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β2AR Antagonist (Timolol) Promote Skin Wound Repair Processes

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Abstract

Background: Wound healing is a complex physiological and dynamic process requiring the coordinated, temporal orchestration of numerous cell types and biological processes to regenerate damaged tissue and initiate repair that is dependent on a number of inter-related factors. Adrenergic receptors are targets for many therapeutic agonists and antagonists in current use including tissue repair and wound healing. Previous work has demonstrated that a functional β-adrenergic receptor autocrine/paracrine network exists in skin, but the role of β2-adrenergic receptor (β2AR) in wound healing is unknown clearly.
Aims: is to demonstrate whether the β2 receptor have role in wound healing and angiogenesis.
Materials and methods: A murine wild-type (*in vivo)*, excisional skin wound model was used to demonstrate that blocking of β2AR accelerate wound repair, twenty no pregnant female albino mice were used to investigate the effect of the drug on experimental wound healing grossly, histopathologically and immunohistochemically compared with vehicle-only controls.
Results: Gross morphological wound healing demonstrates that reduction in the size of the wound and the rate of wound healing was highly significant in timolol group than in control group, also it increase collagen III, smooth muscle actin (SMA) and CD31expression after being followed for 5 and 10 days.
Conclusion: the current study shown that the administration of β_2 adrenergic receptor antagonist (timolol) promoted wound healing through increasing of angiogenesis, collagen III deposition, myofibroblast density and re-epithelization process.

Keywords: Angiogenesis, Collagen III, in vivo, re-epithelialization, timolol, wound healing.

INTRODUCTION

Wound healing is a complex physiological and dynamic process requiring the coordinated, temporal orchestration of numerous cell types and biological processes to regenerate damaged tissue and initiate repair that is dependent on a number of inter-related factors [1]. According to the duration and nature of healing process, the wound is characterized as acute and chronic [2]. All tissues in the body are capable of healing by one of two mechanisms regeneration or repair, Regeneration is the replacement of damaged tissues by identical cells and is more limited than repair [3], while the repair injured or damaged tissue is substituted by connective tissue [4]. Re-epithelializationis regrowth of epithelial cells across the wound surface occurs during the final stage of proliferation. A humid wound environment accelerates this process, allowing epithelial cells to migrate more simply [5]. The epidermis can synthesize and secrete a number of proteins including epinephrine [6], a ligand for the β-adrenergic receptors (βARs): β1-adrenergic receptor (β1AR), β2AR, andβ3AR[7]. They are G protein–coupled receptors highly expressed on all cell lineages in the skin[8,9]; therefore, an autocrine and paracrine βAR network exists in the epidermis and dermis, respectively. In excised human skin, βAR activation delayed wound re-epithelialization, whereas βAR antagonism promoted skin re-epithelialization[6] in an *ex vivo* model of chronic wound reepithelialization [10]. In murine skin wound models, stress-induced increases in epinephrine delayed wound repair, whereas, conversely, βAR antagonism enhanced re-epithelialization in a murine skin burn model *in vivo* [11] and accelerated skin barrier recovery [12]. In addition, a nonselective βAR antagonist improved wound healing in diabetic [13] and burn-injured rats[14].Here the effects of β2AR antagonism (timolol) on the some processes in wound repair were investigated.

MATERIAL AND METHOD

Murine wound model

Twenty four non-pregnant females' albino mice between 8 and 12 weeks of age were used in this study. Mice were fed with standard oxoid pellet and given water ad libitum. All animals kept at 28-30ºC and the experiments were approved by the Institute Review Board (IRB) Al-Nahrain University, College of Medicine. Mice were anesthetized by intraperitoneal injection of ketamine (100mg/ kg)/ xylazine (10mg/ kg). Back skin shaved and 2 full-thickness 6-mm incisional wounds created in each mouse, in the center of the back, using a sterile 6-mm biopsy punch to mark the skin for surgical excision. Wounds are treated topically with Aqua Rosa alone for the control group (12 mice) and freshly prepared Aqua Rosa containing (5 mg/ml) non-selective β-2AR antagonist (timolol) (sigma, Germany) for the study group (12 mice) immediately after wounding and daily thereafter for 5 days. Each mouse housed separately after wounding until wound harvest. Wounds digitally photographed, daily, to determine the difference and to monitor percentage of wound healing over time. Biopsies from the wound was taken from each wound of six animals of the study groups after five days. The other six animals received nothing of the drug's application for further 5 days. On the tenth day a biopsy was taken from each of the remaining wounds. For histological analysis, the wounds tissue sections fixed in 10% formal saline. Four sections, the 5-micrometer thickness was made from each section. One will be stained with the hematoxylin–eosin (H &E) technique to determine the progress of the healing process, and inflammation and the other three sections were immunostained with an antibody against smooth muscle actin (SMA), collagen III and CD31 [an endothelial cell (EC) marker] according to the manufacturer's protocols.The intensity or number of

stained cells/vessels in each image counted in a double-blind manner, and the average will be calculated for each group [6].

Collection of Specimens

This study included a total of twenty four wound samples collected from animals, the work perform at the college of medicine in Babylon University during the period from October -2016 to January-2017.

