophic scar formation.
Salbutamol is a well-known β_2-adrenergic receptor agonist that mainly used in the treatment of asthma (27). It selectively β_2-receptors agonist that inhibits inflammatory processes of CD4 cells, monocytes and macrophages. (26) In addition, anti-inflammatory effects of β_2-receptors agonist on pulmonary inflammation models (28) support the role of activation of these receptors in inflammatory process (29).

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Materials and Methods

Forty healthy male albino rabbits between 10 and 12 months of age were used in the study. The rabbits were housed in the animal house of Al- Nahrain University medical department. The rabbits were left for 2daysat the animal room of controlled temperature (28-30ºC), allowed free access to water ad libitum and food. Protocol of the current research was approved by the committee Institute Review Board at Al-Nahrain University College of Medicine.

Animals Grouping

Forty rabbits were divided into four groups, ten rabbits in each group

Group1. Healthy animal group.

Group2. Hypertrophic scar was induced and the animals left without treatment.

Group3. Hypertrophic scar was induced then the animals treated with intralesional triamcinolone acetonide (standard drug) once weekly for 4weeks.

Group4. Hypertrophic scar was induced scar then the animals treated with intralesional salbutamol 0.2% (investigated drug) once weekly for 4weeks.

Induction of hypertrophic scar in rabbits

Hypertrophic scar model was described by (30)animals were anesthetized with intramuscular injection of ketamine (45 mg/kg) and xylazine (5 mg/kg). Surgical injury were performed on day 0 with an 8-mm biopsy punch .Four wounds were created meticulously on the ventral surface of one ear just down to cartilage. Removal of the perichondrial layer delayed epithelization, after the hemostasis has been achieved with manual pressure, wounds were covered with sterile gauze for 1 day. Each rabbit was housed separately after wounding until hypertrophic scars harvest. On day 30, the eventual scars were resulted.

Parameters of Study

1) Histopathological study of skin sections
2) Study of transforming growth factor beta 1 level (TGF-β1) in skin tissue.
3) Study of collagen 3 alpha1 (COLIIIαI) in skin tissue.

Tissue harvesting and evaluation of scars

The best two Samples were collected from each animal in group of study using 11 mm bunch biopsy with more than 3 mm edge of adjacent skin after anesthetized the animals .Then submitted for histological and immunhistochemical analysis. In histological analysis, the wounds tissue sections were fixed in 10% formalin. Sections was stained with hematoxylin–eosin (H &E) technique to determine inflammatory degree , height and index of scar and size of scar. The other sections were mounted on positively charged slides and immune stained with an antibody against collagen III and transforming growth factor (TGF-β1) marker (31).the average (mean ± SD) will be calculated for each group (32).

Histological Examination

Samples preparation

A 10% formaldehyde solution is used for store wound samples for histopathological and immunohistochemistry study.

Assessment of changes in Histopathology of Skin Sections

Tissues of Rabbits of all groups were harvested at the end of experiment (at day 30) and changes of histopathologicalfeatures of skin were evaluated and scored as follows:

1) Scar elevation index (SEI) is the ratio of the highest vertical height of scar area between perichondrium and dermal surface to the highest vertical height of normal surface around the scar. Each wound was measured by a blinded examiner using a calibrated eyepiece reticule (33)
2) 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 calculated according to the following scores: 0 = absence of fibroblasts; 1 = few fibroblasts; 2 = 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 (34).

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Immunohistochemistry kits for detection of collagen 3α1, transforming growth factor β 1(TGF- β1)
Immunohistochemistry Evaluation
A biotinylated, cross-adsorbed, and affinity purified secondary anti-mouse IgG was used to detect primary antibody-antigen complexes bind to a glass microscope slide, following reaction with an enhanced detection reagent, 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 reactionsuch as collagen was measured by ranking the signal intensities according to the following scale: – (absent), + (mild), ++ (moderate), +++ (marked) (35).
Slides were examined to identify immunoreactivity for TGF-β1. Scoring system was done, and the score recorded was the average intensity of the expression: score 0= Absence of immunoreactivity, score 1= Weak immunoreactivity, score 2 =Moderate immunoreactivity , score 3= Strong immunoreactivity (31).

Statistical analysis
Using two statistical software programs: the statistical package for social science (SPSS version 22) and Microsoft Office Excel 2013 data were collected, summarized, analyzed and presented. All 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 Tukey test and one-way ANOVA. $P \leq 0.05$ was considered significant and highly significant when $p \leq 0.01$ (36).

Results

1. Healing rate
Appearance of untreated induced hypertrophic scar: normal healing process involves three: inflammation (0–3 days), cellular proliferation (3–12 days) and remodeling (3–6 months), so in this study there is inflammatory signs seen from the first day in all rabbits with wound closure starting from the 4th day and excessive formation of fibrosis (100%induction) at 30th day.
Triamcinolone acetonide treated group : healing signs were very clear starting after treatment with fading of inflammatory sign. Finally, a Complete wound closure and decrease thickness of scar (after 30 days of treatment).
Salbutamol treated group: remarkable decrease of inflammatory signs occurred after starting treatment with closure of wound and no sign of thickness
(After 30 days of treatment) .

2.Immunohistochemistry
2.1 . Transforming growth factor (TGF-β1)
Induced hypertrophic scar compared with healthy control and
with other groups in Transforming growth factor (TGF-β1)
Immunohistochemical for transforming growth factor (TGF-β1) is shown in the table (1) and figures (1 , 2, 3) Scores were calculated
according to the method demonstrated in methodology of present study and presented as mean ±SD. The results were as following:
1. There was statistically extremely high significant difference in mean of
immunohistochemical scores of TGF-β between healthy control and
Induced hypertrophic scar control group enrolled in the present study
($P<0.001$).
2. The mean of IHC scores of TGF-β1 expression was effectively
suppressed in all groups as compared with induced hypertrophic scar
group ($P\leq0.05$).
IHC expression scores of TGF-β resulted in highly significant (P=0.002)
decreased with triamcinolone acetonide and salbutamol treated groups **(1.38±0.74)** and (1.5±0.54) receptively compared with (3.0 ±0.0) in induced hypertrophic scar control group.

