Histological study on the effect of topical application of glucosamine on wound healing in rats

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Introduction

Wounds are common in clinical practice, both in outpatient clinics and in hospitals. Poor and/or prolonged wound healing is a clinical problem resulting in long hospital stay and considerable economic costs. Glucosamine (GA) is a water-soluble hexosamine derived from chitin or chitosan. It has been shown to have a stimulatory effect on matrix formation and hyaluronic acid synthesis. However, these results were mainly through systemic administration, with few studies topically conducted. The aim of this study was to determine the effect of topical administration of GA on the wound healing process in a rat model of full-thickness skin wounds.

Materials and methods

Twenty-four male albino rats were divided into two groups: a control group (group I) and an experimental group (group II). The control group (10 rats) received regular dressing in addition to the nonmedicated gel base. The experimental group (14 rats) received 2% GA in gel form. A 1 cm^2 full-thickness skin wound was created on the back of each rat. Treatment was administered daily until the end of the study (for 10 days). The wound closure rate, collagen synthesis, blood vessel formation, and fibroblast proliferation were studied by histological, immunohistochemical, and morphometric methods.

Results

GA significantly enhanced and accelerated the wound closure rate as well as fibroblast proliferation. Collagen synthesis was also enhanced, as well as proliferation of hair follicles, in contrast to the control group. GA also improved the neovascularization process at the wound site. Overall, topical application of 2% GA accelerated the wound healing process and the wound contracting ability.

Conclusion

The results of this study indicated that GA has the potential to be used for treatment of skin wounds as a topical medication; however, further evaluations of its mechanisms of action and its clinical disadvantages should be made.

Keywords:
glucosamine, immunohistochemistry, rats, wound healing

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Introduction

Wounds are common clinical problems resulting in pain, discomfort, long hospital admission, and considerable economic costs [1]. Wounds may be acute or chronic. Causes of wounds are variable and include venous or arterial insufficiency, diabetes mellitus, burns, trauma, chronic pressure, and surgery [2].

The process of wound healing includes several components such as fibroblast activation and migration, re-epithelization, endothelial cell proliferation and angiogenesis, in addition to a local inflammatory response at the wound site [3]. Tissue regeneration begins immediately after injury through different growth factors and cytokines [4]. However, the repair process may be complicated with infection, tissue disruption, and severe or prolonged inflammation [5,6]. These complications may affect the rate and quality of tissue regeneration, resulting in delayed and/or poor wound healing.

Topical application of growth factors to improve wound healing has been met with limited success. This was due to their high diffusibility and their very short half-life [6,7]. Various skin grafting techniques, including xenografts, allografts, and autografts, have also been used for the treatment of deep and full-thickness wounds. However, because of their antigenicity and the limited availability of donor sites, skin grafts are not usually feasible [8]. Therefore, searching for new modalities and agents to promote and enhance a good wound healing is a clinical demand.

Glucosamine (GA) is a hexosamine product made from chitin or chitosan. Chitin is extensively distributed in nature and is the second most plentiful polymer after

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cellulose. Chitosan is a cationic polysaccharide derived from *N*-deacetylation of chitin [9]. GA was reported to possess many biological effects such as membrane stabilization and hepatic protection [10]. It also enhances the synthesis of hyaluronic acid [11] and was used in the treatment of osteoarthritis [12]. It was also shown to have an anti-inflammatory activity [13]. Furthermore, GA has been shown to induce favorable effects on cartilage metabolism *in vitro*. It reduced articular cartilage breakdown and stimulated the synthesis of matrix proteoglycans by chondrocytes, restoring the articular functions [14,15].

The effects of systemic administration of GA on skin wounds were previously investigated [16]. The author demonstrated that GA has accelerated the process of wound healing and the rate of tissue regeneration. In addition, the use of GA resulted in reduced wrinkle and scar tissue formation [11,17–19]. However, Mori *et al.* [20] previously demonstrated that GA had no stimulatory effect on fibroblasts. It even inhibited fibroblast proliferation. Therefore, a debate continues regarding the beneficial effects of GA on the wound healing process. Furthermore, although many studies were conducted to evaluate the dermatological effects of systemic administration of GA, chitin, and chitosan, few investigations were found for the topical effects of GA on wound healing.

The aim of the present study was to determine whether topical application of 2% GA has beneficial effects on the healing process of full-thickness skin wounds in rats.

Materials and methods

Drug preparation

GA was purchased from Sigma (St Quentin Fallavier, France). A 2% GA gel form was made from GA by dissolving 2 g GA in 2 ml distilled water and then transferring the solution into 2% carboxymethylcellulose (CMC) (2 g CMC dissolved in 98 ml distilled water). The gel form was made to facilitate the application of GA during wound treatment. A nonmedicated gel base was also made by creating 2% CMC gel without GA.

The antiseptic solution, bovidone iodine 10% (Betadine), was purchased from Nile Company for Pharmaceuticals and Chemical Industries (Cairo, A.R.E., Egypt). It was used as a regular antiseptic dressing according to the experimental design.

Experimental animals

Twenty-four adult male albino rats, weighing 200–230 g, were obtained from Kasr El-Aini animal House, Faculty of Medicine, Cairo University. The rats were housed under good hygienic conditions at room temperature. They were maintained on a standard diet and allowed water *ad libitum* throughout the study.

Experimental design and excision of wounds

The animals were randomly divided into two groups: a control group (group I, n=10) and an experimental group (group II, n=14). The wounds of the animals of group I were dressed in a regular manner with the antiseptic bovidone iodine in addition to the nonmedicated gel base (CMC, 2 ml gel base twice a day) until the end of the study. The wounds of the animals of the experimental group were dressed only with the 2% GA gel (2 ml twice a day) for the same period.

Experimentally induced excision wounds

General anesthesia was induced using intramuscular injection of ketamine at a dose of 30 mg/kg. The skin was shaved and disinfected with 70% alcohol. In each rat, a single 1-cm^2 circular full-thickness skin wound was created on the posterior surface of the back. The wound dressing procedure was performed in a standard way for all animals and repeated twice a day until the end of the study (the day in which a single wound in any group was closed). At day 5 after surgery, half of the animals from each group were sacrificed with a high dose of ether, and full-thickness skin specimens were taken from the wound sites and fixed in 10% formol saline for histological and immunohistochemical evaluation. The remaining animals continued to receive the treatment as previously described until the end of the experiment, when the animals were sacrificed with a high dose of ether and full-thickness skin specimens were taken from the wound sites and fixed in formol saline for histological evaluation.

Histological and immunohistochemical evaluation was carried out as follows:

All specimens (those taken at day 5 and at the end of the experiment) were subjected to histological examination with H&E and Mallory's trichrome stains.

Specimens taken at day 5 were, in addition, subjected to orcien staining and immunohistochemical analysis for evaluation of early elastic fiber formation and early expression of CD31 and α smooth muscle actin (α-SMA), respectively.

Determination of wound closure rate

To determine the rate of wound closure, digital photographs were captured from the wound surfaces every 3 days with a digital camera. A measuring transparent grid was set on the wound surface, and the wound area (mm^2) at each visit was estimated. The wound closure rate was expressed as the percentage of wound area compared with that on postoperative day according to the following formula as previously reported [21].

Wound closure rate (%)= (initial wound area–wound area at each visit)/initial wound area)×100.

Histological evaluation

The skin tissue samples from all groups were immediately fixed in 10% formol saline, dehydrated, and processed for paraffin sectioning. The paraffin blocks were cut into 5-μm thick perpendicular to the wound, stained with standard H&E, Mallory's trichrome, and orcein stains, and examined. The evaluated parameters were re-epithelialization, inflammatory cell infiltration, fibroblast

proliferation, neovascularization, collagen deposition, and hair follicle production.

Immunohistochemical evaluation

The deparaffinized and rehydrated tissue sections were incubated with 3% H_2O_2 for 10 min. The sections were then put into a 10 mmol/l citrate buffer solution (pH 6.0) and heated twice in a microwave oven to recover the antigen. The slides were then washed twice with PBS (pH 7.2–7.6). Nonspecific binding sites were blocked with 5% BSA in TBS for 20 min. Then, the sections were incubated with mouse anti-CD31 primary antibody (Dako Biotechnology, Denmark) or mouse anti-α-SMA primary antibody (Dako Biotechnology) at 4°C overnight, and then washed with PBS. The slides were then incubated with biotinylated goat anti-mouse secondary antibodies (Dako Biotechnology) for 20 min, followed by incubation with streptavidin-HRP for 20 min. They were then counterstained for 1 min with hematoxylin and dehydrated through sequential ethanol for microscopic examination [22].

Morphometric studies

Images were analyzed using a Leica Qween 500 computer-assisted image analyzer system (Cambridge, UK) consisting of a microscope equipped with a high-resolution video camera. Tissue sections were examined under the microscope and 10 randomly selected fields per animal were examined from each group. The measured parameters were the number of fibroblasts, the number and diameter of blood vessels, the area percentage of collagen fibers, and the area percentages of CD31 and α-SMA expression in both groups.

Statistical analysis

The collected data were reported as mean and SD. Statistical comparisons between the groups were carried out with SPSS statistical software (v.16.0). One-way analysis of variance was used to analyze the data. *P* values less than or equal to 0.05 were considered statistically significant.

Results

The treatment was administered daily until day 10 after surgery, when two wounds of rats from the experimental group were found to be nearly closed.

Wound closure rate

The mean initial area of wounds for all rats was 106.453 ± 9.324 mm^2. There were no significant differences between the two groups regarding the initial wound areas. The average rate of wound closure in the GA-treated group was 7.142 ± 1.863 mm^2, which was significantly higher in comparison with that of the control group (4.586 ± 1.983 mm^2) ($P<0.05$) (Fig. 1b). Ten days after surgery (end of the study), the wounds dressed with 2% GA (group II) showed comparatively less scar width as compared with the control group (Fig. 1a).

Histological results

Wounds at 5 days after surgery

The regenerating dermis: crusts composed of degenerating inflammatory cells, RBCs, and keratin were frequently present on the wound surfaces of both groups (Fig. 2). However, the formed crusts in the experimental group were thicker compared with that of the control group at day 5 after surgery (Fig. 2).

There was inflammatory cell infiltration in both groups. The inflammatory cellular infiltration consisted mainly of polymorphonuclear cells, some lymphocytes, and macrophages. RBCs were also frequently observed among the inflammatory cells in both groups. The inflammatory cell infiltration was less intense in the control group (group I) compared with the experimental group (group II) (Figs 2 and 3). In group II, dressing wounds with GA did not inhibit the inflammatory cell infiltration and there was an apparent increase in the number of inflammatory cells mainly in the crusts (Fig. 2). The difference in inflammatory cell infiltration between the two groups was apparently significant.

