ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

B2 agonist (Salbutamol) modulate skin wound healing processes

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Article History:

Received on: 06.01.2018
Revised on: 17.02.2018
Accepted on: 22.02.2018

Keywords:

Wound healing
Angiogenesis
Salbutamol
Collagen III
re-epithelialization

ABSTRACT

Wound healing is a complex physiological and dynamic process required the coordination of numerous cell types and biological processes to regenerate damaged tissue and initiate repair which is dependent on a number of inter-related factors. This study was aimed to demonstrate whether the β2 receptor has role in wound healing and angiogenesis. A murine wild-type (*in vivo)*, excisional skin wound model was done to demonstrate that activation of β2AR delay wound repair, twenty-four male albino mice were used to investigate the effect of the drug on experimental wound healing grossly, histopathologically and immune-histochemically compared with vehicle-only controls. The results showed that the rate of wound healing was significantly slower in salbutamol group than in control group (P<0.05) after being followed for 5 days for some of the animals and for 10 days for other animals from the third day and thereafter. The results also revealed that 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 salbutamol significantly reduced collagen III, SMA and CD31 mean immunohistochemical score in days 5 and 10 when compared to control group (P<0.05). The current study concluded that the administration of β_2 adrenergic receptor agonist (salbutamol) delays wound healing through reduction of angiogenesis, collagen III deposition, myofibroblast density and re-epithelialization process.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v9i1.1169

Production and Hosted by

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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 (Shaw and Martin, 2009). According to the duration and nature of healing process, the wound is characterized as acute and chronic (Robson *et al.,* 2001). 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 (Eming *et al.,* 2014), while the repair injured or damaged tissue is substituted by connective tissue (Demidova-Rice *et al.,* 2012). Re-epithelialization is regrowth of epithelial cells across the wound surface occurs during the final stage of proliferation (Ortiz-Urda *et al.,* 2005). The epidermis can synthesize and secrete a number of proteins including epinephrine (Pullar *et al.,* 2006) which is a ligand for the β-adrenergic receptors (βARs): β1-adrenergic receptor (β1AR), β2AR, andβ3AR (Walluka, 2007), which are G protein-coupled receptors highly expressed on all cell lineages in the skin (de Coupade *et al.,* 2004; Iaccarino *et al.,* 2002); explaining the presence of an autocrine and paracrine βAR network in the epidermis and dermis, respectively. In excised human skin,

βAR activation delayed wound re-epithelialization, whereas βAR antagonism promoted skin re-epithelialization (Pullar *et al.,* 2006) in an *ex vivo* model of chronic wound re-epithelialization (Kratz, 1998). In murine skin wound models, stress-induced increases in epinephrine delayed wound repair (Sivamani *et al.,* 2009), conversely, βAR antagonism enhanced re-epithelialization in a murine skin burn model *in vivo* (Sivamani *et al.,* 2009) and accelerated skin barrier recovery (Denda *et al.,* 2003). In addition, a nonselective βAR antagonist improved wound healing in diabetic (Romana-Souza *et al.,* 2014) and burn-injured rats (Romana-Souza *et al.,* 2008). The β2AR agonist Salbutamol is a safe and widely used in asthma medication (Boskabady *et al.,* 2003). So, this study was aimed to investigate the effect of β2AR agonist (salbutamol) on some processes in wound repair.

MATERIALS AND METHODS

Plant material

Twenty-four male albino mice between 8 to 12 weeks of age were used in this study. They were fed with standard oxoid pallet and given water ad libitum. All animals kept at 28-30ºC and the experiments were approved by the Institute Review Board (IRB) in Al-Nahrain University, College of Medicine, Iraq. 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 were treated topically with Aqua Rosa alone for the control group (12 mice) and freshly prepared aqua rosa containing (5 mg/ml) selective β-2AR agonist (salbutamol) (O'Leary *et al.,* 2015) 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 differences and to monitor the percentage of wound healing over time. A biopsy 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 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 wound biopsy. One was stained with the hematoxylin-eosin (H &E) technique to determine the progress of the healing process while the other three sections were immune-stained with antibodies 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 (mean ± SD) were calculated for each group (Pullar *et al.,* 2012).