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Groups	Mean ± SD	**P** value
healthy control	1.13±1.35	<0.001
Induced hypertrophic scar	3.0±0.0	<0.001
Intralesional triamcinolone	1.38±0.74	0.002*
Intralesional salbutamol	1.5±0.54	<0.001*

Table (1). Mean TGF- β1 scores in control and study groups
Maanwhitney test. SD =standard deviation; P=level of significance at (P≤0.05);
***** Comparison between induced hypertrophic scar and other group.

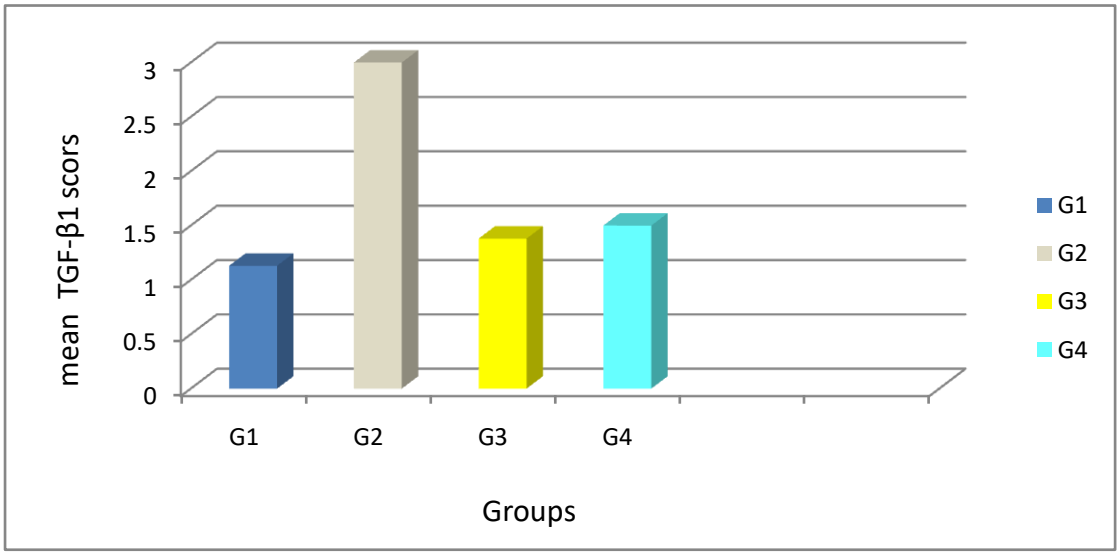

Figure (1). Mean TGF-β1 scores in control and study groups .
(G1) healthy control, (G2) Induced hypertrophic scar, (G3) Triamcinolone, (G4) salbutamol

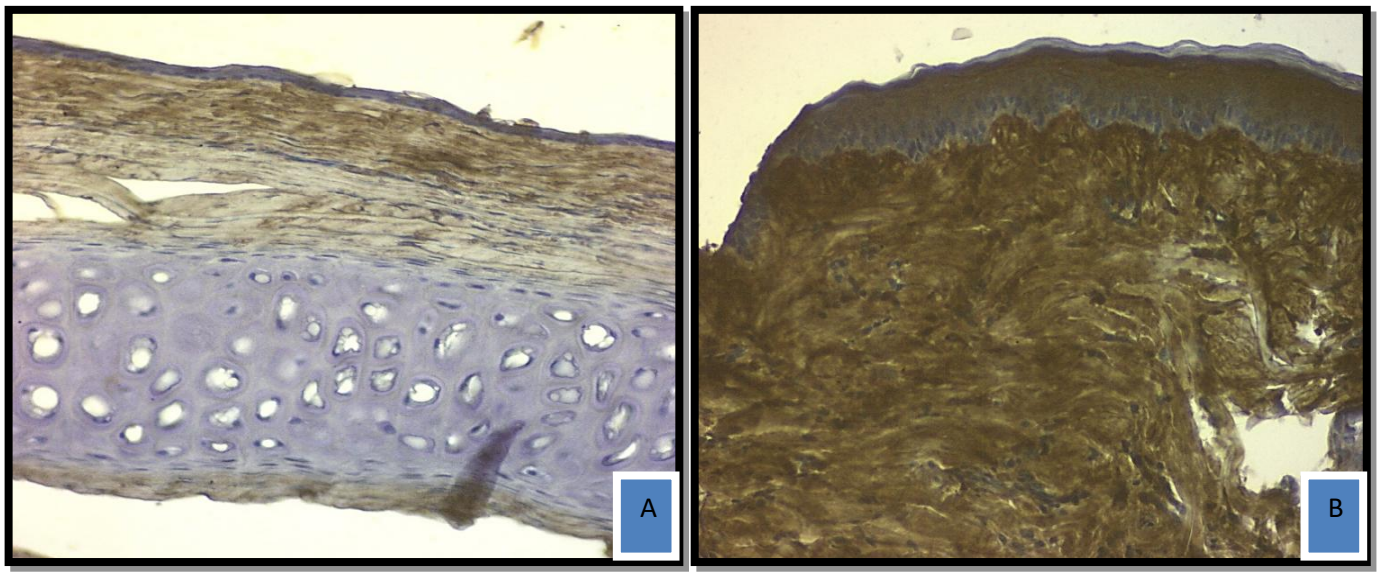

Figure (2). Cytoplasmic immunohistochemical expression of TGF-β1 in dermis(x20). A.normal tissue shown low intensity of TGF-β1 B. Induce hypertrophic scar shown high intensity of TGF-β1