Granulation tissue formation in the regenerating dermis was generally mild. That of the experimental group was more abundant as compared with the control group. The vertical thickness of the entire wound was greater in the GA-treated group compared with the control group (Fig. 7).

The dermis of animals in the GA-treated group showed marked proliferation of blood vessels as compared with the control group (Fig. 4). In the GA-treated group, most of the apparent capillaries showed opened lumens and some contained blood (Fig. 4b). In the control group the dermis, however, showed less capillary proliferation (Fig. 4a). The increased proliferation of blood capillaries and increased angiogenesis were apparently more in group II compared with group I. The dermis also appeared more dense in the experimental group as compared with the control group as more fibrous tissue was apparently demonstrated in the GA-treated group compared with the control group (Fig. 4). The fibroblasts appeared as elongated flat cells with basophilic cytoplasm, showing few processes and containing a single euchromatic oval nucleus (Fig. 5). The difference in appearance of the deep areas of the dermis in both groups was not marked (Fig. 6).

Mallory trichome-stained sections of the dermis in Group II demonstrated more collagen fiber deposition as compared with group I (Fig. 7).

Hair follicles and pilosebaceous units in wound areas were absent in the GA-treated group as well as in the control group at day 5 after surgery. The number of hair follicles and pilosebaceous units at wound edges was apparently similar in both groups (Fig. 8).

The epithelium: re-epithelialization of wounds occurred in the control and GA-treated animals. Both groups at 5 days after surgery showed an apparently similar degree of re-epithelialization (Fig. 9). However, the epithelium in

the GA-treated animals was thicker and more hyperplastic with apparently greater cell number as compared with the epithelium in the control group (Fig. 10).

Wounds at 10 days after surgery (end of the study)

The regenerating dermis: inflammatory cellular infiltration was markedly ceased in the GA-treated group compared with the control group. The surface of wounds in the control group still showed more macrophages, neutrophils, and many degenerating inflammatory cells (Fig. 11). In the GA-treated group, the dermis appeared more dense with little spaces and the collagen fibers appeared more regularly arranged compared with the control group (Fig. 12). The presence of proliferating capillaries was apparently diminished in the GA-treated group as compared with the control group (Fig. 12). The GA-treated group showed more fibroblasts, which appeared more regularly arranged, increased in size, and more elongated. Their cytoplasm became more abundant and deeply basophilic and they showed larger nuclei compared with fibroblasts from the control group (Fig. 13). More collagen fibers were demonstrated in the GA-treated group as compared with the control group in the H&E-stained sections (Fig. 13) as well as in the Mallory trichrome-stained sections (Fig. 14). In the GA-treated group, the deep dermis of some wounds demonstrated more dense, thicker, and wavy collagen fibers, which were arranged in thick bundles as compared with the control group (Fig. 15). In other wounds, more mature collagen in bundles appeared at wound edges in the GA-treated group as compared with the control group (Fig. 15). Hair follicles and pilosebaceous units were detected in the wound areas of both groups and their number was greater in the GA-treated group when compared with the control group, which demonstrated markedly less content of these structures at 10 days after surgery (Fig. 16).

The epithelium: re-epithelilization continued in both groups. However, it was greater and tended to be complete in the GA-treated group as compared with the control group. The control group demonstrated more hyperplastic and thicker epithelium with wider wound areas when compared with the GA-treated group. In the GA-treated group, the epithelium was apparently normal and showed uniform thickness (Fig. 17).

Effect of treatment on elastic fibers: this study is not a mere comparison between wounds at day 5 (early wound healing) and at day 10 (late wound healing and near wound closure). Rather, it examines the effect of GA on the healing process. Therefore, it was more relevant to study the effect of GA on elastic fiber formation and on immunoexpression of the markers CD31 and α-SMA at an early stage of the healing process. Hence, these parameters were evaluated only at day 5 after surgery.

To examine any effect of GA on early elastic fiber formation, specimens of wounds at 5 days after surgery were stained with orcein. The orcein-stained sections from the GA-treated group as well as those from the control group revealed no significant difference in elastic fiber content (Fig. 18).

Immunohistochemistry

Increased expression of CD31 was observed in the GA-treated group compared with its expression in the control group at day 5 after surgery (Fig. 19).

Similarly, the expression of α-SMA was higher in the GA-treated group in comparison with the control group (Fig. 20).

Morphometric results (Figs 21 and 22)

Number of fibroblasts (Fig. 21):

The average number of fibroblasts at day 5 after surgery was 102.5 ± 17.26/high-power field (×400) in the GA-treated group, compared with 83.75 ± 9.16 in the control group. At day 10 after surgery, it was 118.08 ± 7.83 in the GA-treated group compared with 88.1 ± 12.94 in the control group. The difference in the number of fibroblasts between the two groups was statistically significant at day 5 as well as at day 10 (P<0.001) after surgery.

The number and diameter of blood vessels (Fig. 21):

The average number of blood capillaries at day 5 after surgery was 8.58 ± 1.16/high-power field (×400) in the GA-treated group compared with 5.91 ± 1.2 in the control group. The difference in the number of blood capillaries between the two groups was statistically significant (P<0.001). At day 10 after surgery, the mean number of blood capillaries was reduced to 7.25 ± 1.54 in the GA-treated group compared with 9.91 ± 2.02 in the control group. This difference between the two groups was also statistically significant (P=0.007). The mean diameter of blood vessels in the control and experimental groups at day 5 and at day 10 after surgery was nearly similar. Figure 21 shows the mean diameter of blood vessels in the two groups. The difference in the mean diameter of blood capillaries between the two groups was not statistically significant (P=0.131).

The area percentage of collagen fibers (Fig. 22):

The mean area percentage of collagen fibers was 33.55 ± 2.18% in the control group compared with 37.43 ± 3.12% in the GA-treated group, which was statistically significant (P=0.02).

Area percentages of CD31 and α-SMA expression (Fig. 22):

The area percentage of CD31 expression was 17.791 ± 1.372% in the GA-treated group compared with 4.774 ± 1.133% in the control group. The difference in area percentages of CD31 expression between the two groups was statistically significant (P<0.001).

The area percentage of α-SMA expression was 34.313 ± 1.560% in the GA-treated group compared with 13.334 ± 1.046% in the control group. Expression of α-SMA was statistically significant in the GA-treated group compared with the control group (P<0.001).

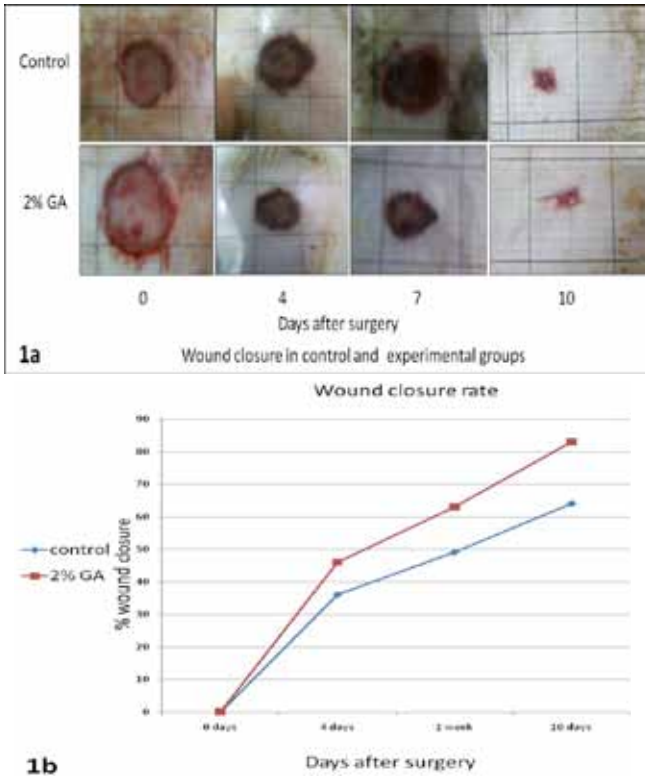


Figure 1. Photographs (a) and a linear chart (b) of skin wounds from the control and glucosamine (GA)-treated groups showing wound closure and wound closure rate, respectively. The rate of wound closure is enhanced in the GA-treated group.

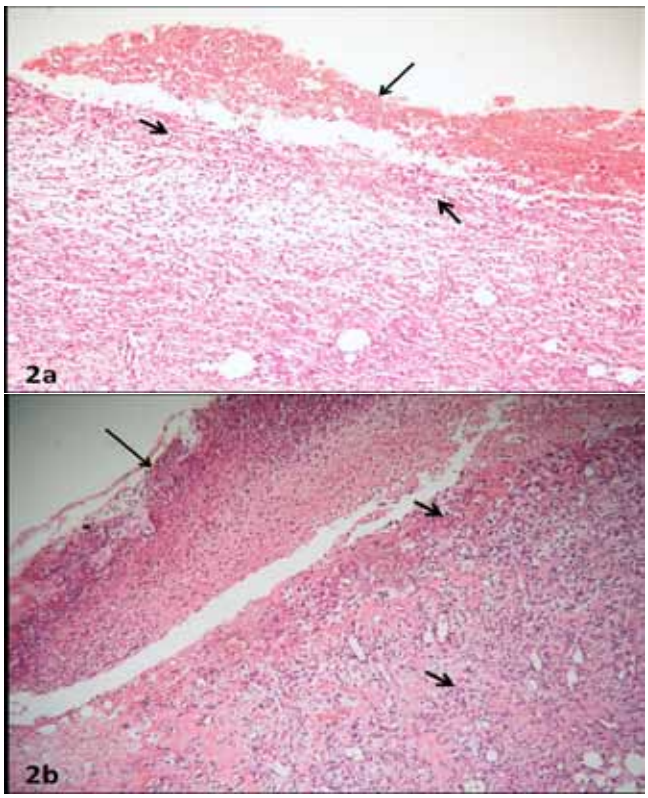


Figure 2. Photomicrographs of skin wounds 5 days after surgery from the control group (a) and glucosamine (GA)-treated group (b) showing crusts covering both wound surfaces (long arrows). The crusts are formed of degenerating inflammatory cells, RBCs, and keratin. Crusts in the GA group are thicker compared with that of the control group. Inflammatory cell infiltration of the dermis in both groups is also observed (short arrows).

H&E, × 100.