Preparation of the samples

Each wound tissue sample was stored in 10% formaldehyde solution to use for histopathological and immunohistochemistry study.

Preparation of Formalin-fixed paraffin-embedded tissues (FFPE):

Tissue Fixation

Sections Transferred into formalin (10%); Fixative volume was 20 times that of tissue on a weight per volume, tissue was fixed for a minimum 48 hours at room temperature and then processed, using gentle agitation[15], then tissues embedded in paraffin blocks.

Tissue sectioning and slide preparation:

Serial sections (3-5 µm) thickness were obtained using microtome, from each wound paraffin block, 5 slides were prepared. Sections were mounted on ordinary slides (to be used in Haematoxylin and Eosin staining system) and on positively charged slides (to be used for immunohistochemistry) using a water bath of 45C^{o} to prevent tissues sections folding during mounting procedure, each slide was labeled using a pencil to carry the same number on its paraffin block.

Haematoxylin and Eosin (H & E) staining of paraffin sections:

The Haematoxylin and Eosin staining system were used for histopathological examination of the wound sample to confirm healing ,as in [16].

Immunohistochemistry IHC detection of collagen III, smooth muscle actin(SMA) and CD31expression:

I. Anti-collagen III antibody: Rabbit polyclonal antibody to collagen III (Code number: ab7778) (Abcam, UK).
II. Anti-alpha smooth muscle actin antibody:Rabbit polyclonal to alpha smooth muscle actin (Code number: ab5694) (Abcam, UK).
III. Anti-CD31 antibody: Rabbit polyclonal to CD31 cellular localization membrane and cell junction (Code number: ab28364) (Abcam, UK).

Immunohistochemistry IHC procedutre:

5 µm thick sections were made on positively charged slides and the staining procedure was perform as in manufacture protocol (Abcam, UK) , using ab80436 staining kit.

Evaluation of IHC results:

The extent of presence of polymorphonuclear leukocytes (PMNL) and fibroblasts were measured in a blinded manner according to a semi-quantitative scoring system: - (absent), + (minimal), ++ (mild), +++ (moderate), and ++++ (marked)[17,18].

The extent of the immunohistochemical reaction of ECM proteins, such as collagen and fibronectin, was measured by ranking the signal intensities according to the following scale, (–)absent, (+) mild, (++) moderate, (+++) marked [19] or 0= undetected, 1= low density, 2= medium density, 3=dense, to 4=very dense as defined by [20],quantification of collagen III protein expression was evaluated under light microscopy at X40.CD31 is often presented as a number of microvessels per square millimeter or mean value with standard deviations. [21,22].

Statistical analysis:

Data were collected, summarized, analyzed and presented using three statistical software programs: SPSS (version 22), Microsoft Office Excel 2013 and MedCalc 2014. Numeric variables were presented as the mean and standard deviation (SD). Comparison of mean values between two groups was carried out using Mann Whitney U test. Comparison of mean values within the same group on different occasions was carried out using Wilcoxon test. P-value was considered significant when it was equal to or less than 0.05 and highly significant when it was equal to or less than 0.01[23].

RESULTS

Wounds were followed up for healing process which was measured as the reduction in the size of the wound as demonstrated in above. Table (1) and (2) and figures (1) and (2) showed that the rate of wound healing was highly significant faster in timolol group than in control group ($P<0.01$) after being followed for 5 days for 6 animals and for 10 days for 6 animals from the second day and thereafter.

Inflammation was assessed by an expert pathologist and was graded as mild, moderate and severe. Mild inflammation was given a score 1, moderate was given a score of 2 and severe was given a score of 3. as in table (3) and figure (3). Although there was some variation in inflammation severity between 5 days and 10 days samples, the differences in severity of inflammation at the same day and for different groups (control and timolol) were not significant ($P>0.05$).

Immunohistochemical for collagen III expression is shown in the table (4) and figure (5) and (6), Immunohistochemical for (SMA) expression is shown in the table (5) and figure (7) and (8), Immunohistochemical for CD31 is shown in the table (6) and figure (9) and (10). The results were as following: In 10 days the mean immunohistochemical scores were significantly higher than that in 5 days for all groups and for all markers enrolled in the present study ($P<0.05$). Adding timolol resulted in a highly significant increased collagen III, SMA and CD31 immunohistochemical score in 5 and 10 days ($P<0.01$).

Table 1: Mean area wound healing (mm^2) for 5 days

Groups	Day 1 (%)	Day 2 (%)	Day 3 (%)	Day 4 (%)	Day 5 (%)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Control 5	0	21.40 ±7.33 A,d	30.60 ±13.97 A,c	40.20 ±9.09 A,b	55.00 ±8.94 A,a
Timolol 5	0	45.80 ±6.42 B,d	61.00 ±4.64 B,c	75.00 ±3.39 B,b	86.40 ±2.97 B,a

The capital letter indicates comparison among groups (Mann Whitney U test); small letters indicate a comparison between days in the same groups (Wilcoxon test); different letters indicates significant variation at (P≤0.05); the letters A and a indicates highest values.