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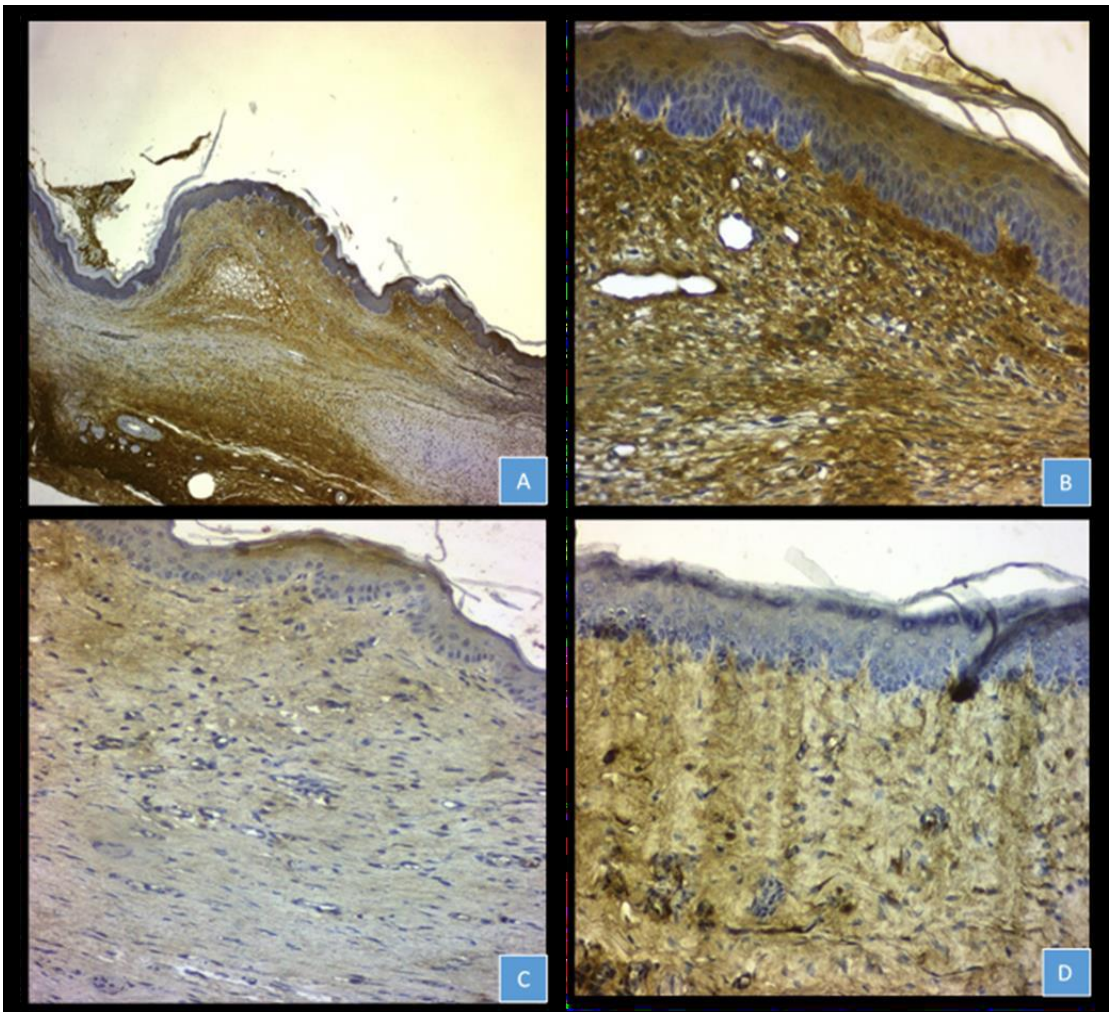

Figure (3). Cytoplasmic immunohistochemical expression of TGF-β1 in treatment groups
A& B hypertrophic scar shown mild intensity TGF-β1 in TAC treated group(X20)
C& D. hypertrophic scar shown mild intensity TGF-β1in salbutamol treated group(X20)

2.2. Collagen III

Induced hypertrophic scar compared with healthy control and with other groups in Collagen III

Immunohistochemical stain for collagen III are depicted in the table (2) and figures (4 , 5 , 6). Scores were calculated according to the method that demonstrated in chapter two and presented as mean ±SD. The results were as following:

1. The mean of immunohistochemical scores of collagen III in induced hypertrophic scar group was (3.0±0.0) which is significantly higher than healthy control group enrolled in the present study (P≤0.001).
2. The mean of IHC scores of collagen III expression was effectively suppressed in all groups as each compared with induced hypertrophic scar group (P≤0.05).

IHC expression scores of collagen III was highly significant (P=0.01) decreased with triamcinolone acetonide treatment (1.5±0.54) compared with (3.0 ±0.0) in induced hypertrophic scar control group.
Moreover treatment with intralesional salbutamol caused highly significant (P =0.002) decreased IHC expression scores of collagen III (1.75±0.46) compared to (3.0 ±0.0) in induced hypertrophic scar control group.

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Group	Mean ± SD	P value
healthy control (G1)	1 ±0	**<0.001**
Induced hypertrophic scar	3.0 ±0.0	
Triamcinolone treated group	1.5 ±0.54	**<0.001***
Salbutamol treated group	1.75 ±0.46	**<0.001***

Table (2). Mean collagen III in study groups

Mann whitney test.SD: Standard deviation;P : level of significance at (P≤0.05);
***** Comparison between induced hypertrophic scar and other group.