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

Wound samples transferred into formalin (10%); Fixative volume was 20 times that of tissue, tissue was fixed for a minimum 48 hours at room temperature the fixed tissue was processed, using gentle agitation (Weiss *et al.,* 2011), then embedded in paraffin blocks.

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

The Hematoxylin and Eosin staining system were used for histopathological examination of the wound sample to confirm healing (Anderson and Gordon, 1996) as showed in figure (3).

Immunohistochemistry for detection of collagen III, smooth muscle actin (SMA) and CD31(endothelial cell marker)

I. Anti-collagen III antibody ab7778: Rabbit polyclonal antibody to collagen III (Abcam, UK).

II. Anti-alpha smooth muscle actin antibody ab5694: Rabbit polyclonal to alpha smooth muscle actin (Code number: ab5694) (Abcam, UK).

III. Anti-CD31 antibody ab28364: Rabbit polyclonal to CD31, cellular localization membrane and cell junction (Code number: ab28364) (Abcam, UK).

Immunohistochemistry IHC Methods:

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

Evaluation of IHC results

The extent of presence polymorphonuclear leukocytes (PMNL) and fibroblasts were measured in a blinded manner according to a semi-quantitative scoring system: - (absent), + (minimal), ++ (mild), +++ (moderate), and ++++ (marked) (Gal *et al.,* 2008; Lacjakova *et al.,* 2010). 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) (Gal *et al.,* 2011) or 0= undetected, 1= low density, 2= medium density, 3=dense, to 4=very dense as defined by (Souil *et al.,* 2011). CD31 is often presented as a number of micro-vessels per square millimeter or mean value with standard deviations (Pullar *et al.,* 2012; Weidner et

al, 1991). Quantification of collagen III protein expression was evaluated under light microscopy at X40.

Statistical analysis

Data were collected, summarized, analyzed and presented using three statistical software programs: the statistical package for social science (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 (Daniel, 2005).

RESULTS

Gross morphological wound healing: Wounds were followed up for healing and the rate of the process was measured in the unit area of reduction in the size of the wound as demonstrated above. Table (1) and (2) and figures (1) and (2) showed that the rate of wound healing was significantly slower in salbutamol group than in control group ($P<0.05$) after being followed for 5 days for some of the animals and for 10 days for other animals from the third day and thereafter. Immunohistochemistry for collagen III expressions are shown in the table (3) and figure (4) and (5), Immunohistochemistry for (SMA) expression is shown in the table (4) and figure (6) and (7), Immunohistochemistry for CD31 is shown in the table (5) and figure (8) and (9). The results were as following: In 10 days the mean immunohistochemistry 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 salbutamol significantly reduced collagen III, SMA and CD31 mean immunohistochemical score in days 5 and 10 when compared to control group ($P<0.05$).