Table 2: Mean area wound healing (mm^2) for 10 days

Groups	Day 1 (%)	Day 2 (%)	Day 3 (%)	Day 4 (%)	Day 5 (%)	Day 10 (%)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean± SD
Control 10	0	22.00 ±6.20 B,d	30.00 ±8.60 B,d	46.60 ±6.31 B,c	56.40 ±7.83 B,b	71.80 ±7.12 B,a
Timolol 10	0	36.60 ±11.04 A,e	58.00 ±17.42 A,d	68.20 ±13.27 A,c	79.60 ±8.41 A,b	95.40 ±2.70 A,a

The capital letter indicates comparison among groups (Mann Whitney U test); small letters indicate a comparison between days in the same groups (Wilcoxon test); different letters indicates significant variation at (P≤0.05); the letters A and a indicates highest values.

Table 3: Mean inflammation score in control and study groups

Groups	Day	Mean ±SD
Control	5 days	1.60 ±0.55 B
	10 days	1.40 ±0.40 B
Timolol	5 days	2.60 ±0.55 A
	10 days	1.80 ±0.45 B

SD: Standard deviation; Capital letters indicate the level of significance at (P≤0.05); different letters indicate significant variation; (A) indicates the highest value.

Table 4: Mean collagen III scores in control and study group

Group	Day	Mean ±SD
Control	5 days	1.20 ±0.44 D
	10 days	1.60 ±0.55 C
Timolol	5 days	2.80 ± 0.45 B
	10 days	3.80 ±0.45 A

SD: Standard deviation; Capital letters indicate the level of significance at (P≤0.05); different letters indicate significant variation; (A) indicates the highest value.

Table 5: Mean SMA score in control and study group.

Groups	Day	Mean ±SD
Control	5 days	4.80 ±1.30 C
	10 days	6.40 ±0.89 B
Timolol	5 days	7.40 ±0.55 B
	10 days	17.60 ±2.07 A

Capital letters indicate the level of significance at (P≤0.05); different letters indicate significant variation; (A) indicates the highest value.

Table 6: Mean CD31IHC score in control and study group

Groups	Day	Mean ±SD
Control	5 days	3.00 ±0.71 C
	10 days	3.80 ±0.84 B
Timolol	5 days	4.80 ±0.84 B
	10 days	9.40 ±0.89 A

Capital letters indicate the level of significance at (P≤0.05); different letters indicate significant variation; (A) indicates the highest value.

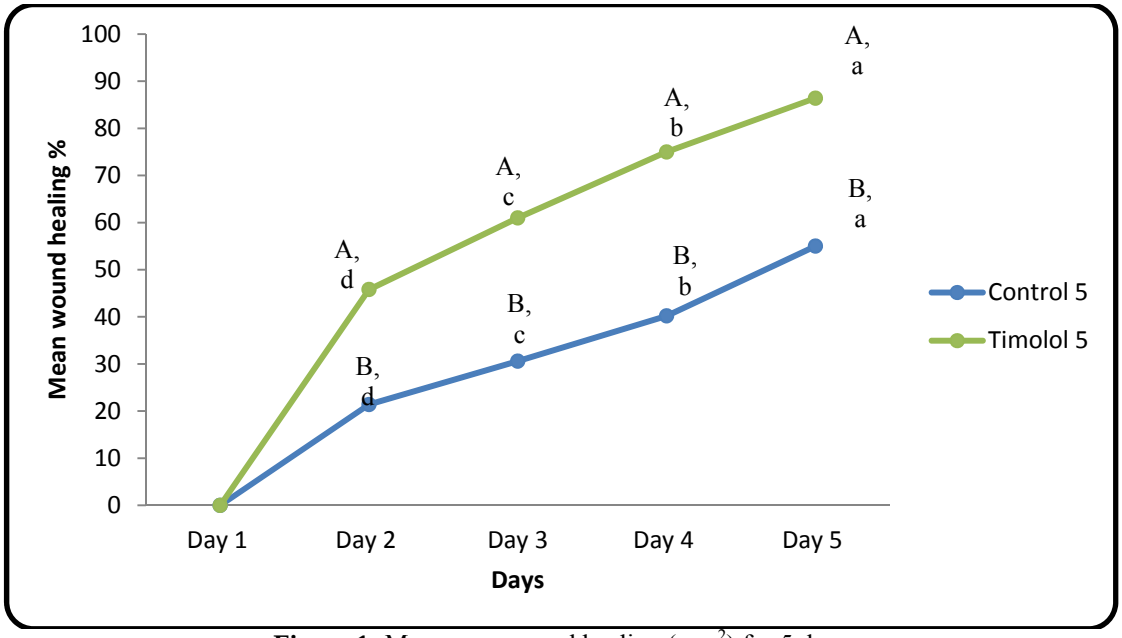

Figure 1: Mean area wound healing (mm^2) for 5 days

The capital letter indicates comparison among groups (Mann Whitney U test); small letters indicate a comparison between days in the same groups (Wilcoxon test); different letters indicates significant variation at (P≤0.05); the letters A and a indicates highest values.