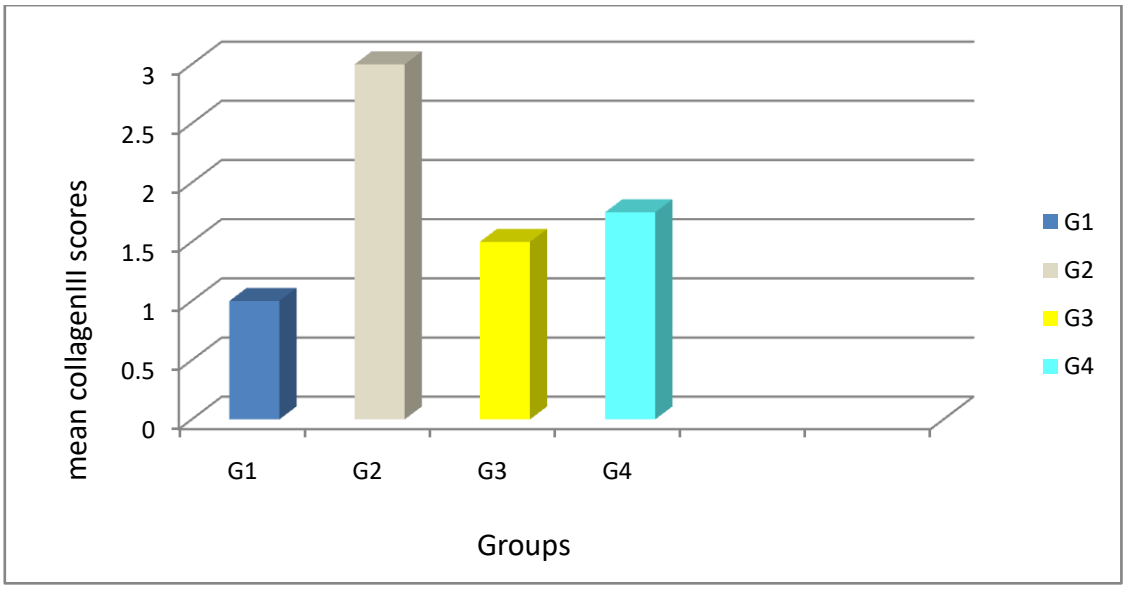

Figure (4). Mean collagen III scores in study groups
(G1) healthy control, (G2) Induced hypertrophic scar, (G3) Triamcinolone, (G4) salbutamol

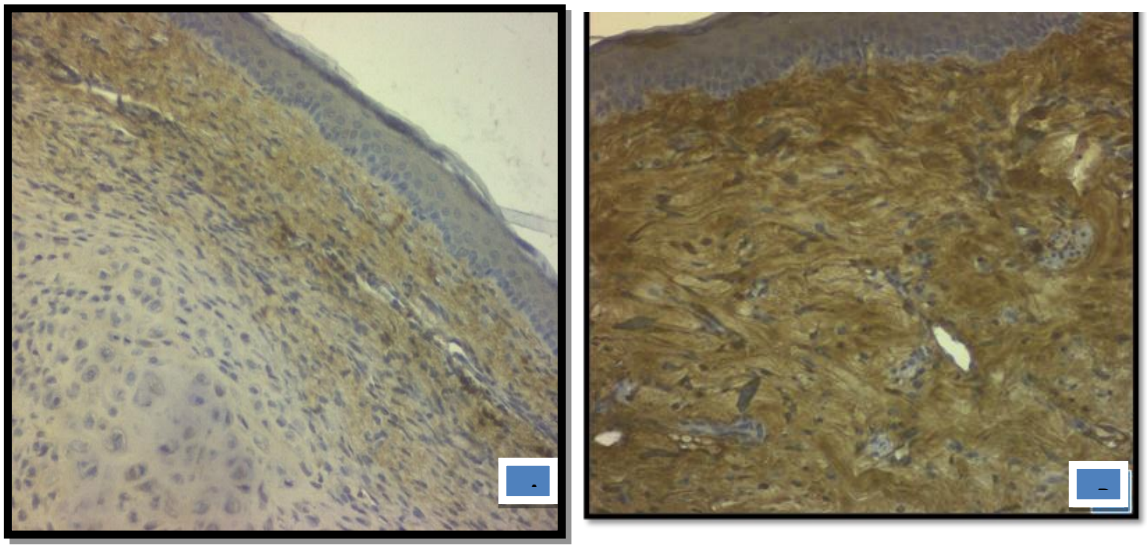

Figure (5).cytoplasmicimmunohistochemical expression of collagen III (x20)
A.normal tissue shown low intensity of collagen III
B. Induce hypertrophic scar shown high intensity of collagen III