DISCUSSION

Wound healing is a complex process that involves interaction among cellular and extracellular matrix elements. The healing process is affected by local and systemic factors. The rate of wound healing was significantly slower in salbutamol group than in control group after being followed for 5 days for some of the animals and for 10 days for other animals from the third day and thereafter. Le Provost and Pullar (2015) found that, after 14 days, β2AR agonist-treated open wound area was 23% larger than the control area, which is in accordance with the finding of the present study in that β2AR agonist delays wound healing, but the ratio in the present study was 15% after 10 days of administration of β2AR agonist and this may have attributed to the time, drug, dose and or rout of administration (Eming *et al.,* 2014). In the present study collagen immunohistochemistry showed that salbutamol significantly reduced collagen formation in wounds after being followed up for 5 and 10 days. Pullar and Isseroff (2005) studied the effect of the β2AR agonist on fibroblast activity and collagen synthesis and deposition in Fibroblast-seeded collagen gels (an *in vitro* media) and found that a beta-agonist (isoproterenol) reduced fibroblast collagen formation in a dose-dependent manner (Pullar and Isseroff, 2005). This finding supports the finding of the present study in that β2AR agonist causes reduction in collagen synthesis in wound healing. Le Provost and Pullar (2015) found that application of salbutamol and formoterol (β2AR agonist) caused a significant reduction in collagen synthesis in wound healing, a result that is in accordance with the findings of the present study. The explanation of the effect β_2AR activation and inhibition on collagen deposition can as following: Fibroblasts express β_2AR on their surface (Kämpfer *et al.,* 2014; Rehsia, and Dhalla, 2010; Romana-Souza *et al.,* 2014). Activation and inhibition of β_2AR cause modulation of intracellular c-AMP (Kämpfer *et al.,* 2014). It was found that increased c-AMP is associated with less collagen formation by fibroblast and vice versa (Pullar and Isseroff, 2005). 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 [Ding *et al.,* 2014; Rao *et al.,* 2014]. The result of the present study showed that adding salbutamol significantly reduced mean SMA immunohistochemical score in days 5 and 10 when compared to control group ($P<0.05$). Le Provost and Pullar (2015) found that application of salbutamol and formoterol (β2AR agonist) caused a significant reduction in SMA expression in wound healing, a result that is in accordance with the findings of the present study. The increase in SMA immunohistochemical expression is an indirect marker of myofibroblast density in examined skin tissue. In conclusion, the administration of β_2AR agonist causes decrease in myofibroblast density and subsequently reduces 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 [Ibrahim *et al.,* 2015; Raut *et al.,* 2012]. It should be

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

Groups	Day 1 (%) Mean ± SD	Day 2 (%) Mean ± SD	Day 3 (%) Mean ± SD	Day 4 (%) Mean ± SD	Day 5 (%) 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
Salbutamol 5	0	21.20 ±2.39 B,c	23.00 ±5.05 C,c	25.40 ±4.34 C,b	39.60 ±7.16 C,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 an indicates highest values.

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

Groups	Day 1 (%) Mean ± SD	Day 2 (%) Mean ± SD	Day 3 (%) Mean ± SD	Day 4 (%) Mean ± SD	Day 5 (%) Mean ± SD	Day 10 (%) 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
Salbutamol 10	0	22.20 ±4.76 B,c	24.20 ±5.63 B,c	28.00 ±8.37 C,c	36.40 ±9.34 C,b	67.00 ±7.78 C,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 an indicates highest values.

Table 3: Mean collagen III scores in control and study groups

Groups	Day	Mean ±SD
Control	5 days	1.20 ±0.44 D
	10 days	1.60 ±0.55 C
Salbutamol	5 days	1.00 ±0.31 D
	10 days	1.40 ±0.40 C

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 SMA score in control and study groups

Groups	Day	Mean ±SD
Control	5 days	4.80 ±1.30 C
	10 days	6.40 ±0.89 B
Salbutamol	5 days	2.60 ±0.55D
	10 days	4.20 ±1.30 C

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

Table 5: Mean CD31IHC score in control and study groups

Groups	Day	Mean ±SD
Control	5 days	3.00 ±0.71 C
	10 days	3.80 ±0.84 B
Salbutamol	5 days	1.40 ±0.55 D
	10 days	2.60 ±0.55 C

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 agonist (salbutamol) has been found to reduce SMA expression and hence they worked as anti-angiogenic markers. 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 (Basilio-de-Oliveira *et al.,* 2015; Haber *et al.,* 2015). For that reason, it was used in the present study as a marker of angiogenesis. The present study showed that adding salbutamol (β_2AR agonist) significantly reduced mean immunohistochemical CD31 score in days 5 and 10 when compared to control group (P<0.05). O'Leary et al. (2015), assessed angiogenesis in murine wound