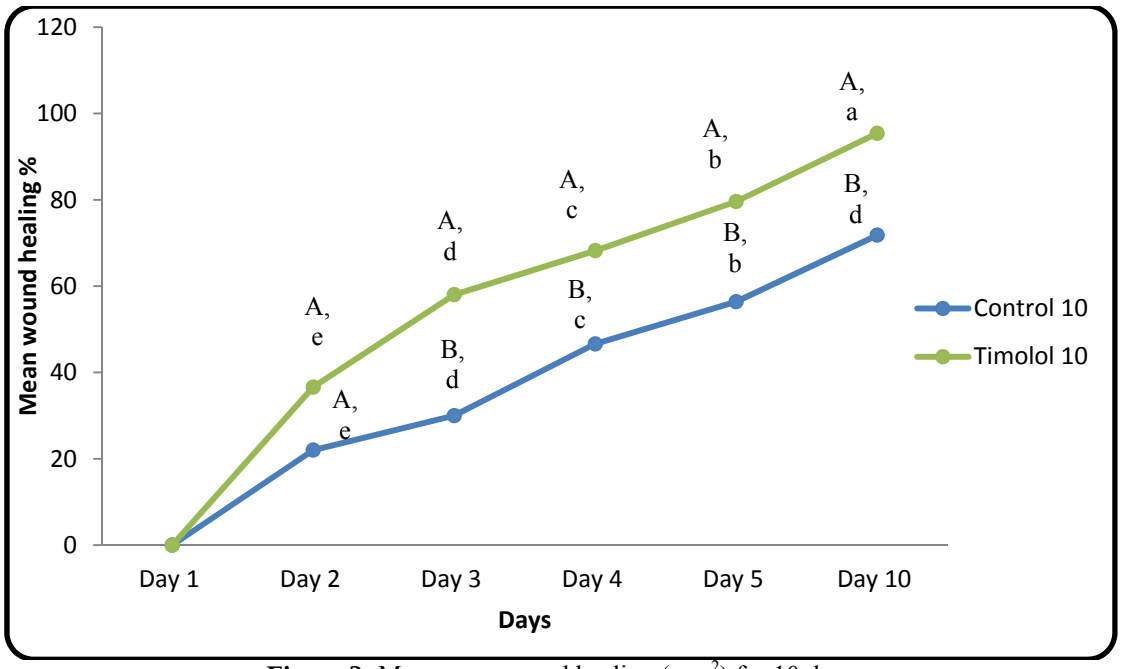

Figure 2: Mean area wound healing (mm^2) for 10 days

The capital letter indicates comparison among groups (Mann Whitney U test); small letters indicate a comparison between days in the same groups (Wilcoxon test); different letters indicates significant variation at (P≤0.05); the letters A and a indicates highest values.

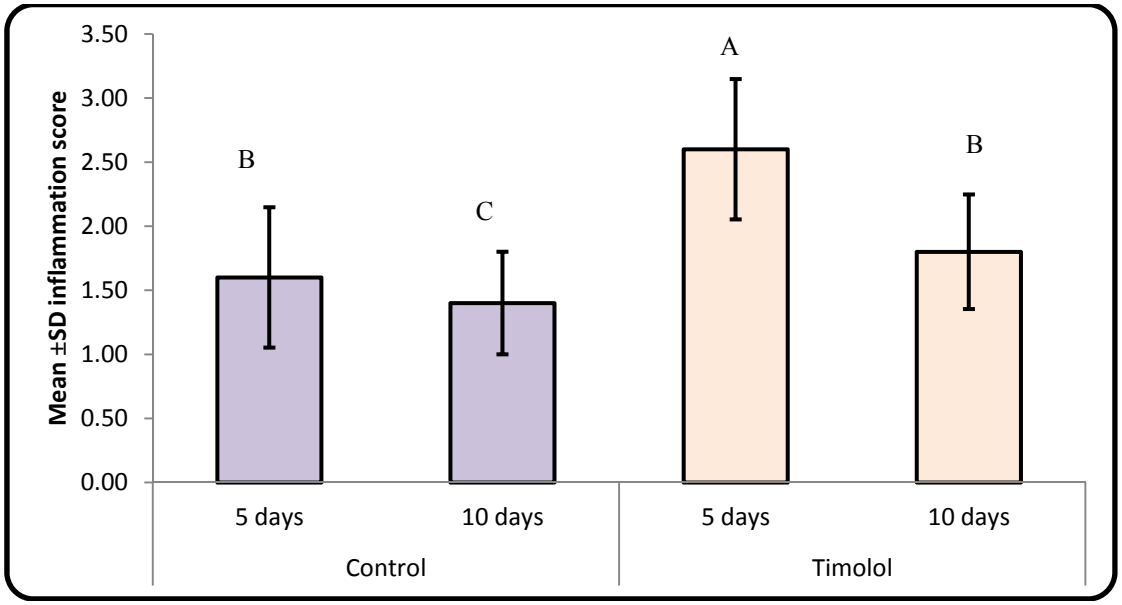

Figure 3: Mean inflammation score in control and study group

Capital letters indicate the level of significance at (P≤0.05); different letters indicate significant variation; (A) indicates the highest value.