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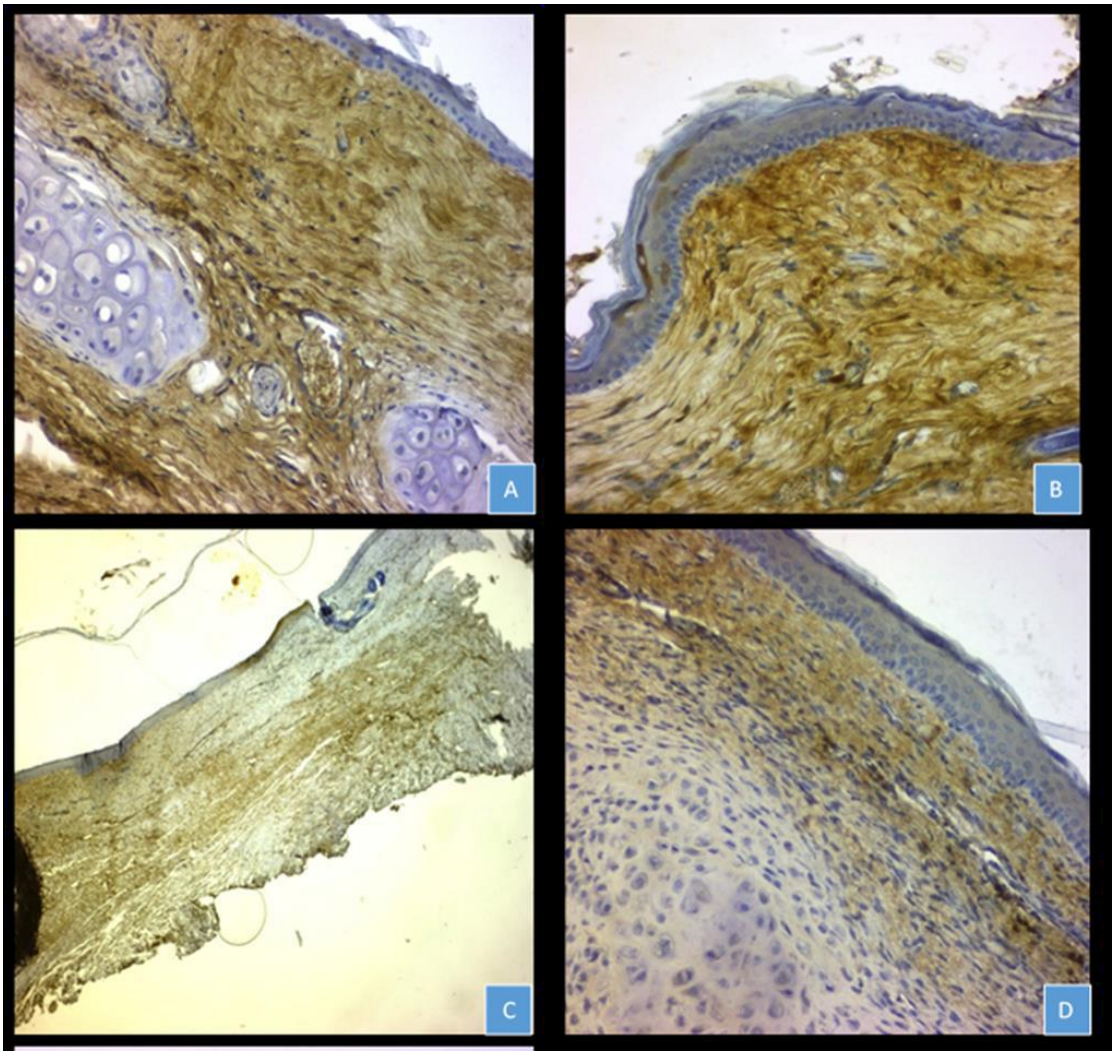

Figure (6) Extracellular immunohistochemical expression of COLIII in treatment groups(X20).
A&B hypertrophic scar shown mild intensity collagen III in triamcinolone treated group.
C& D hypertrophic scar shown mild intensity collagen III in salbutamol treated group.

3 -Histological finding

3.1 Inflammation

Induced hypertrophic scar compared with healthy control and with other groups in inflammation

A biopsy was taken from animals . Inflammation was evaluated by an expert pathologist and graded as mild, moderate and severe. Where mild inflammation was given a score 1, moderate inflammation was given a score 2 while severe inflammation was given a score 3. All Results are shown in the table (3) and figures (7 , 8 ,9).

Histopathological score reflective of scar in experimentally induced hypertrophic scar was shown to be extremely high significant ($P \leq 0.001$) increased in induced hypertrophic group without treatment (2.75 ±0.46) as compared with (0.0±0.0) healthy control group.

Histopathological score reflective of scar injury was very high significant ($P \leq 0.001$) decreased with intralesional salbutamol and triamcinolone treatment with mean of (1.13±0.35) and (0.75±0.46) respectively compared with (2.7 ±0.45) in induced hypertrophic scar control group.

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Group	Mean ± SD	P value
healthy control	0±0	**<0.001**
Induced hypertrophic scar	2.75 ±0.46	
Triamcinolone treated group	0.75±0.46	**<0.001***
Salbutamol treated group	1.13±0.35	**<0.001***

Table (3). Mean inflammation score in study groups .Mann whitney test.SD: Standard deviation;P : level of significance at (P≤0.05);
***** Comparison between induced hypertrophic scar and other group