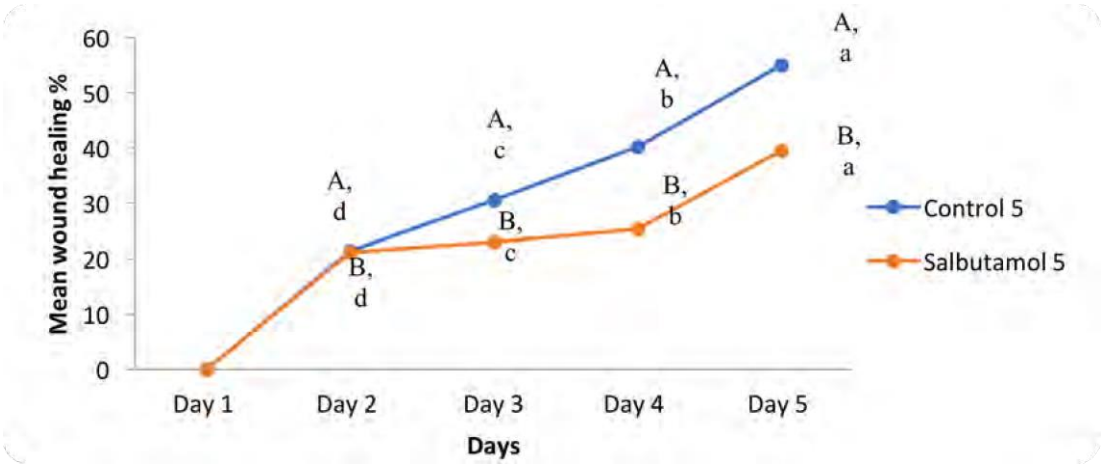

Figure 1: Mean area wound healing (mm2) for 5 days

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 an indicates highest values.

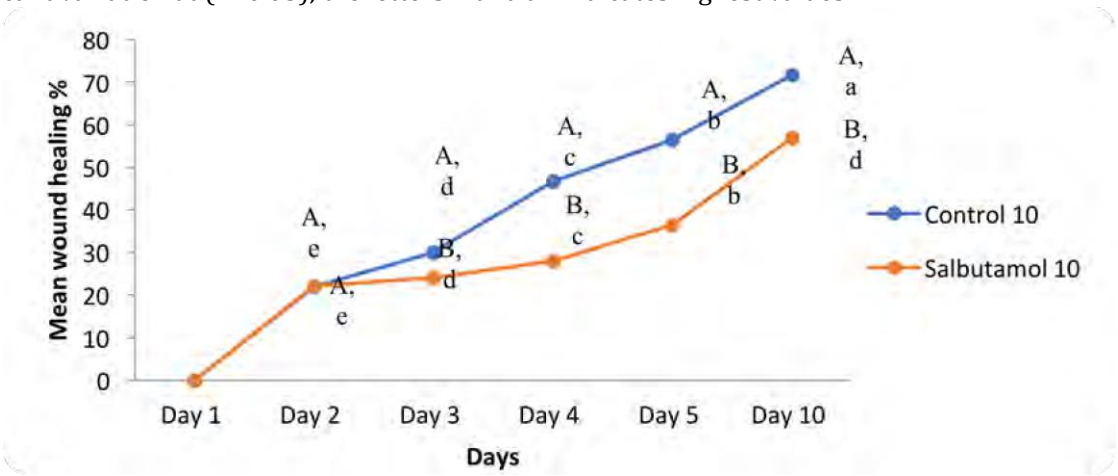

Figure 2: Mean area wound healing (mm^2) for 10 days

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 an indicates highest values.