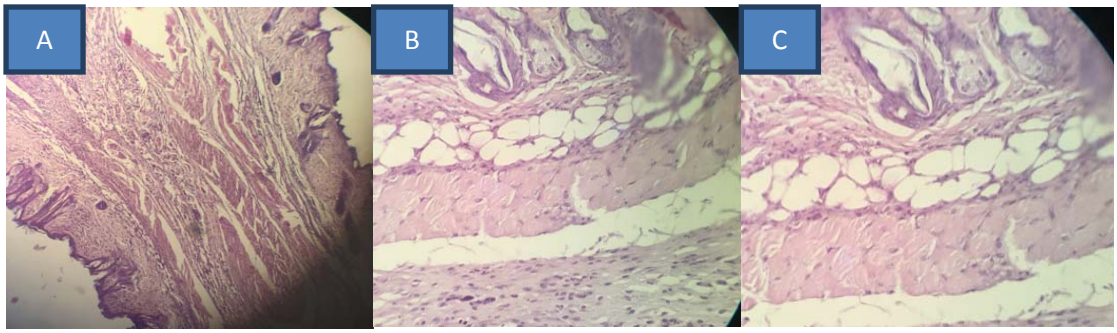

Figure 4: Some of the histological sections that were stained with H and E stain and examined for inflammation, necrosis, and presence of epithelial cells; (A) 10X; (B) and (C) 40X.

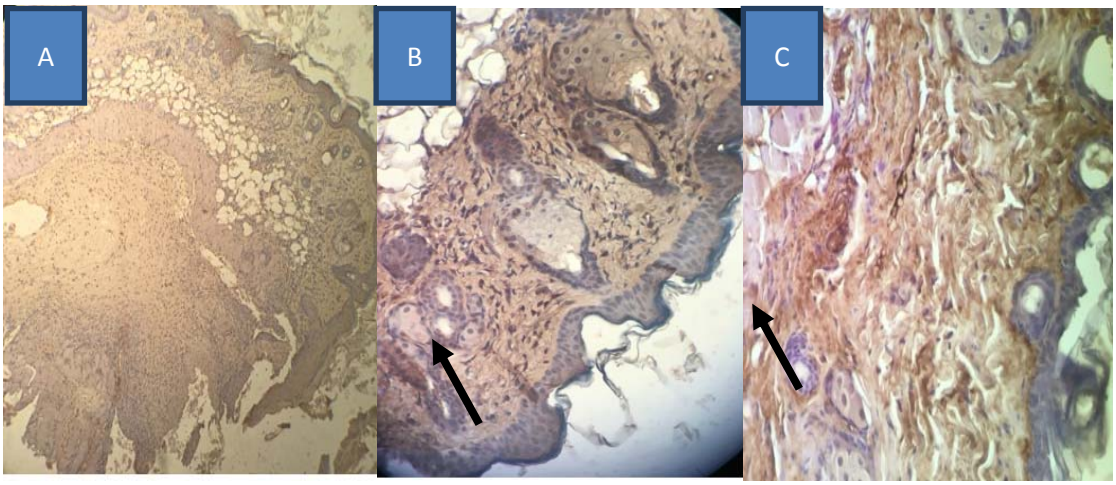

Figure 5: Extracellular immunohistochemical expression of collagen III within the dermis (black arrow). (A) 10X; (b) and (C) 40X.

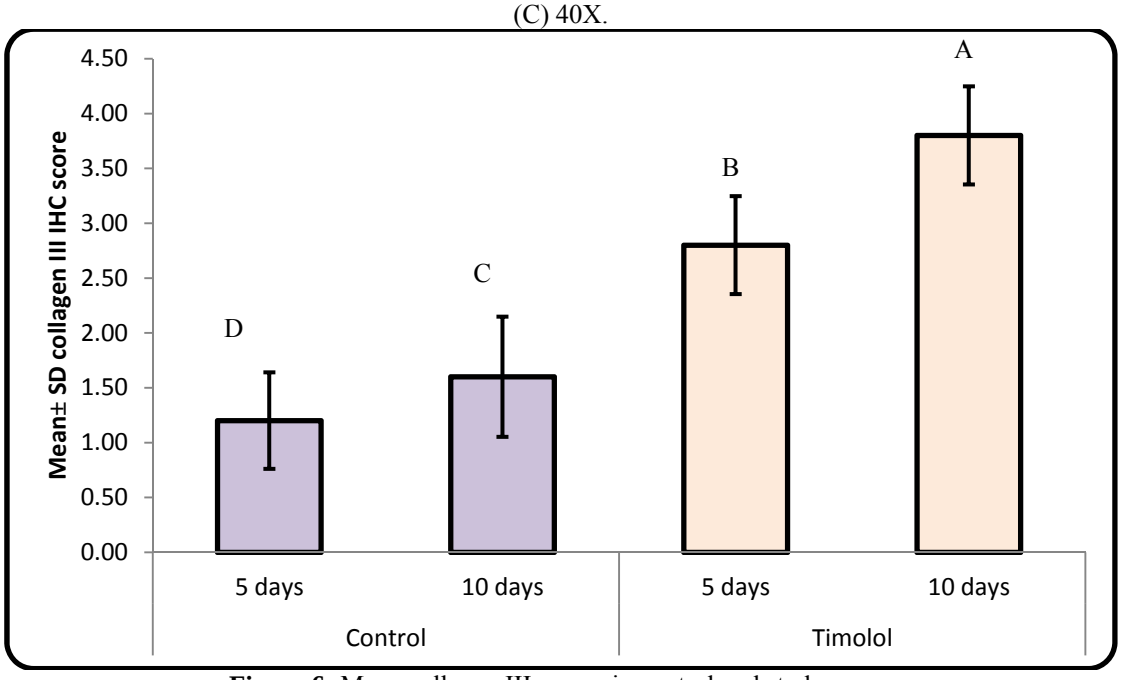

Figure 6: Mean collagen III scores in control and study group

Capital letters indicate the level of significance at (P≤0.05); different letters indicate significant variation; (A) indicates the highest value.