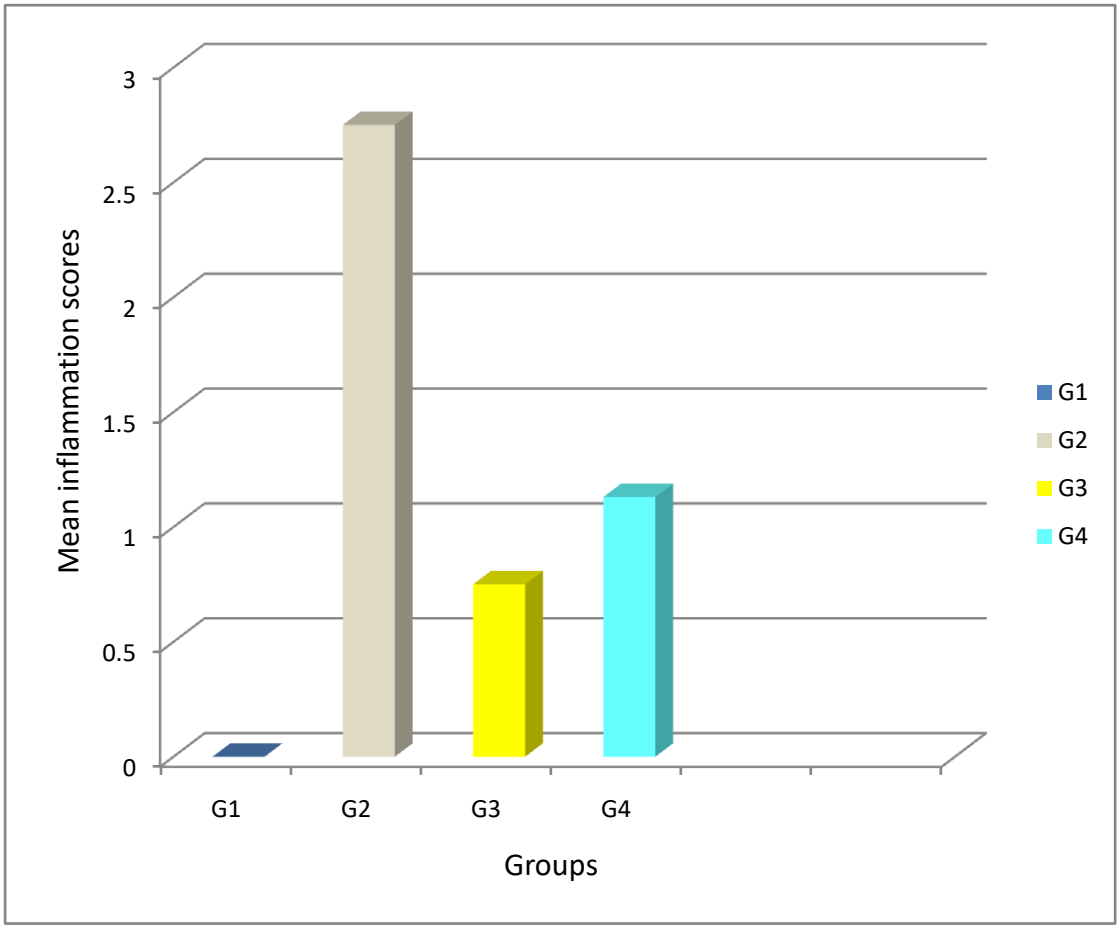

Figure (7). Mean inflammation score in study groups
(G1) healthy control, (G2) Induced hypertrophic scar, (G3) Triamcinolone, (G4) salbutamol

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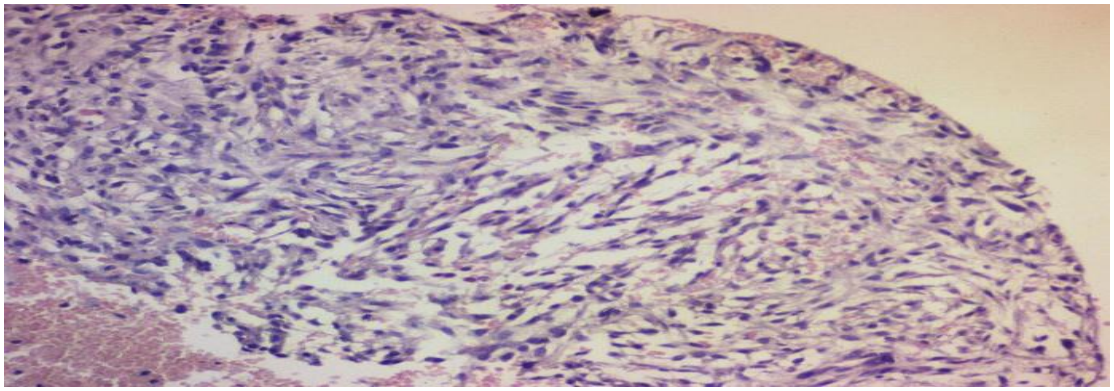

Fig (8-A) . Induced hypertrophic scar tissue represent with sever inflammation and high number of polymorph nuclear cells , also Dermis cellularity increases , fibroblasts was sever and arranged in disorganized manner (x20)

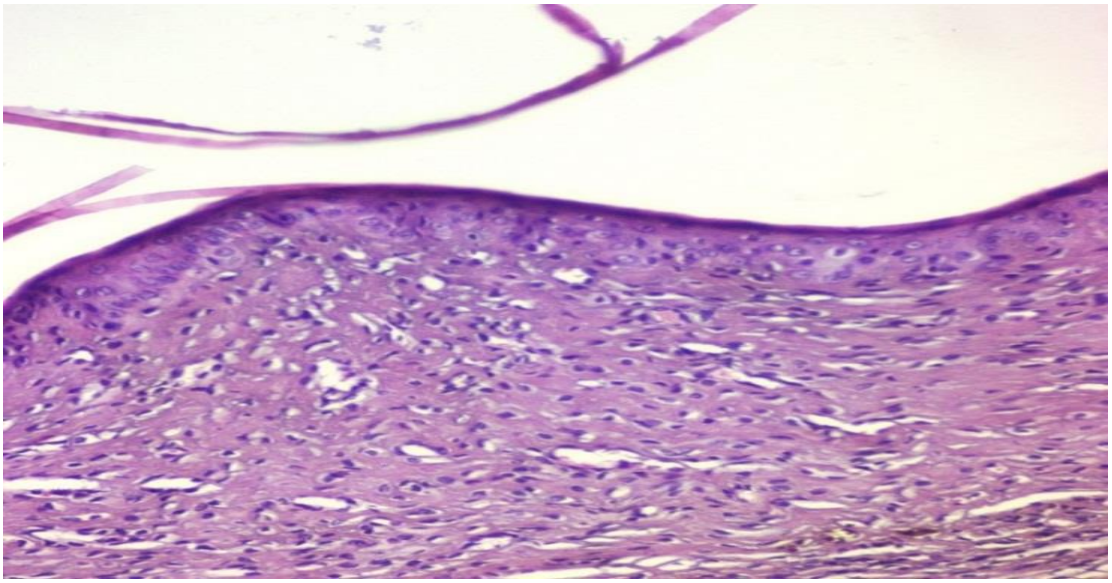

Figure (8-B). Triamcinolone treatment group , staining with H and E stain and evaluated for inflammation , fibroblast count and arrangement (X20).