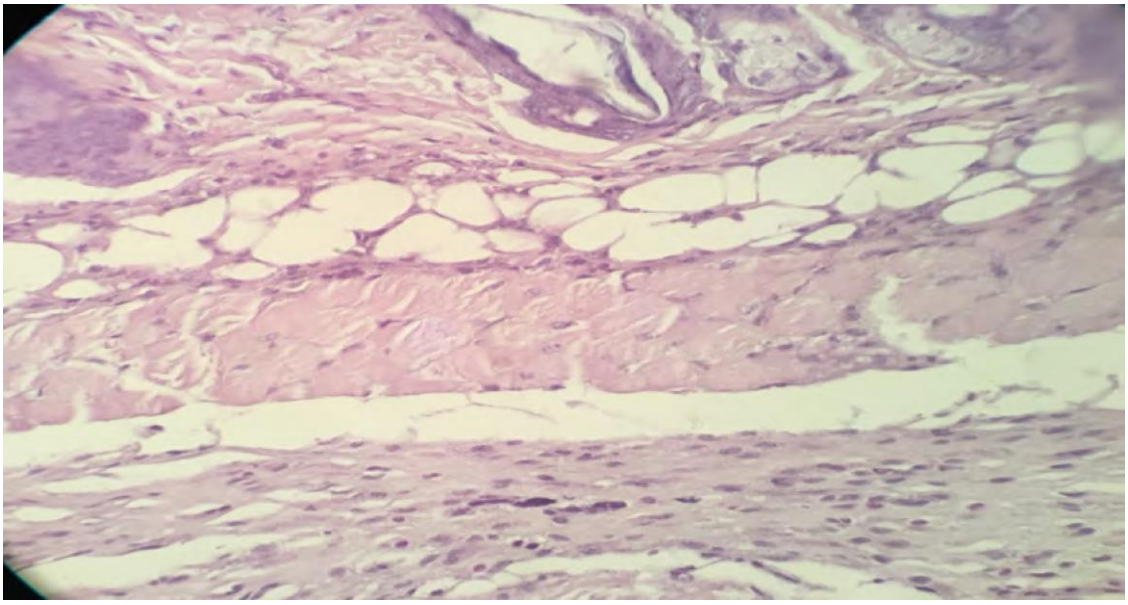

Figure 3: A Histological section that was stained with H and E stain (40X).

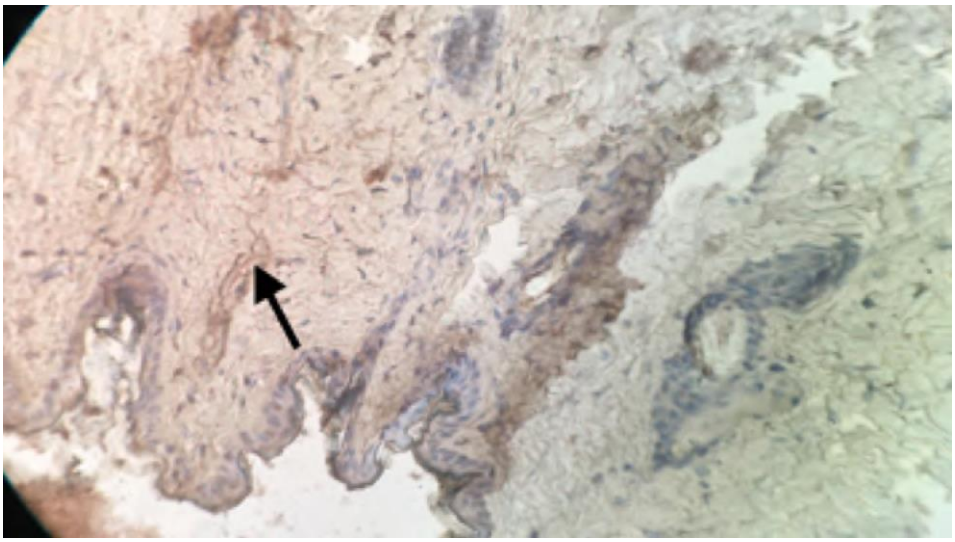

Figure 4: Extracellular immunohistochemical expression of collagen III within the dermis (black arrow) in salbutamol group (40X).