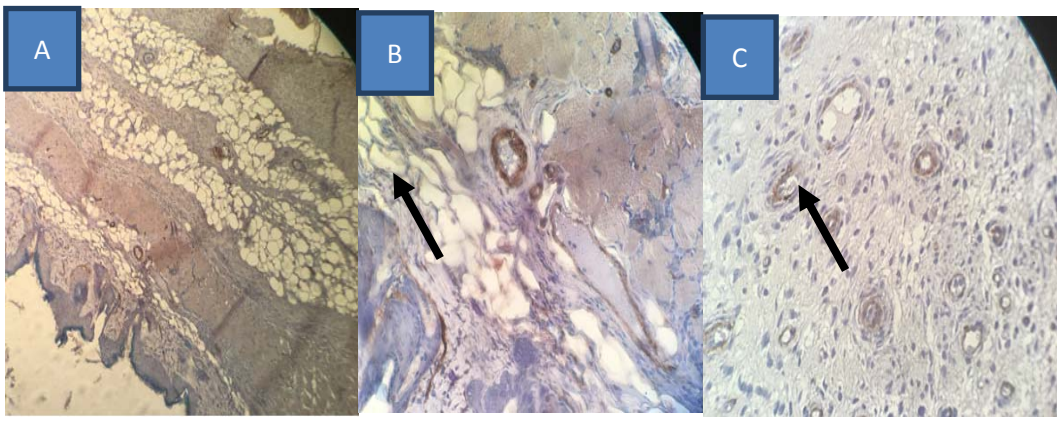

Figure 7: Cytoplasmic immunohistochemical expression of SMA in the wall of blood vessels (black arrow). (A) 10X; (b) and (C) 40X.

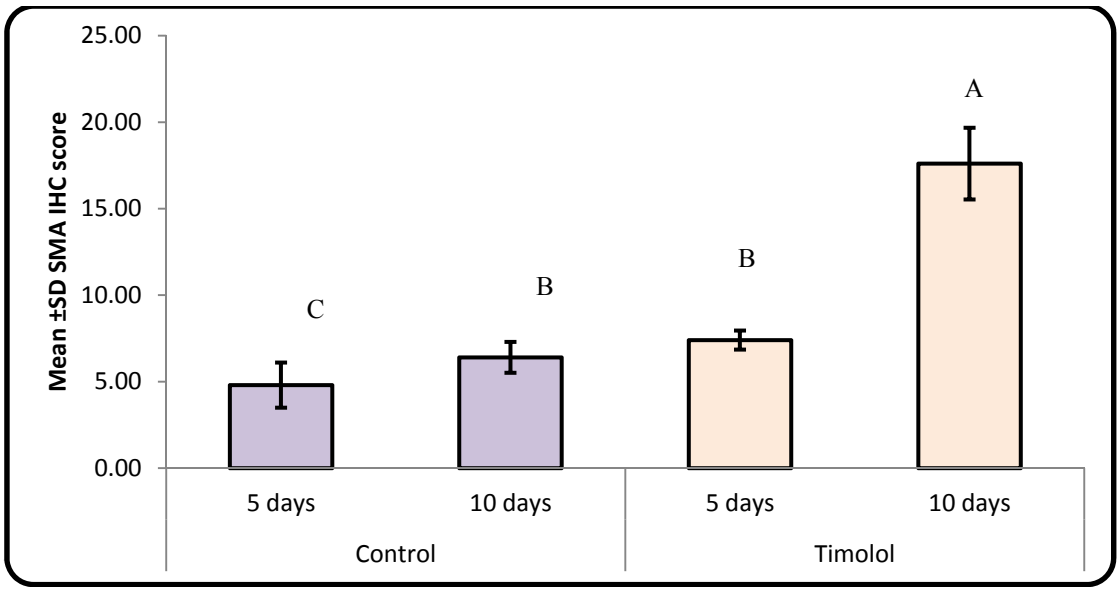

Figure 8: Mean SMA score in control and study groups
Capital letters indicate the level of significance at (P≤0.05); different letters indicate significant variation; (A) indicates the highest value.