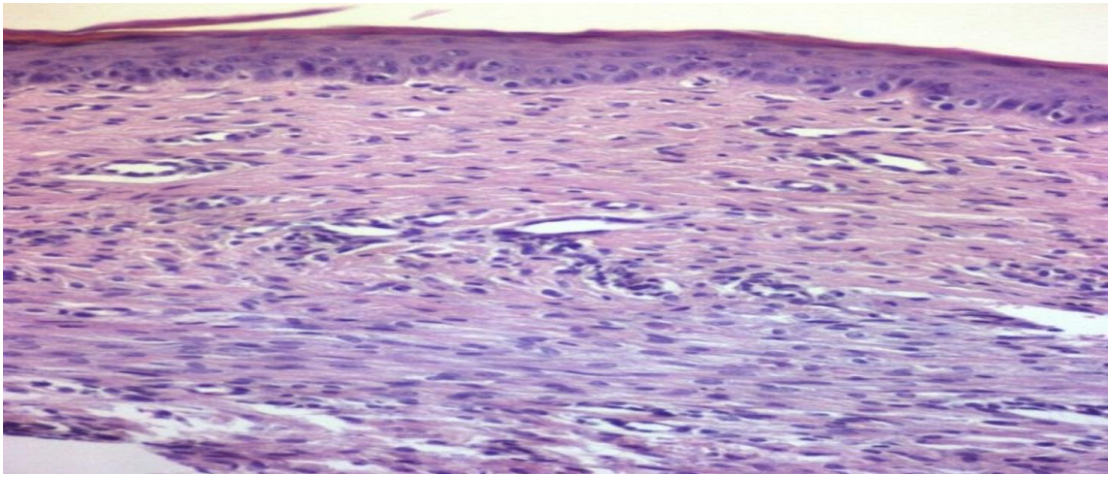

Figure (8-C) B. hypertrophic scar of salbutamol group.

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3.2 Height and index of scar

Induced hypertrophic scar compared with healthy control and with other groups in Height and index of scar

SEI is obtained as following:

1. There was high significant difference in mean of height and Scar elevation index between healthygroup and hypertrophic scar group in the present study (P≤0.001).
2. There was high significant difference (P≤0.001) in mean of height and index in two groups: triamcinolone acetonide group and salbutamol group as each compared to induced hypertrophic scar group. Data obtained are shown in table (4) and figures (9, 10) .

Parameters		Healthy	Hypertrophic	Triamcinolone	Salbutamol
Height of scar	Mean	95.0	756.25	285.0	370.0
	SD	9.87	40.7	27.91	92.58
	P value	**<0.001**		**<0.001***	**<0.001***
Scar elevation index	Mean	1.0	8.03	3.03	3.94
	SD	0.0	0.87	0.42	1.11
	P value	**<0.001**		**<0.001***	**<0.001***

Table (4). Mean of height of scar elevation index control and study groups Unpaired t test .SD: Standard deviation;P = level of significance at (p≤0.05);
***Comparison between induced hypertrophic scar and other group.

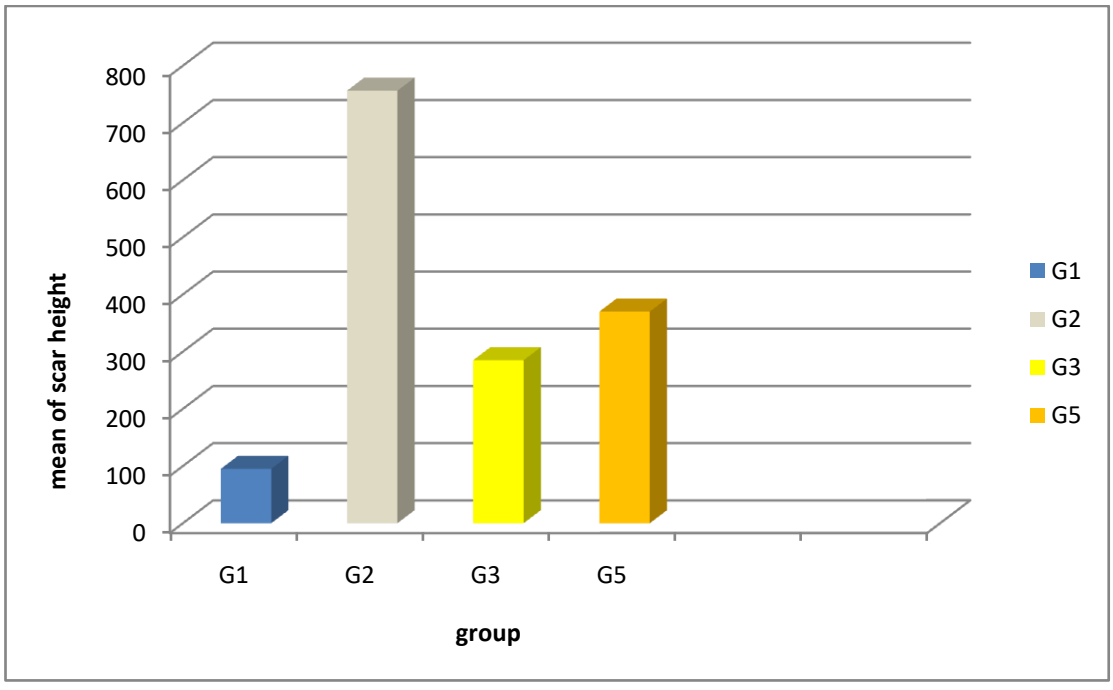

Figure (9). Mean of scar height in control and study groups

(G1) healthy control, (G2) Induced hypertrophic scar, (G3) Triamcinolone, (G4) salbutamol