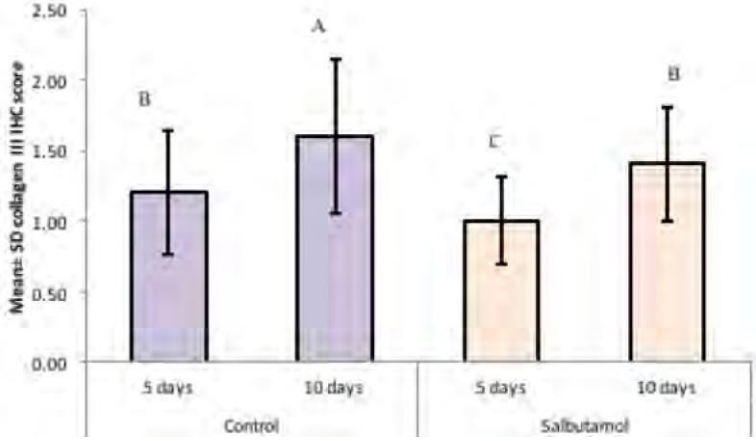

Figure 5: Mean collagen III scores in control and study groups

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

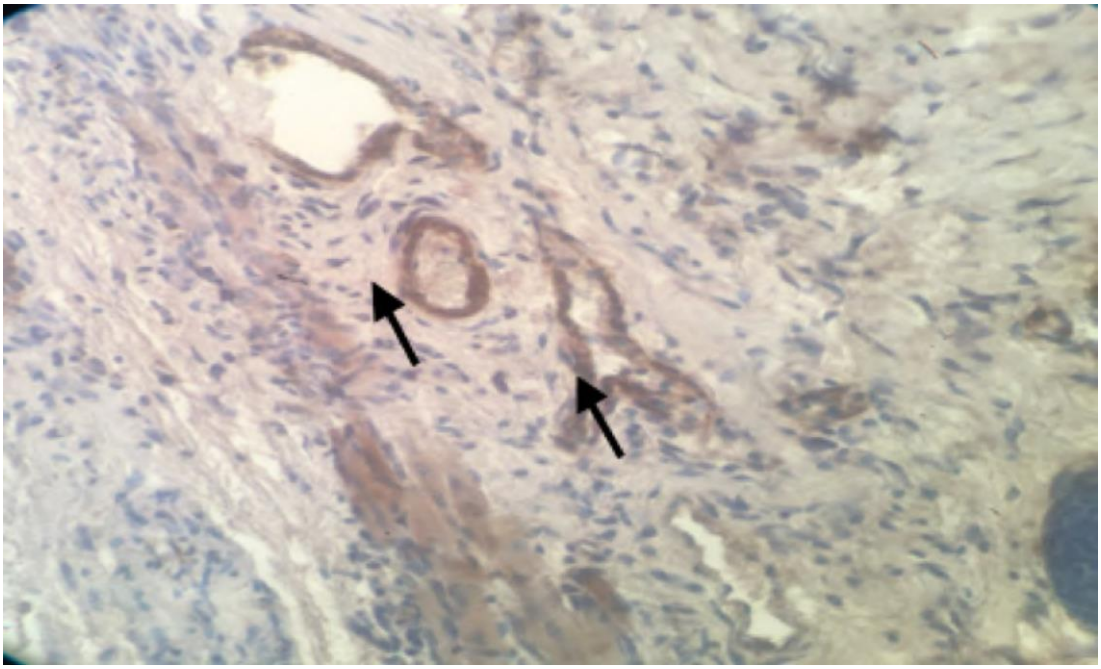

Figure 6: Cytoplasmic immunohistochemical expression of SMA in the wall of blood vessels