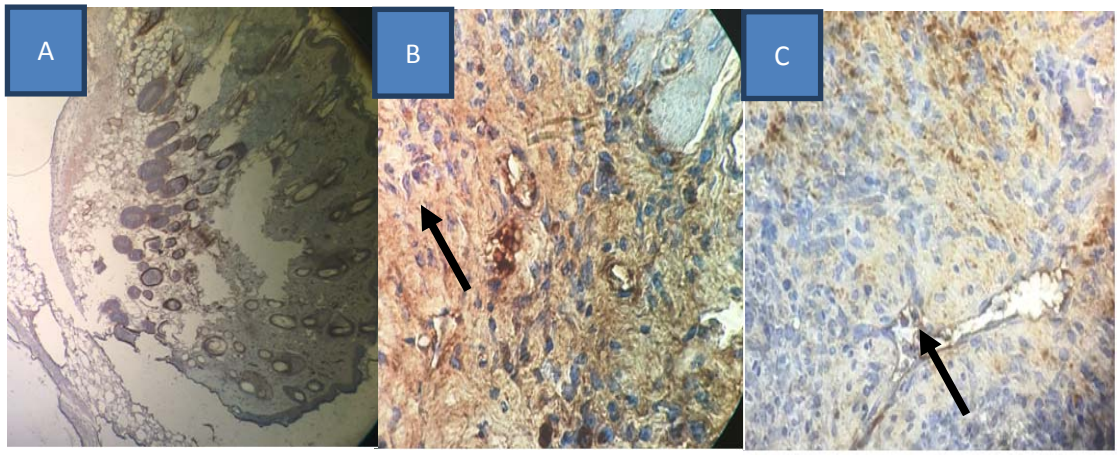

Figure 9: Cytoplasmic immunohistochemical expression of CD31 by vascular endothelial cells (black arrow) . (A) 10X; (b) and (C) 100X.

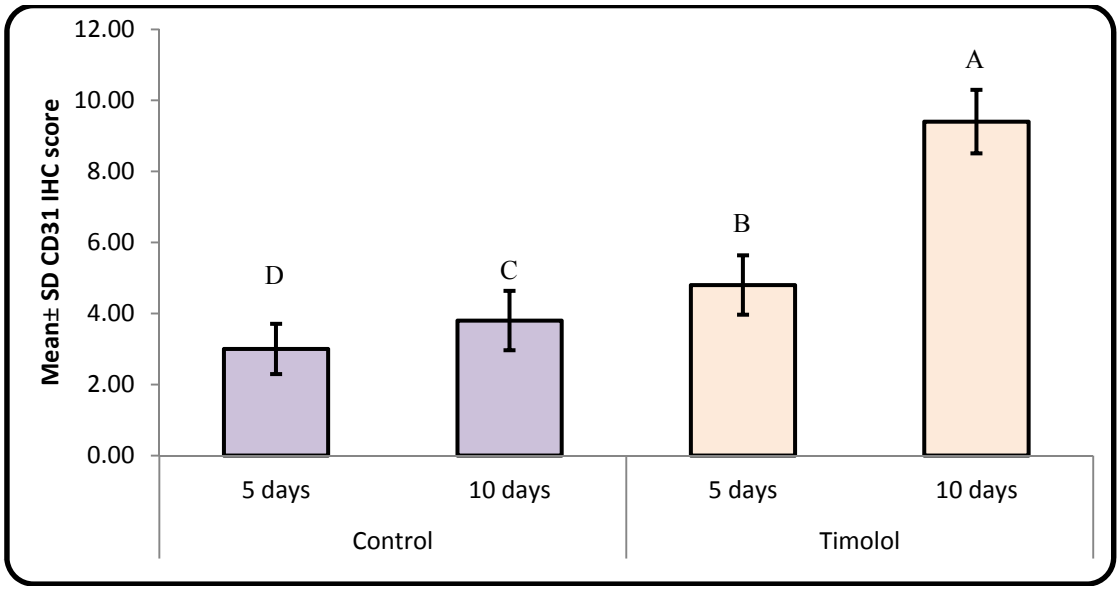

Figure 10: Mean CD31IHC score in control and study groups
Capital letters indicate the level of significance at (P≤0.05); different letters indicate significant variation; (A) indicates the highest value.

DISCUSSION

Current study showed that the rate of wound healing was highly significant faster in timolol group than in control group. Romana-Souza *et al*., 2014 mentioned that the wound area appeared to be smaller in the adrenoceptor(AR) knockout (KO) mice than in the wild-type mice 7 and 10 days after wounding [13]. The observation of increased rate of wound healing in the present study and also in previous study [13] following treatment with β2AR antagonist and in AR KO mice, may be attributed to the fact that angiogenesis process is accelerated and hence there will be increase in nutrient and oxygen supply and also increased rate of removal of metabolic waste products and also to the ability of β2AR antagonist in increasing keratinocyte migration speed[22]. Natural wound healing proceeds involve an inflammatory response and associated cellular migration, proliferation, matrix deposition, and tissue remodeling[24,25]. It has been shown that increased proinflammatory cellular infiltrates composed largely of neutrophils and macrophages contribute to delayed healing in chronic ulcers [26]. Pullar *et al*., in 2012 studied the role of β2AR on inflammatory process involved in wound healing and concluded that topical β2AR antagonist treatment had no effect on the number of polymorphonuclear cells or macrophages recruited to the wound site either 3 or 5 days post wounding [22]and this supports the finding of the present study in that β2AR antagonist have no significant effect on inflammatory response accompanying wound healing in mice. In this study it was found that inflammation severity on day 10 was significantly less than that on day 5and this may be due to the natural process of wound healing in which inflammation severity becomes less toward the end of the healing process and being replaced by the formation of granulation tissue (angiogenesis and fibroblast proliferation).