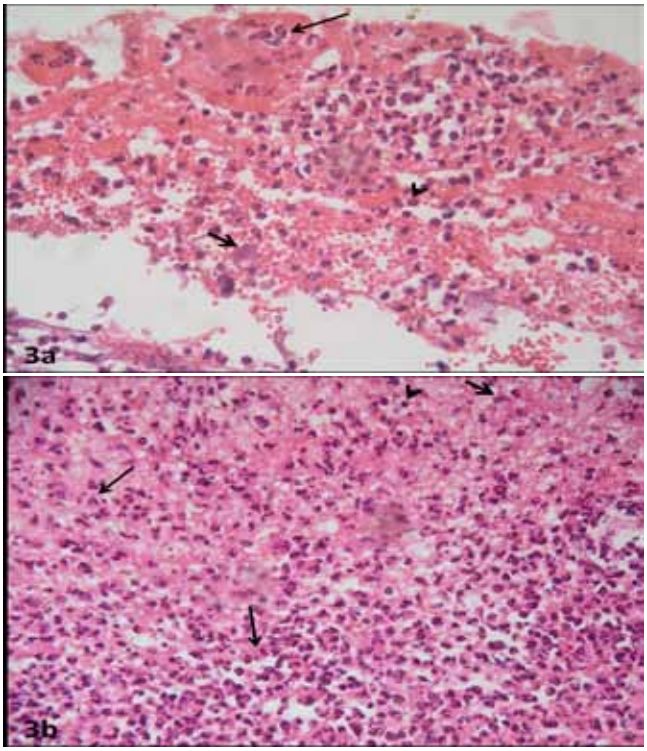


Figure 3. Photomicrographs of skin wounds 5 days after surgery from the control group (a) and glucosamine-treated group (b) showing inflammatory cellular infiltration that mainly consisted of polymorphonuclear cells (long arrows). Some lymphocytes (arrow heads) and macrophages (short arrows) are also present. RBCs are prominent among the inflammatory cells in both groups.

H&E, × 400.

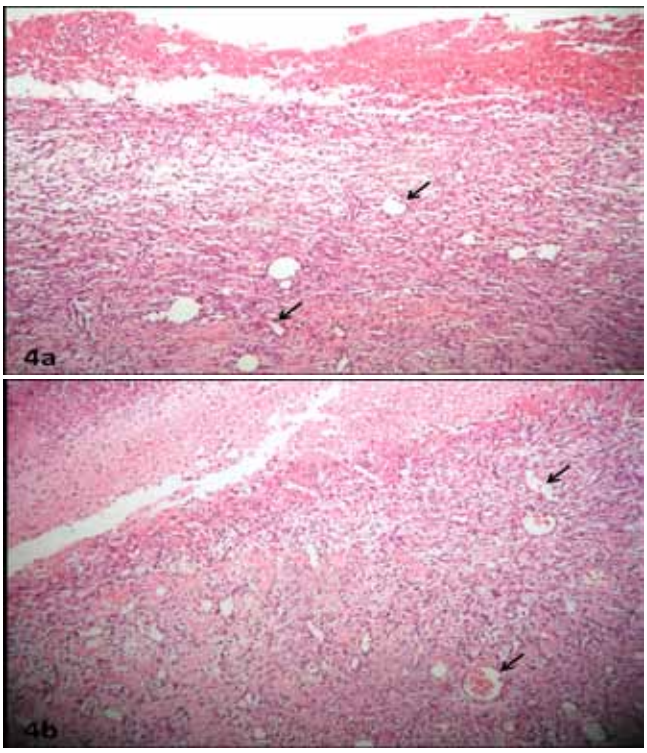


Figure 4. Photomicrographs of skin wounds 5 days after surgery from the control group (a) and glucosamine (GA)-treated group (b) showing marked proliferation of blood vessels (arrows) in the GA-treated group compared with the control group. Some blood capillaries are filled with blood in the GA-treated group. The dermis in the GA-treated group is more dense compared with that of the control group because of the apparent increase in fibrous tissue.

H&E, × 100.

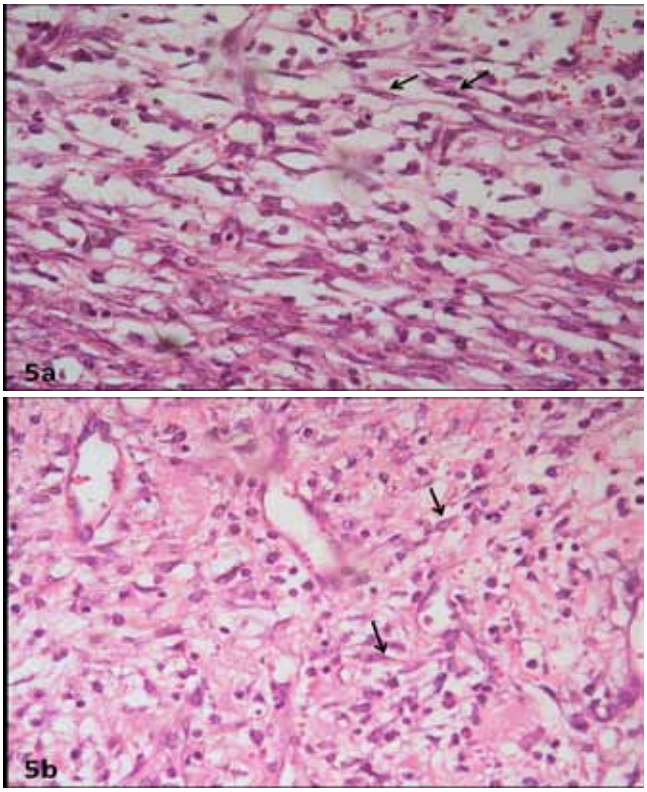


Figure 5. Photomicrographs of skin wounds 5 days after surgery from the control group (a) and glucosamine-treated group (b) showing fibroblasts as elongated flat cells having basophilic cytoplasm with few processes and containing euchromatic oval nuclei (arrows).

H&E, × 400.

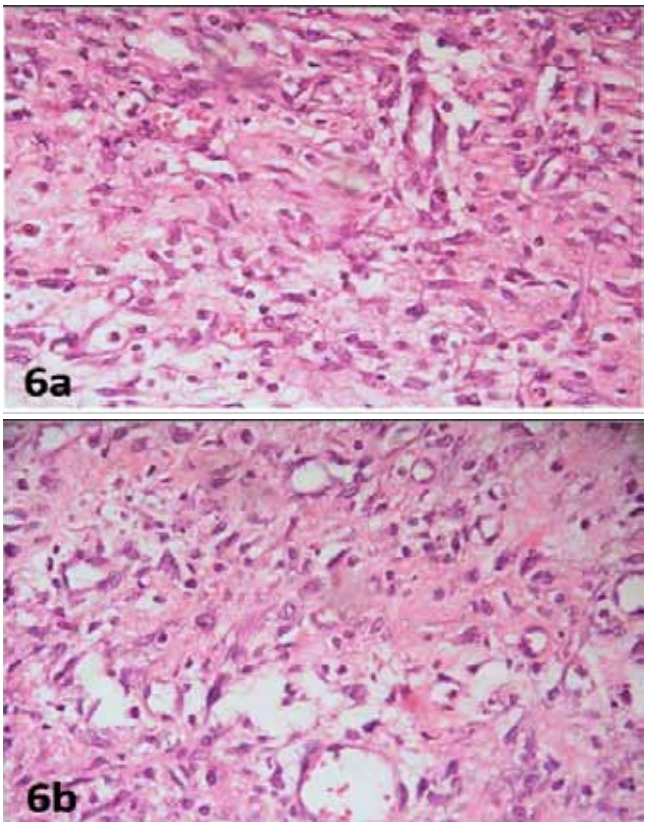


Figure 6. Photomicrographs of skin wounds 5 days after surgery from the control group (a) and glucosamine-treated group (b) showing similarity in appearance of the deep portions of the dermis in the two groups.

H&E, × 400.