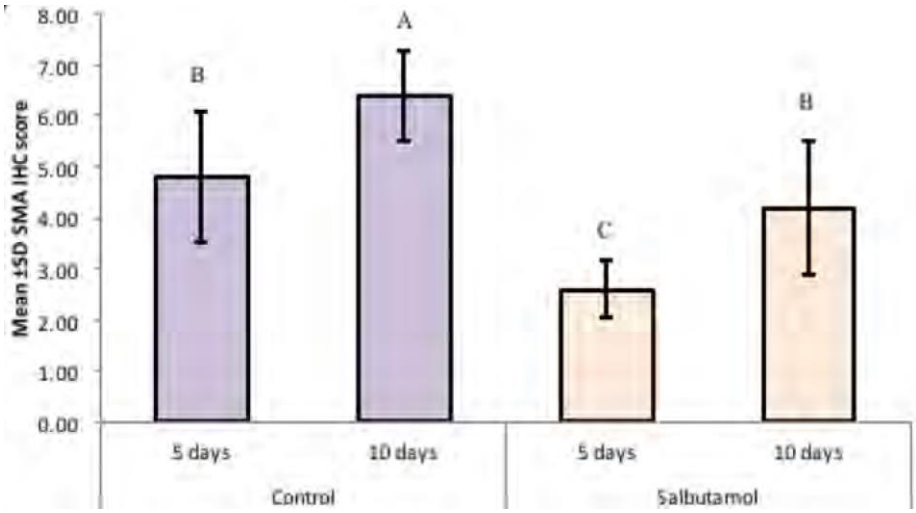

Figure 7: Mean SMA score in control and study groups

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

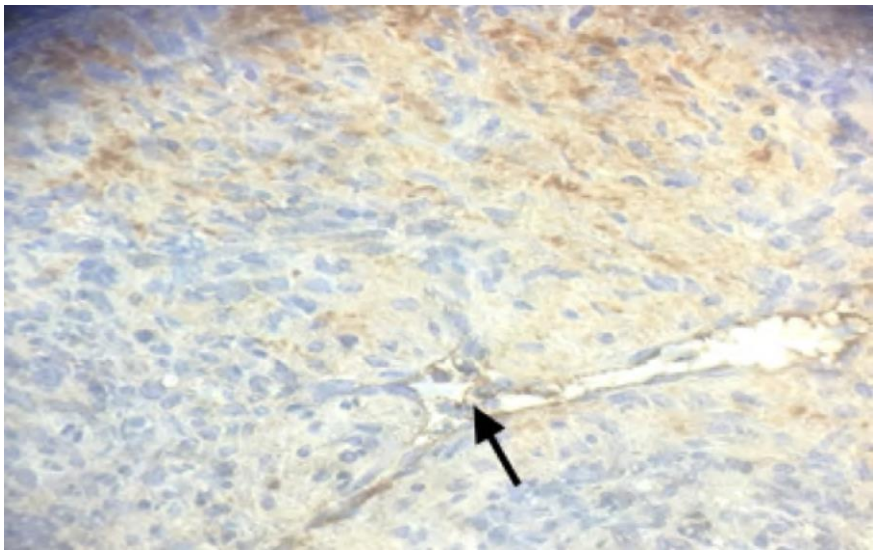

Figure 8: Cytoplasmic immunohistochemical expression of CD31 by vascular endothelial cells (black arrow) in control group (100X).

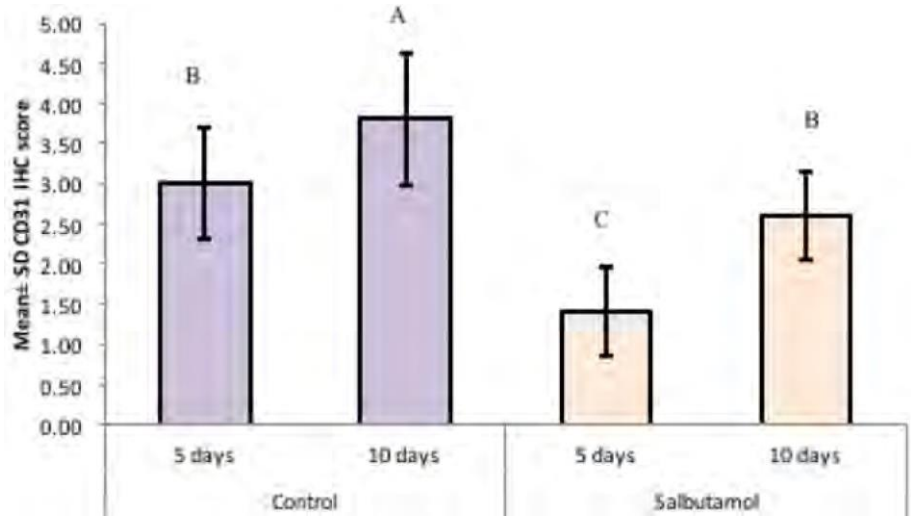

Figure 9: Mean CD31IHC score in control and study groups

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

healing by measuring immunohistochemical CD31 expression and found that administration of the β_2AR agonist (Salbutamol) significantly reduced CD31 expression and hence angiogenesis. This result supports the finding of the present study that Salbutamol is an anti-angiogenic factor in wound healing. The mechanism by which β_2AR activation or inhibition modulates angiogenesis has been fully discussed previously above. The present study showed that in 10 days the mean immunohistochemical collagen III, SMA and CD31 expressions were 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 (Dakhil, 2017).

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