The current study showed that using the β_2AR antagonist (timolol) resulted in significant increase in collagen deposition in comparison with control group, which is agreed with the findings of Pullar *et al*. (2012) who stated that administration of β_2AR antagonist resulted in a significant increase in collagen III depositions in wounds after being followed for 5 days [22]. Pullar and Isseroff in 2005 studied the β2AR antagonist effects on fibroblast activity and collagen synthesis and deposition in Fibroblast-seeded collagen gels (an in vitro media) and found that it increased fibroblast collagen formation [27]. This finding supports the results of the present study. while Raut *et al*., in 2012 studied the effect of two β_2AR antagonist agents (propranolol and metoprolol) on collagen deposition in wound healing and found that these two β_2AR antagonists significantly reduced collagen deposition[28], a result which is in contrary to the finding of the present study ,this controversy may be attributed to different approaches in assessing collagen deposition; Raut *et al*. assessed collagen deposition in wound healing in rats after examination of formalin fixed paraffin embedded tissue sections that have been routinely stained with H and E stain, where as in the present study, immunohistochemistry using collagen III type specific primary antibodies were used to assess collagen III status; it is well known that during healing process the first type of collagen deposited is collagen III then it is later on replaced by collagen I. and routine H and E stained section permit a difficult chance to differentiate between them. Also, the present study showed a marked increase in collagen III in relation to duration so that longer duration (10 days) was associated with significantly more deposition of collagen. This phenomenon may be due to the fact that early in wound healing inflammation is more marked than fibroblast proliferation and activation; however, when the time elapsed fibroblast proliferation, action and collagen deposition predominates[29]. In the present study, the density of myofibroblast was assessed by measuring the immunohistochemical expression of SMA because it is a reliable marker for myofibroblast differentiation and its expression is a directly correlated with myofibroblast density in tissues [30,31]. The result showed that adding timolol resulted in highly significant increased SMA immunohistochemical score in 5 and 10 days, in agreement with the findings of the present study, Pullar *et al*. (2012) stated that administration of β_2AR antagonist caused a significant increase in SMA immunohistochemical expression in wounds after being followed for 5 and 10 days. Romana-Souza *et al*., (2008) found that propranolol significantly increased SMA expression [14]. This result again is in accordance with the result of the present study. Raut *et al*., in 2012 studied the effect (propranolol and metoprolol) on myofibroblast density in wound healing and found a significantly reduced in it, a result which is in contrary to the finding of the present study. this controversy also may be due to different approaches in assessing myofibroblast density, It is obvious that routine H and E stained section which was used by Raut permit a difficult chance to differentiate between ordinary fibroblast and those exhibiting myofibroblast differentiation. The increase in SMA expression is an indirect marker of myofibroblast density in examined skin tissue. The addition of β_2AR antagonist causes an increase in myofibroblast density and hence promotes significant wound contraction. Myofibroblast-mediated contraction is the major mechanism of wound contraction; the interaction between myofibroblasts and the surrounding extracellular matrix (ECM) plays an important role in this phenomenon; myofibroblast differentiation, collagen fiber deposition and myofibroblast–ECM interaction is the most important determinant of wound contraction[28,32,33]. It should be mentioned here that SMA is also a marker of smooth muscles within the wall of newly formed blood vessels and may indirectly speculate the degree of angiogenesis in wound healing. β_2AR antagonist (timolol) has been found to increase SMA expression in wound healing in the present study and so by this way they are pro-angiogenic agents. The current study also showed immunohistochemical SMA expression increases significantly with time. This phenomenon may be due to the fact that early in wound healing inflammation is more marked than fibroblast and myofibroblast proliferation; however when the time elapsed fibroblast and myofibroblast proliferation predominates[29].

The immunohistochemical CD31 expression is a reliable marker of endothelial cells lining newly formed blood vessels and hence predicting the degree of angiogenesis in wound healing [34,35]. For that reason, it was used in the present study as a marker of angiogenesis. The current study showed that adding timolol (β_2AR antagonist) resulted in a highly significant increased CD31 immunohistochemical score in 5 and 10 days. In agreement with the findings of the present study, Pullar *et al*. (2012) stated that administration of β_2AR antagonist caused a significant increase in CD31 immunohistochemical expression in wounds after being followed for 5 and 10 days. The mechanism by which β_2AR inhibition modulates angiogenesis has been fully discussed previously above. The present study showed that in 10 days the mean immunohistochemical CD31 expression was significantly duration of wound healing. This phenomenon may be due to the fact that early in wound healing inflammation is more marked than endothelial cell proliferation and migration; however when the time elapsed endothelial cell proliferation and migration predominate[29].

CONCLUSION

In conclusion, the current study shown that the administration of β_2 adrenergic receptor antagonist (timolol) promotes wound healing through increased angiogenesis, collagen III deposition and myofibroblast density.

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