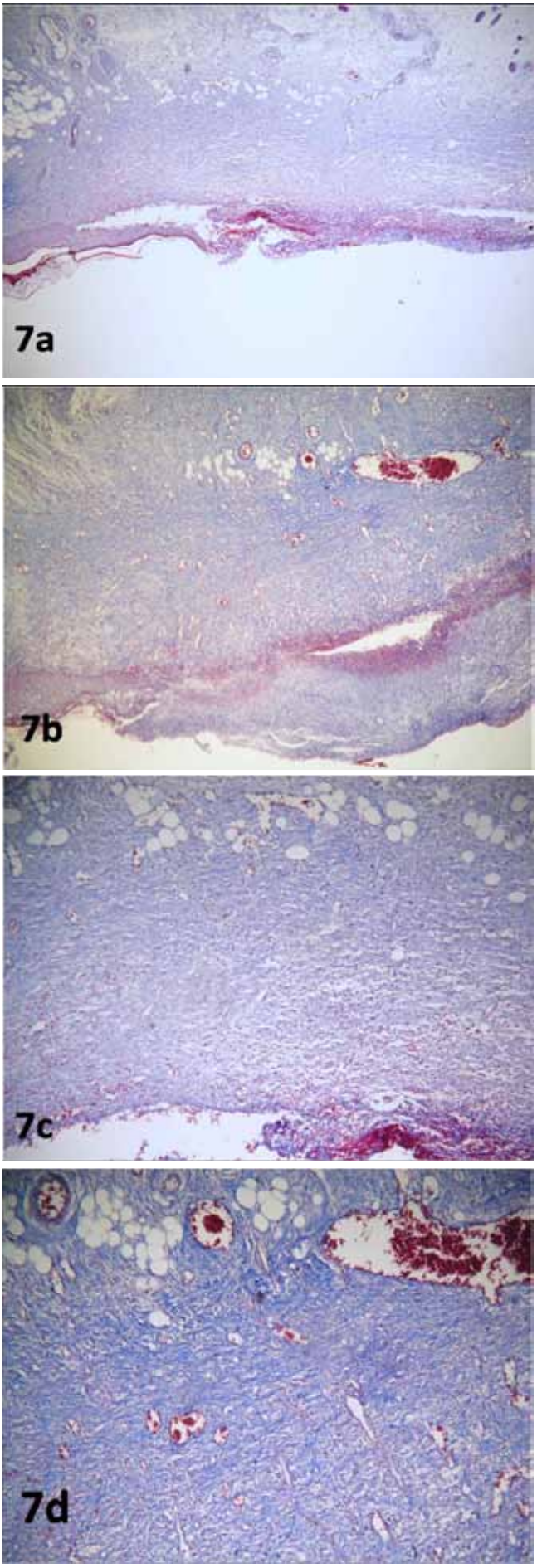


Figure 7. *(Continued)*

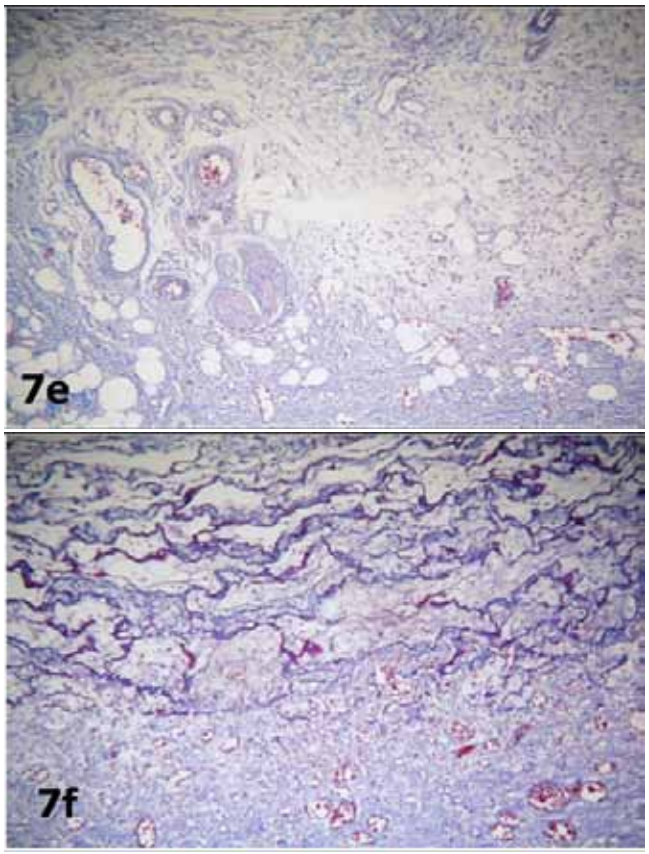


Figure 7. Photomicrographs of skin wounds 5 days after surgery from the control group (a, c, and e) and glucosamine (GA)-treated group (b, d, and f) showing more collagen fiber deposition in the dermis of the GA-treated group compared with the control group. The vertical dimension of the wound (wound thickness) appears greater in the GA-treated group (b) compared with the control group (a).
Mallory trichome, (a, b) × 40; (c–f) × 100.

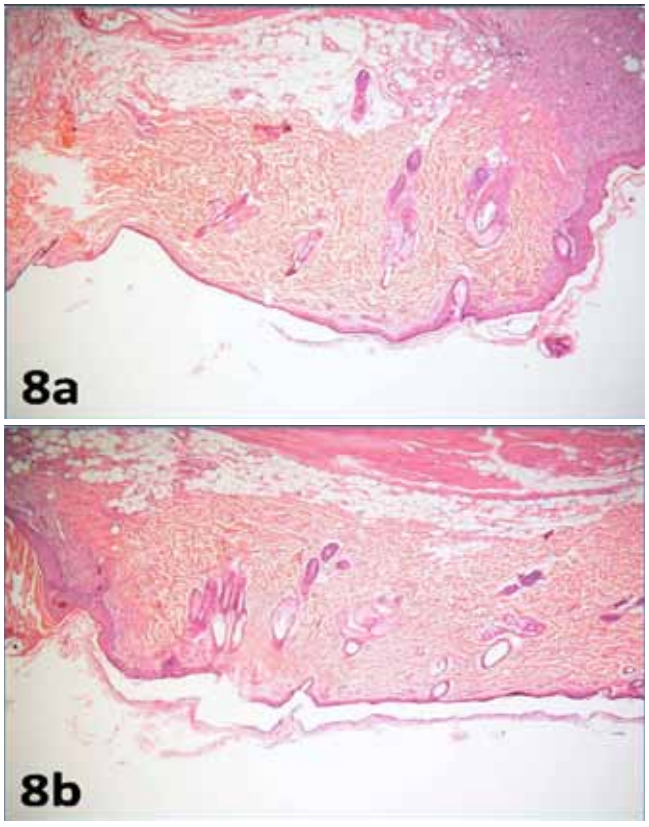


Figure 8. *(Continued)*

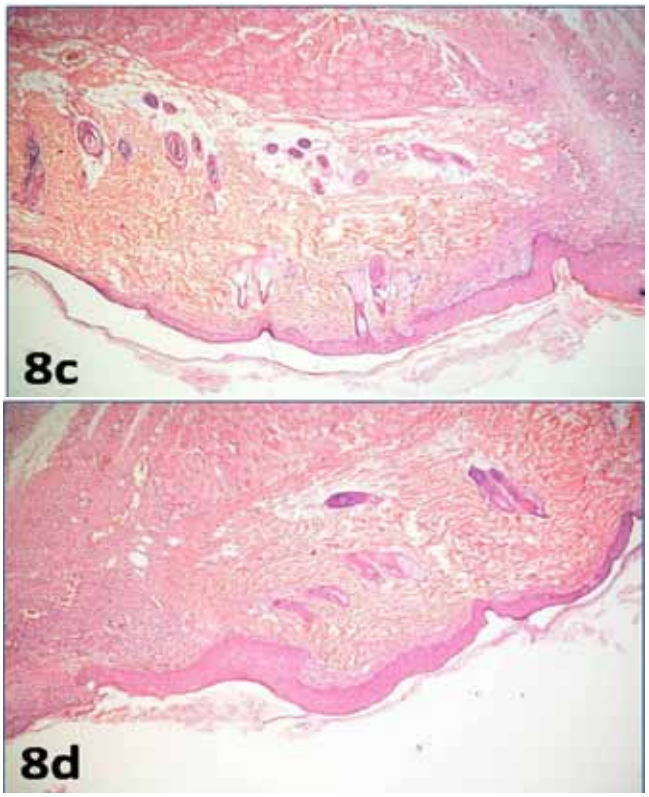


Figure 8. Photomicrographs of skin wounds 5 days after surgery from the control group (a, b) and glucosamine-treated group (c, d) showing absence of hair follicles and pilosebaceous units in wound areas in both groups. At wound edges the difference in the number of these structures in the two groups is not significant.
H&E, × 40.

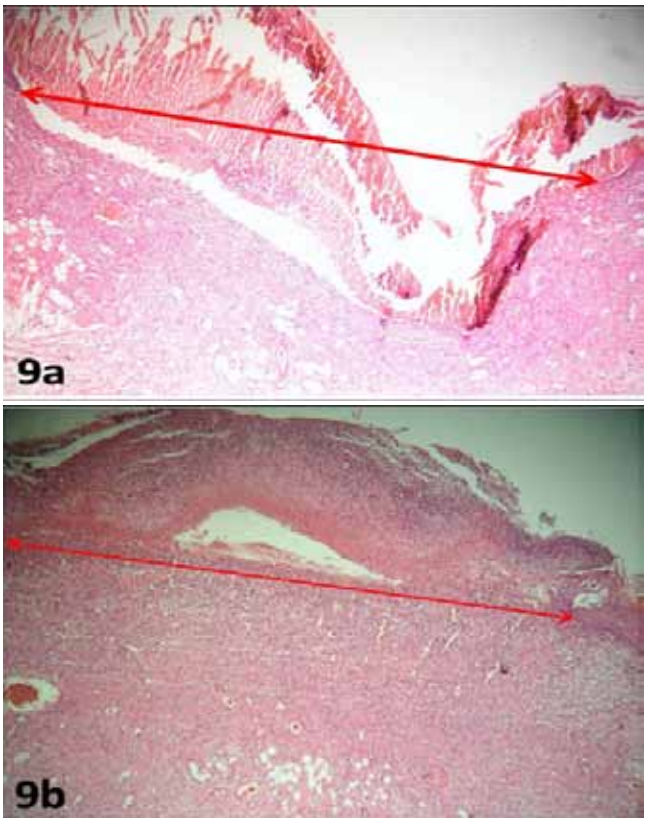


Figure 9. Photomicrographs of skin wounds 5 days after surgery from the control group (a) and glucosamine-treated group (b) showing apparently similar degree of re-epithelialization (arrows).
H&E, × 40.

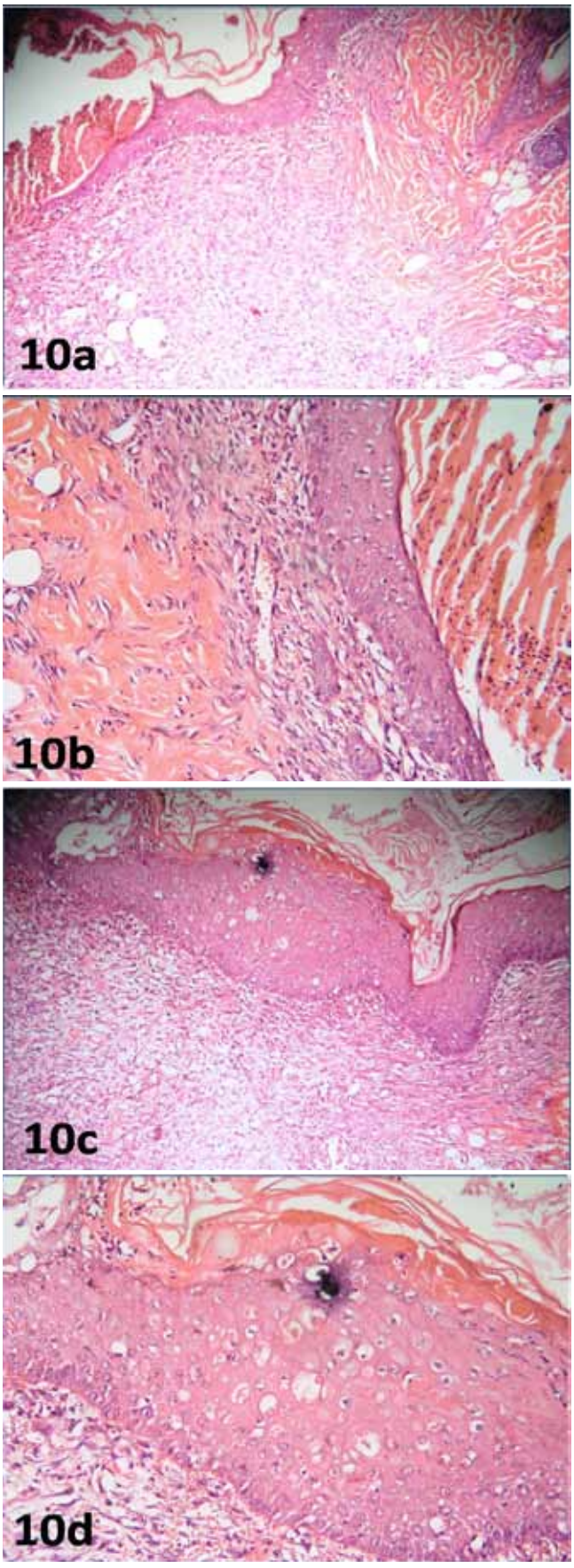


Figure 10. Photomicrographs of skin wounds 5 days after surgery from the control group (a, b) and glucosamine (GA)-treated group (c, d) showing a thicker and more hyperplastic epithelium in the GA-treated animals with apparently greater cell number compared with the epithelium in the control group.