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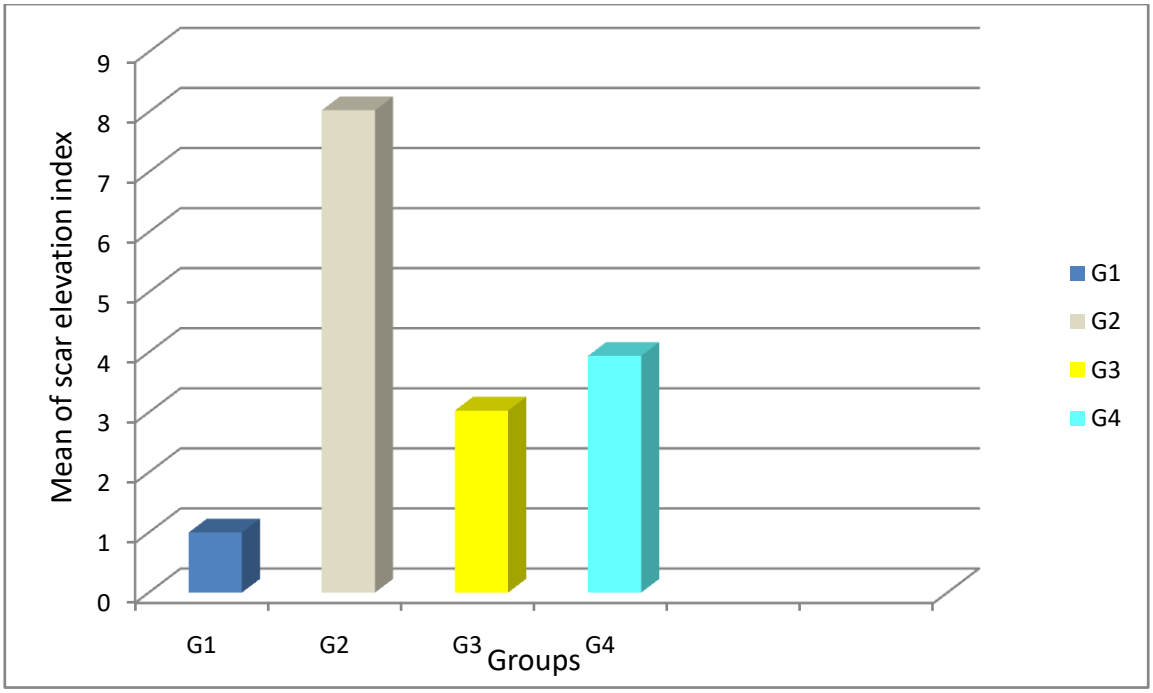

Figure (10). Mean of scar elevation index in control and study groups (G1) healthy control, (G2) Induced hypertrophic scar, (G3) Triamcinolone, (G4) salbutamol

Discussion

Hypertrophic scars are a pathologic condition that characterized by proliferation of the dermal tissue, deposition of excessive fibroblast derived extracellular matrix and persistent fibrosis (37).This disordermay lead to functional disorders, psychological morbidity, and costly price of long term health care (38). Different methods have been demonstrated for the treatment of hypertrophic scars, however to date, the optimal treatment strategy has not been established (39).Moreover there are several challenges aspect which is embodied in the treatment time is too long , lack of specific remedies , Several drugs have side-effects, , lack of early interventions may deprive the optimal time for treating (40) for this reasons the present study had strong stimulus to find out a new relatively safe and effective modality of treatment that may considered alternative to corticosteroids and chemotherapeutic agents . The rabbit dermal model was used and validated in a variety of studies for evaluation and treatment of experimental hypertrophic scar. (41).

Immunohistochemical expression of TGF-β1

TGF-β can mediate fibroblast proliferation, angiogenesis, and re-epithelialization in the healing process of wound- (42).

In the present study there were significant differences between induced hypertrophic scar and normal skin in cellular response to growth factors (TGF-β) which is in accordance with finding of (43).

The effect of intralesional injection of triamcinolone acetonide significantly reduced TGF-β as compared to induced scar non treated group after 30 days of treatment ($P \leq 0.05$) that is in accordance with Sari *et al* (44)

In current study, intralesional salbutamol injection significantly reduced TGF-β as compared to induced scar group after 30 days of treatment ($P \leq 0.05$) , this effect probably explained by the fact that salbutamol potential inhibitor to the expression of TGF-B (45) . Moreover this effect was comparable to the therapeutic effect of triamcinolone .

Immunohistochemical expression of collagen III

Result of present study showed the elevation of collagen III expression in induced hypertrophic scar group, which is consistence with finding Oliveira *et al* (46).This study also showed significant reduction of collagen III in triamcinolone group as compared to induced scar group after 30 days of treatment **.**

Intralesional salbutamol injection showed significant ($P \leq 0.05$) decrease in collagen III compared to control scar group , this results are in consisting with Pablo *et al* study that showed degradable effect of salbutamol on cardiac collagen III (47).Moreover this effect was comparable to the therapeutic effect of triamcinolone .

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Histomorphological finding

The present studyshowed characteristic histological changes in controlled nor treated hypertrophic scar, essentially increase in inflammation degree, fibroblast count, height and index of scar also increase scar size.

Inflammation

Current study showed that intralesional injection of triamcinolone significantly reduced the infiltration of inflammatory cell induced by punch biopsy.

The probable anti-inflammatory mechanism of triamcinolone against hypertrophic scar primarily due to inhibition of leukocyte,monocyte migration and phagocytosis (48)**.**

Intralesional salbutamol injection possess marked anti-inflammatory effect comparable to that of triamcinolone treated group .

Height and index of scar

Intralesional triamcinolone injection was shown significant decrease in height measurement and SEI that agree with Çaliskan*et al* (49)

Regarding salbutamol treated group established significant reduction of height and index of scar in comparable level to the action of triamcinolone.

Conclusions

Intralesional triamcinolone andsalbutamol injections are comparatively and potentially effective fordiminish thickness of hypertrophic scar. The therapeutic molecular mechanisms of these drugs weredemonstrated in this study however further studies are required .

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