H&E; (a, c) × 100; (b, d) × 200.

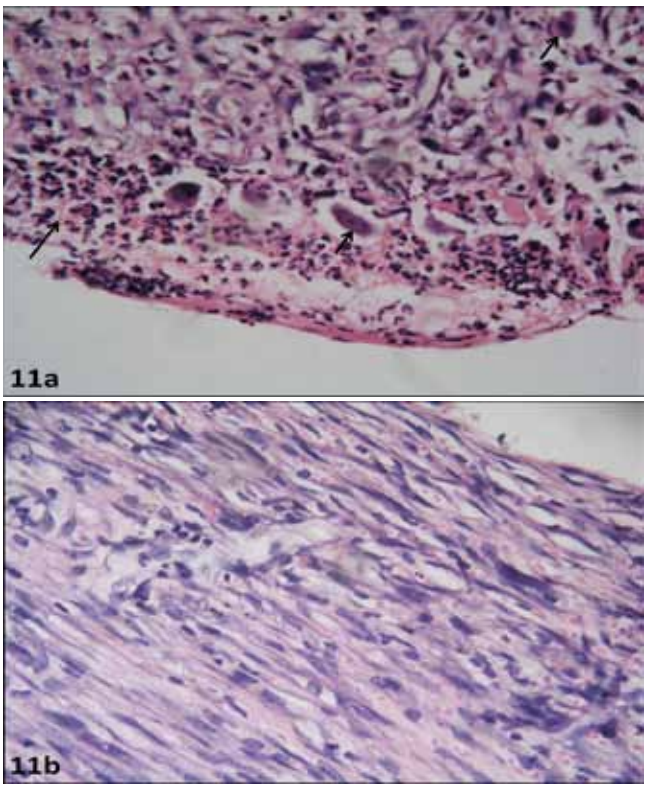


Figure 11. Photomicrographs of skin wounds 10 days after surgery from the control group (a) and glucosamine (GA)-treated group (b) showing the presence of degenerating inflammatory cells (long arrow), neutrophils, and macrophages (short arrows) in the control group and their near absence in the GA-treated group.

H&E, × 400.

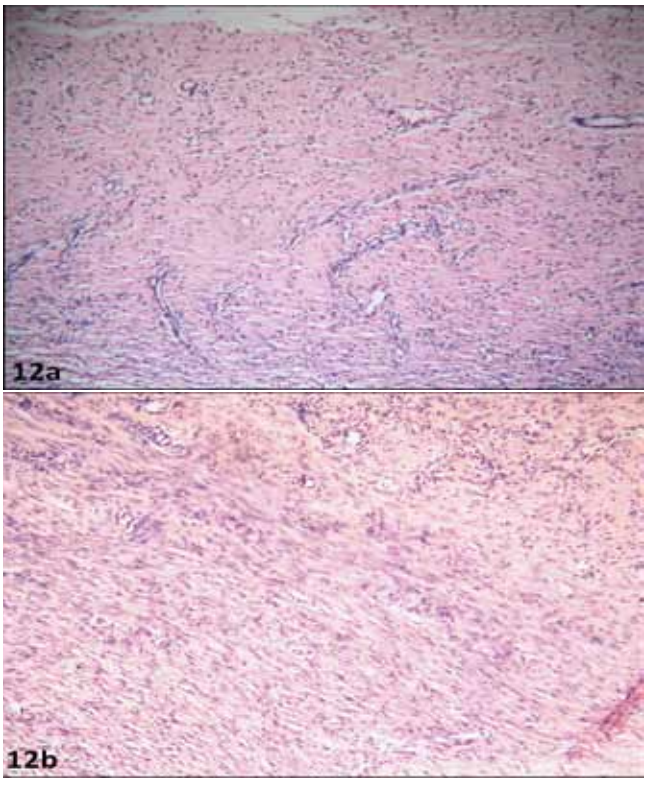


Figure 12. Photomicrographs of skin wounds 10 days after surgery from the control group (a) and glucosamine (GA)-treated group (b). The dermis in the GA-treated group is more dense with little spaces and the collagen fibers are more regularly arranged compared with the control group. The proliferating capillaries are apparently fewer in the GA-treated group.

H&E, × 100.

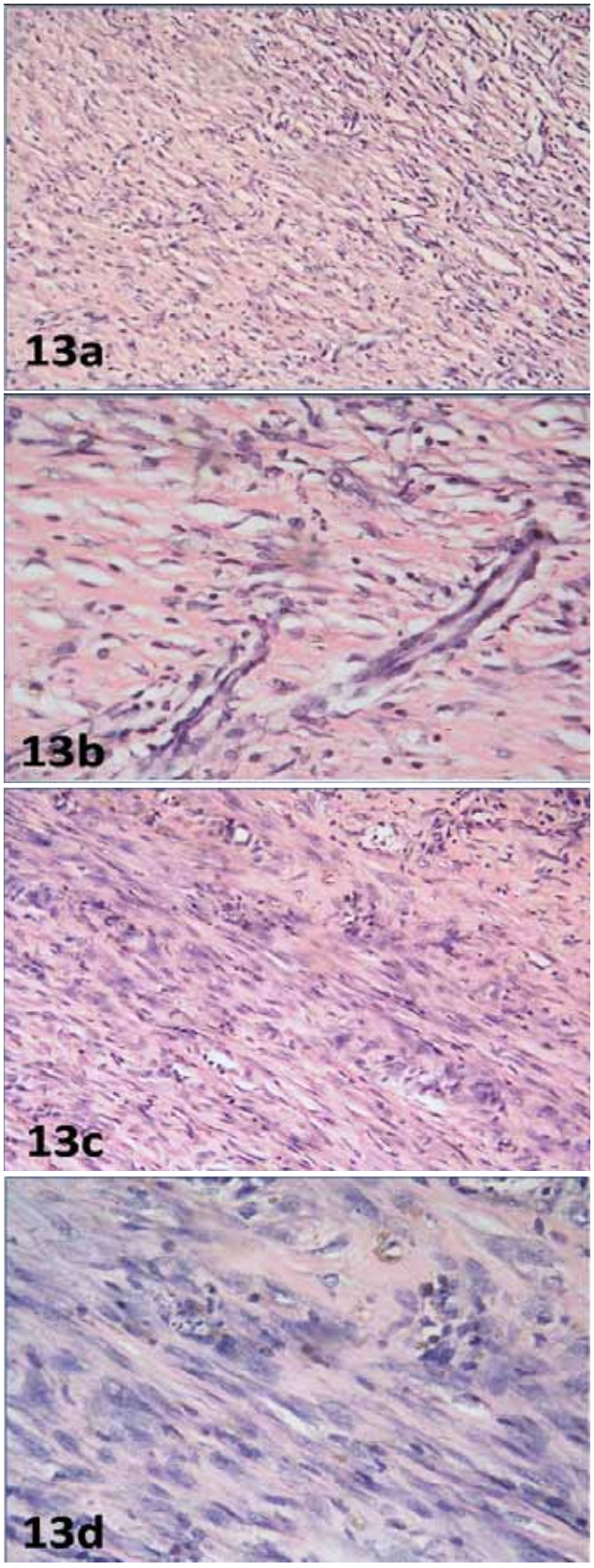


Figure 13. Photomicrographs of skin wounds 10 days after surgery from the control group (a, b) and glucosamine (GA)-treated group (c, d). The GA-treated group shows more fibroblasts, which are regularly arranged, increased in size, and more elongated. They also show more abundant deeply basophilic cytoplasm and larger nuclei compared with fibroblasts from the control group. The GA-treated group contains apparently more collagen fibers when compared with the control group.
H&E, (a, c) × 200; (b, d) × 400.

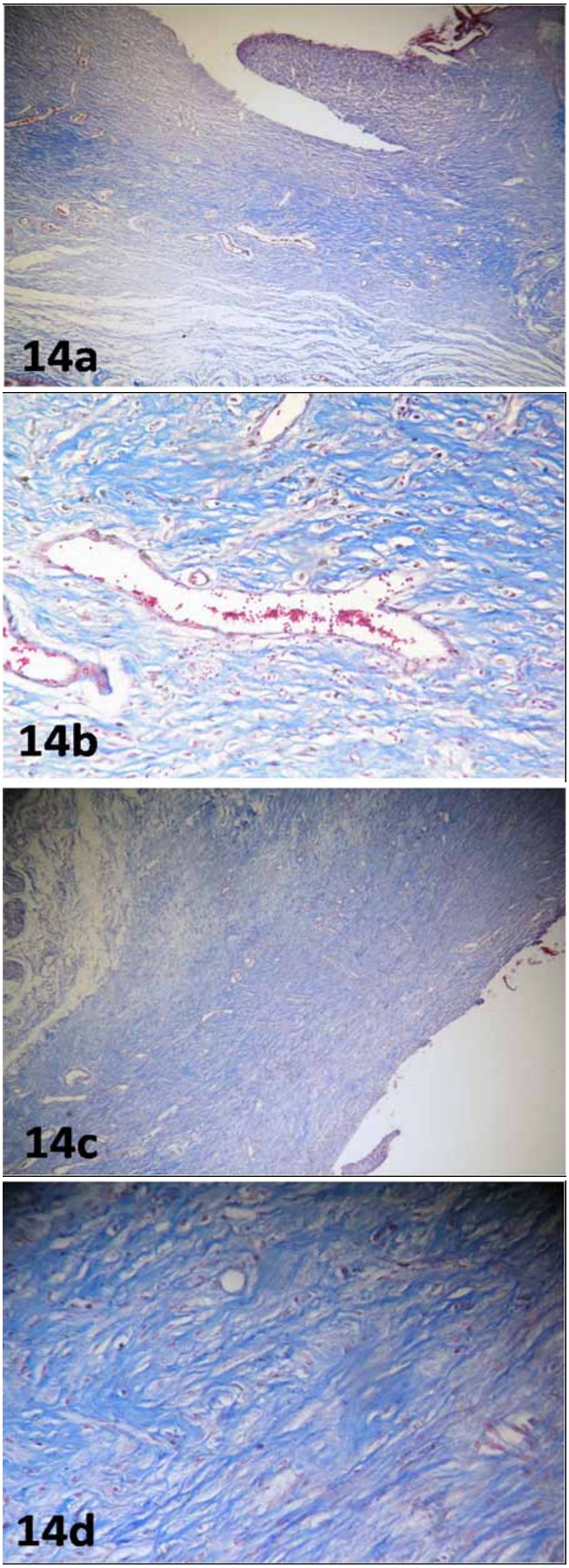


Figure 14. Photomicrographs of skin wounds 10 days after surgery from the control group (a, b) and glucosamine (GA)-treated group (c, d). More collagen fibers are present in the GA-treated group compared with the control group.
Mallory trichrome, (a, c) × 40; (b, d) × 200.

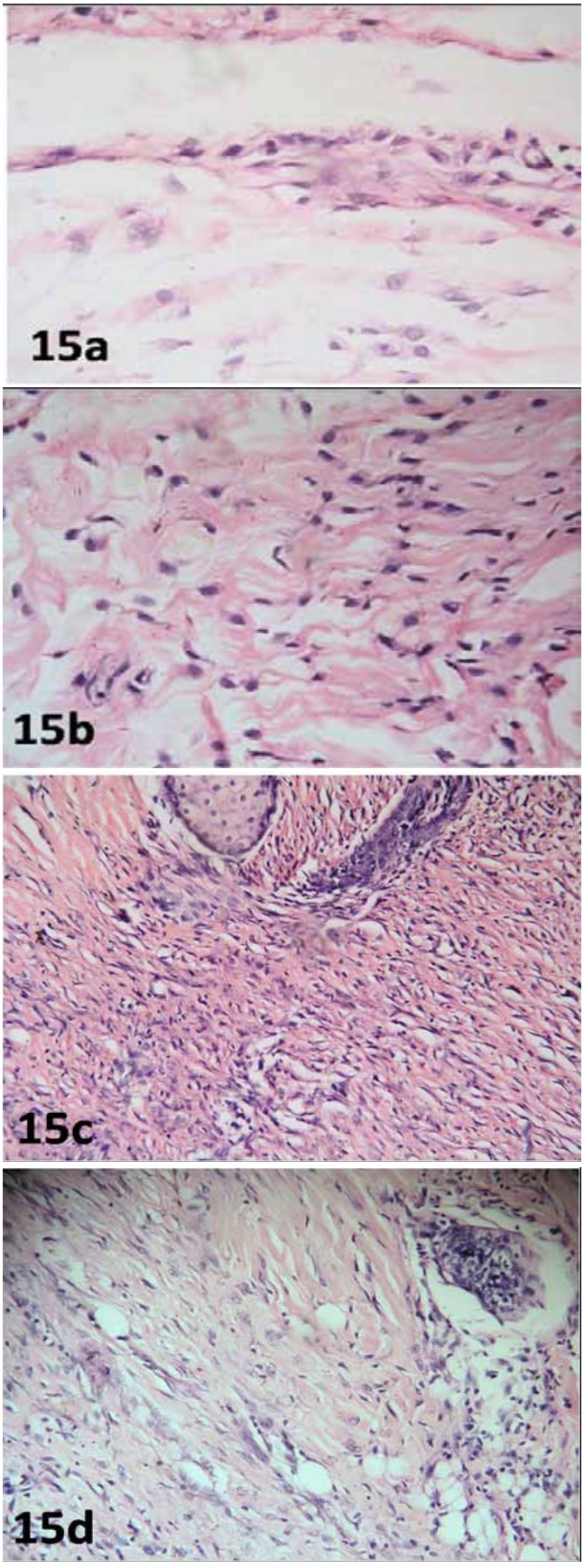


Figure 15. Photomicrographs of skin wounds 10 days after surgery from the control group (a, c) and glucosamine (GA)-treated group (b, d). The deep dermis (upper photos) and the dermis at the wound edges (lower photos) in the GA-treated animals show greater number of collagen bundles, which are more dense, thicker, and wavy when compared with the control group.

H&E, upper photos, × 400; lower photos × 200.

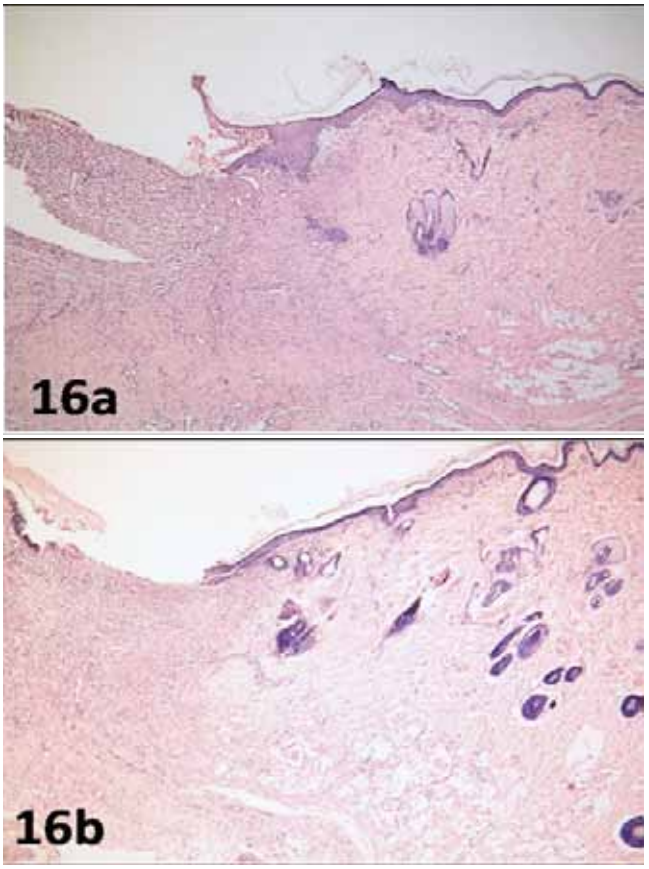


Figure 16. Photomicrographs of skin wounds 10 days after surgery from the control group (a) and glucosamine (GA)-treated group (b). Hair follicles and pilosebaceous units are present in the wound area in both groups. The control group has markedly less content of these structures compared with the GA-treated group.

H&E, × 40.

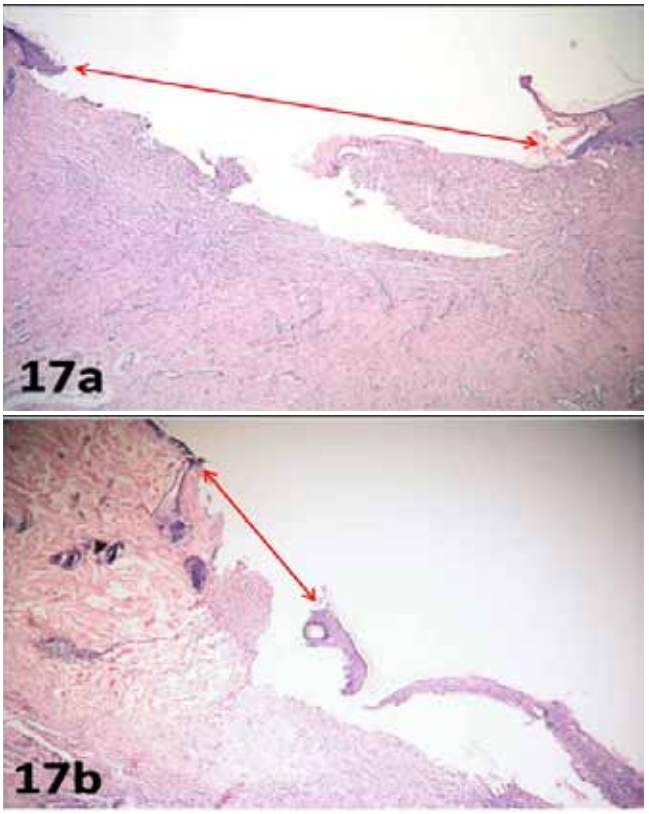


Figure 17. *(Continued)*

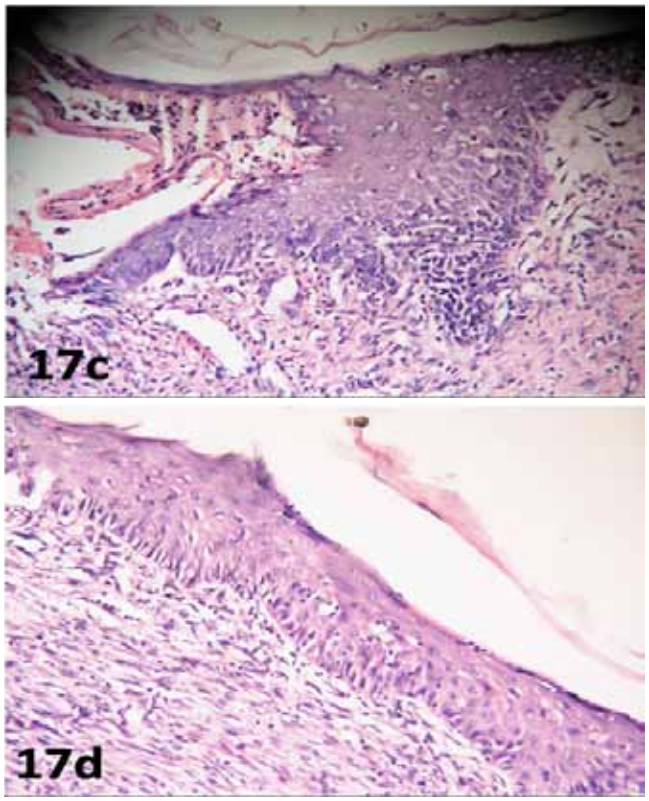


Figure 17. Photomicrographs of skin wounds 10 days after surgery from the control group (a, c) and glucosamine (GA)-treated group (b, d). The photos above show re-epithelilization, which is greater and nearly complete in the GA-treated group compared with the control group. The photos below show the epithelium, which is more hyperplastic and thicker in the control group than in the GA-treated group. The epithelium of the GA-treated group has a normal appearance and is uniform in thickness.
H&E, (a, b) × 40; (c, d) × 200.

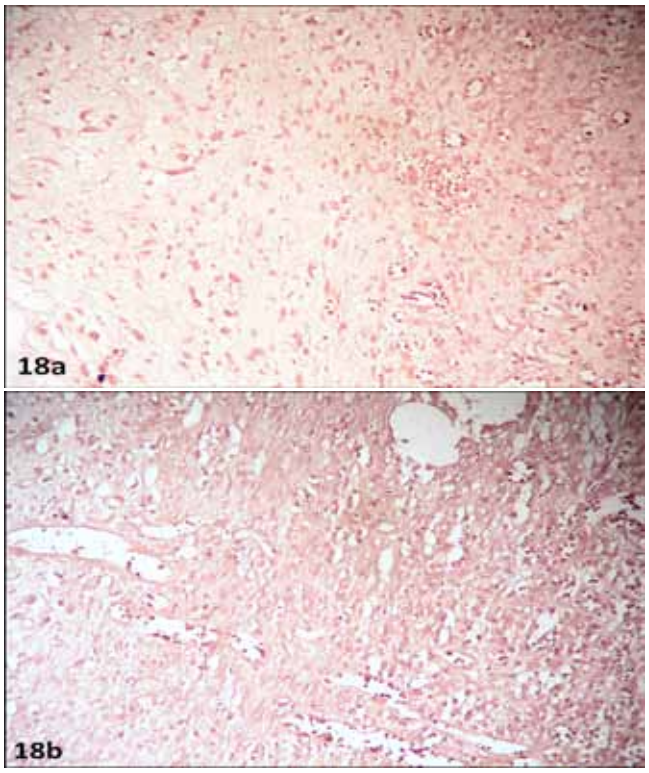


Figure 18. Photomicrographs of skin wounds 5 days after surgery from the control group (a) and glucosamine-treated group (b) showing no significant difference in elastic fiber content between the two groups.
Orcein stain, × 200.

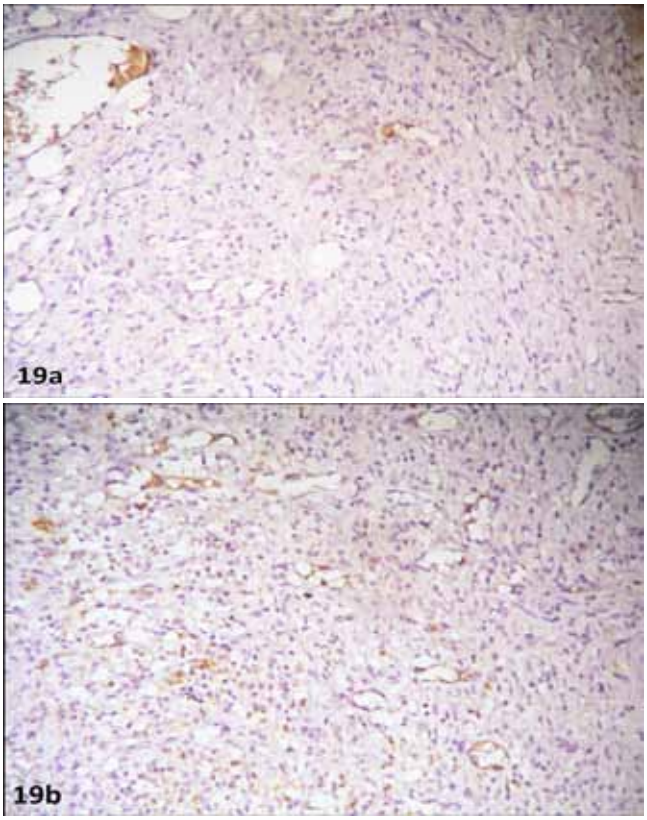


Figure 19. Photomicrographs of skin wounds 5 days after surgery from the control group (a) and glucosamine (GA)-treated group (b) showing increased expression of CD31 in the GA-treated group compared with its expression in the control group.
CD31 immunostaining, × 200.

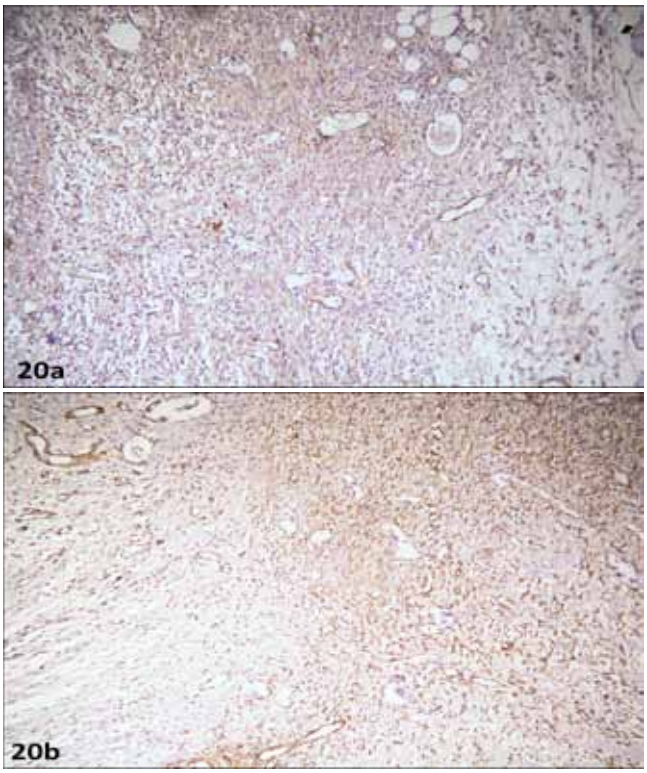
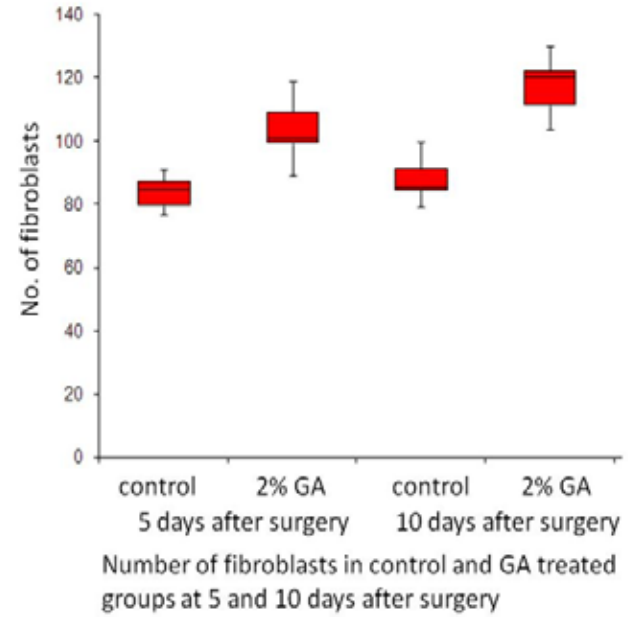
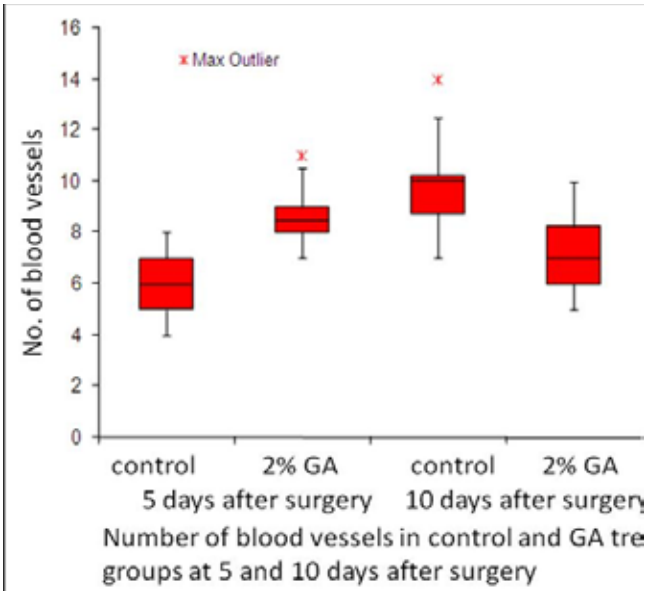


Figure 20. Photomicrographs of skin wounds 5 days after surgery from the control group (a) and glucosamine (GA)-treated group (b) showing increased expression of α smooth muscle actin (α-SMA) in the GA-treated group compared with its expression in the control group.
α-SMA immunostaining, × 200.





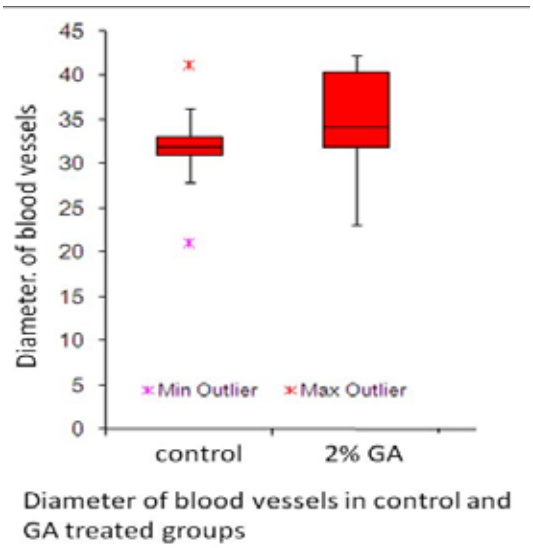
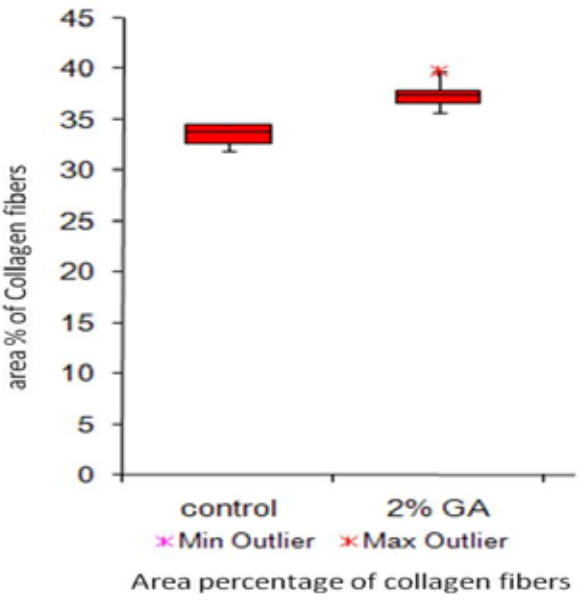
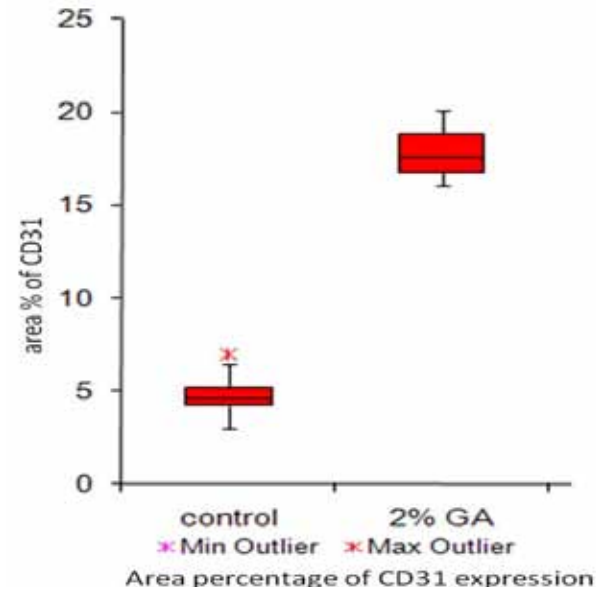


Figure 21. Box plots for the number of fibroblasts and the number and diameter of blood vessels in the control and experimental groups.





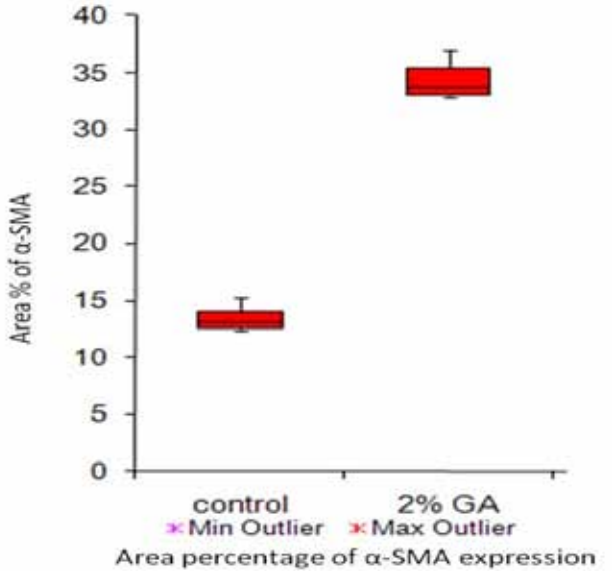


Figure 22. Box plots showing the area percentages of collagen fiber content, expression of CD31, and expression of α-SMA in the control and experimental groups.

Discussion

Skin wounds are encountered in daily life, in outpatient facilities and in hospital healthcare settings. Finding more beneficial agents to enhance and improve wound healing has always been a concern. In this study an excision skin wound model was used for evaluation of the effect of topical application of 2% GA on the wound healing process in rats.

Wound healing is a systematic process starting at the moment of injury, with an inflammatory phase including homeostasis and inflammation, a proliferation phase with granulation tissue formation, tissue contraction, and epithelialization, and finally a remodeling phase, which determines the strength and appearance of the healed tissue [23,24].

In the present study, inflammatory cell infiltration was noticed in the control group as well as in the GA-treated group. However, inflammatory cell infiltration at day 5 after surgery was more evident in the GA-treated group compared with the control group. This was due to the use of the antiseptic bovidone iodine in the control group as a regular dressing, which helped reduce inflammatory cells. In contrast, at day 10 after surgery, inflammatory cell infiltration had mostly ceased in the wounds treated with GA but was still seen in wounds from the control group. This may support the hypothesis that GA possesses an immune-modulatory effect reducing inflammation as reported previously by Yan *et al.* [25]. In addition, Kim *et al.* [26] found that GA had an immune-modulatory influence in atopic dermatitis-like skin lesions by reducing the release of the cytokines interleukin-4 (IL-4) and IL-5 by T-helper 2 cells, improving the healing process of these lesions. Other studies suggested that GA has an anti-inflammatory effect [27,28]. This anti-inflammatory ability of GA was through the downregulation of COX-2 and MMP-13 expression by human skin fibroblasts [27]. In contrast to our findings, other previous studies demonstrated that chitosan, from which GA was derived, topically enhanced wound infiltration by neutrophils (PMN) and macrophages [29]. In addition, it has also been demonstrated that the inflammatory cytokines involved in the process of wound healing, such as tumor necrosis factor-α, IL-1 and IL-8, and transforming growth factor-β1, were facilitated by chitosan [30]. These parameters, however, have not been estimated in the present study.

The histological results of this study showed comparatively more collagen deposition in the GA-treated group compared with the control group. Ueno *et al.* [29] demonstrated that chitosan results in improved granulation tissue formation and increased collagen type III synthesis. In the present study, in the control group there were wider wound areas, more inflammatory cells, and a lesser degree of vascularization in the early stage of wound healing. These findings indicated that the healing process was incomplete. Similar findings have been reported by Ashkani-Esfahani *et al.* [31].

H&E-stained sections on day 10 after injury showed that the GA-treated wounds had smaller wound size compared with the control group. The extent of epithelialization, fibroblast proliferation, collagen deposition, and neovascularization was enhanced by GA treatment. Histological analysis showed that the area percentage of collagen fibers in the two groups was significantly different. Similar results have been reported by other authors [32]. They stated that improved blood supply, granulation tissue formation, and re-epithelialization of wounds were promoted by using chitosan mesh membrane as a wound dressing.

The promoted angiogenesis observed in the present study (in wounds treated with GA) was statistically higher compared with that in the control group. However, Ashkani-Esfahani *et al.* [31] found that the difference in angiogenesis between the GA-treated and control groups was not statistically significant. In accordance with the results of the present study, a previous study demonstrated that chitosan indirectly promotes angiogenesis through complement activation and secretion of IL-8, a neutrophil chemoattractant, by fibroblasts [33].

In the present study, GA treatment stimulated fibroblast proliferation and activation. This was obvious from the increased number of fibroblasts and the associated increase in their size and the deeply stained cytoplasm. Fibroblast proliferation was indirectly enhanced by chitin and chitosan and by their derivatives in previous studies [10,29]. In contrast to these findings, Mori *et al.* [20] demonstrated that chitin and its derivative GA reduces the rate of fibroblast proliferation. This may be explained by the difference in the used GA concentrations in these studies. Mori *et al.* [20] used a high concentration of GA compared with the concentration used in the present study. The present study also demonstrated an earlier regular arrangement of fibroblasts and collagen in both fibers and bundles. This was observed only in wounds from the GA-treated group at day 10 after surgery.

In order to evaluate the enhancement of wound healing by GA, it was important to examine elastic fiber formation and expression of some important markers involved in wound healing, such as CD31 and α-SMA, at an early stage in the healing process rather than toward its end, near wound closure. Enhancement of expression of these markers at an early stage of wound healing will be an indication of the accelerated healing process. The present study detected a significant and early increase in the expression of both CD31 and α-SMA denoting a beneficial effect of GA on the wound healing process.

The present study demonstrated that the wound contracting ability under treatment with 2% GA was significantly greater than that of controls. This has been estimated from the increased expression of α-SMA and consequently the increased content of contracting myofibroblasts. Similar findings have been demonstrated by Ashkani-Esfahani *et al.* [31]. In the present study, over time, there was early fibroblast proliferation, more neovascularization, and early regular arrangement of

fibroblasts and collagen fibers at day 5 after surgery in the GA-treated group. Meanwhile, the number of blood vessels was reduced in the GA-treated group at day 10 after surgery and increased in the control group. This indicates that *2%* GA treatment accelerates the wound healing process.

The beneficial effects of GA on wound healing may be due to its immune modulating ability that results in reduced inflammation, neutrophil recruitment, and, consequently, reduction in oxidative stress. Hwang *et al.* [34] showed that GA inhibited the production of reactive oxygen species in immortalized human skin fibroblasts (HS68) exposed to UVB irradiation. This antioxidative activity of GA was beneficial for wound healing and was also demonstrated by other investigators [25,35].

Conclusions

This study demonstrates that GA significantly enhances wound healing and increases the wound closure rate by intensifying collagen synthesis, hair follicle production, fibroblast proliferation, and re-epithelialization. The topical application of *2%* GA had an immune modulating effect and enhanced new vessel formation early in the process of wound healing. Overall, the wound healing process, wound contracting ability, and wound remodeling were accelerated by the topical application of *2%* GA. Further studies may be required to determine the underlying mechanisms through which GA in a topical form affects wound healing.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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الملخص العربى

دراسة هستولوجية لتثير التناول الموضعي للجلوكوزامين علي التئام الجروح في الفئران

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المقدمة: ان الجروح شائعة في الممارسة الاكلينيكية في العيادات الخارجية و في المستشفيات. و تعتبر ردائة أو طول عملية التئام الجروح مشكلة اكلينيكية تؤدي الي اقامة طويلة بالمستشفي و زيادة في التكلفة الاقتصادية. ان الجلوكوزامين هو مركب مستخلص من الكايتين أو الشيتوزان و لقد شوهد أن له تاثيرا منشطا علي تكوين الأنسجة و كذلك صناعة حمض الهياليورونيك. و لكن هذه الدراسات كانت بالاساس عن طريق التناول العام للعقار و قلة عن طريق التناول الموضعي. و تهدف هذه الدراسة الي تحديد تاثير التناول الموضعي للجلوكوزامين علي عملية التئام الجروح في نموزج من الفئران لجرح جلدي كامل السمك.

طريقة البحث: لقد أجريت هذه الدراسة علي 24 من ذكور الفئران البيضاء البالغة و التي تم تقسيمها الي مجموعتين: المجموعة الضابطة (المجموعة الأولي،10 فئران) ومجموعة التجارب (المجموعة الثانية،14 فئران). تم عمل جرح بمساحة 1سم2 علي ظهر كل فئر من المجموعتين. ثم تم علاج الجروح في المجموعة الضابطة بالطريقة العادية بالاضافة الي استخدام قاعدة الجل بدون عقار. و تم علاج مجموعة التجارب بواسطة جلوكوزامين2% في صورة جل. و استمر العلاج يوميا لمدة 10 ايام. ثم تم دراسة معدل التئام الجروح-صناعة الكولاجين-تكوين الأوعية الدموية- نمو خلايا الأرومة اليفية دراسة هستولوجية و هستوكميائية مناعية و قياسية.

النتائج: لقد أدي استخدام الكلوكوزامين الي زيادة كبيرة و سرعة في معدل التئام الجروح و نمو خلايا الأرومة اليفية. كما زادت صناعة الكولاجين و نمو بصيلات الشعر مقارنة بالمجموعة الضابطة. و لقد حسن الجلوكوزامين تكون الاوعية الدموية الجديدة في مكان الجرح. و بصورة عامة فان التناول الموضعي للجلوكوزامين 2% قد سرع من عملية التئام و انقباض الجروح.

الاستنتاج: تشير نتائج هذه الدراسة الي أن الجلوكزامين له القدرة التي تؤهله للاستخدام الموضعي في علاج جروح الجلد ولكن من الضروري اجراء دراسات اضافية لتقييم طرق عمله و مضاعفاته الاكلينيكية.