folia medica

Folia Medica 67(1)
DOI: 10.3897/folmed.67.e130272

Original Article

Enhancing wound healing with synthetic hyaluronic acid injection in sutured incisions on BALB/c mice

Elham Aryanfar[1], Mahdi Ghoncheh[2], Mohammad Afshar[3,4], Mahboobe Chahkandi[5], Elahe Allahyari[6], Mehri Shadi[3]

1 *Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran*
2 *Department of Surgery, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran*
3 *Department of Anatomy, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran*
4 *Medical Toxicology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran*
5 *Department of Pathology, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran*
6 *Department of Epidemiology and Biostatistics, Faculty of Health, Birjand University of Medical Sciences, Birjand, Iran*

Corresponding authors: Mohammad Afshar, Department of Anatomy, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran; Email: afshar_md@yahoo.com; Tel.: +98 56323815122.
Mahdi Ghoncheh, Department of Surgery, Faculty of Medicine, Birjand University of Medical Sciences, Birjand, Iran; Email: mahdighoncheh@gmail.com; Tel.: +98 5632381504.

Received: 22 June 2024 ♦ **Accepted:** 19 January 2025 ♦ **Published:** 24 February 2025

Citation: Aryanfar E, Ghoncheh M, Afshar M, Chahkandi M, Allahyari E, Shadi M. Enhancing wound healing with synthetic hyaluronic acid injection in sutured incisions on BALB/c mice. Folia Med (Plovdiv) 2025;67(1): e130272. doi: 10.3897/folmed.67.e130272.

Abstract

Introduction: Hyaluronic acid has been shown to possess notable properties in wound healing, skin regeneration, and anti-inflammatory effects. The formation of scar tissue is a common and unintended phenomenon during the wound-healing process, potentially leading to diverse consequences for individuals.

Aim: The objective of this study was to investigate the effect of injecting synthetic hyaluronic acid on the wound healing process in BALB/c mice subjected to suture wounding.

Materials and methods: In this experimental study, 18 adult male BALB/c mice were utilized and distributed into two groups (9 mice each): an experimental group and a control group. A full-thickness incision wound, one centimeter in length, was created in the skin of the back neck area for both groups. After being wounded, all wounds were sutured. However, only mice in the experimental group had 0.05 mL of hyaluronic acid on both sides of the wound. On days 7, 14, and 21 following the injection of hyaluronic acid, three mice from each group were anesthetized, and skin samples were collected for histological evaluation. After staining the tissue slides with hematoxylin-eosin and Masson's trichrome, a comprehensive analysis was conducted by a pathologist to quantify key histological parameters, including the number of fibroblasts, collagen fiber density, granulation area, and epithelium thickness.

Results: The experimental group demonstrated an increased number of fibroblasts, higher collagen density, and a more organized arrangement of collagen fibers when compared to the control group. On day 21, the experimental group exhibited a significantly higher average thickness of the epithelium and greater hair growth when compared to the control group.

Conclusion: Our findings suggest that hyaluronic acid exerts a positive influence on the healing process of sutured incisions, positioning it as a promising candidate for preventing the formation of abnormal scars at the site.

Keywords

hyaluronic acid, mice, scar, wound healing

Introduction

The formation of scars has been shown to have a significant impact on patients' physical, social, and psychological well-being, often diminishing their quality of life and imposing substantial costs on healthcare systems worldwide. Despite the advancements in surgical techniques and wound management that have led to a reduction in the prevalence of severe scarring, the prevention and treatment of scars continue to pose significant challenges in clinical practice. Therefore, understanding the physiology of wound healing and scarring is essential for minimizing surgical scar formation.[1]

The wound healing process comprises four phases: hemostasis, inflammation, proliferation, and maturation.[2] Hemostasis initiates within seconds to minutes following the initial onset of bleeding. Activated platelets release granules that initiate both external and internal coagulation cascades.[3] In the second phase, known as inflammation, white blood cells such as neutrophils and lymphocytes along with macrophages, a crucial phagocytic component, migrate to the wound site. Their primary roles include phagocytosis and infection prevention.[4] The release of growth factors, such as Platelet-Derived Growth Factor (PDGF), Vascular Endothelial Growth Factor (VEGF), and other cytokines, is a hallmark of activated macrophages. These factors are critical for the formation of granulation tissue and the proliferation of fibroblasts.[5] In the third phase, recognized as the proliferation phase, new blood vessels develop, fibroblasts proliferate, and collagen is synthesized. Together, these processes contribute to the formation of granulation tis sue, facilitating the development of epithelium.[6] During the remodeling phase, the extracellular matrix (ECM) self-organizes to generate new functional tissue and mature collagen fibers. In wound healing, the two primary collagen types are collagen I and III. Collagen type III, a fibril-forming collagen, holds crucial significance in fibrillogenesis, influencing the fibril diameter of collagen I.[7]

Scar formation occurs as a natural consequence of the wound-healing process, often triggered by mechanical factors such as tissue retraction during surgery or suture application.[8] The resulting scars, marked by contraction and stiffness, can lead to functional loss, restricted mobility, itching, and pain. Although scars are composed of the same ECM molecules that replace tissue, the spatial relationships between tissues in scars differ from those in natural tissue. [9] In recent times, significant strides have been made in comprehending the mechanisms that impact scarring and fibrosis as well as in their prevention.[1]

Hyaluronic acid (HA) possesses unique properties that make it an exceptionally versatile biomolecule. As a glycosaminoglycan without sulfate bonds, hyaluronan is typically formed from multiple subunits, each composed of repeating disaccharide units of D-glucuronic acid and N-acetylglucosamine, carrying negative charges. This unique structure enables effective water absorption and retention.[10] HA is a vital component of the natural extracellular matrix found in the skin, joints, eyes, and various organs and tissues.[11] Furthermore, it plays a crucial role in all stages of wound healing, from inflammation to remodeling. HA contributes to cellular processes and events within the ECM during each phase of wound healing. Numerous studies have demonstrated that CD44 is a cell surface receptor abundantly expressed on keratinocytes, the predominant cell type in the epidermis. The interaction between hyaluronic acid (HA) and CD44 activates several intracellular signaling pathways that regulate essential cellular functions, including proliferation and migration.[12,13] Furthermore, during the remodeling process, a matrix enriched with HA can influence collagen deposition and contribute to reduced scarring. The effective treatment of wounds and the proper management of sutures continue to pose significant challenges in contemporary clinical medicine.[12] As a biologically active molecule that regulates tissue healing, HA offers a safe and effective approach to skin regeneration.[14]

Topical applications of HA, particularly in cream formulations, have been extensively studied for their efficacy in enhancing wound healing. For example, a 0.2% HA-based cream has been shown to significantly reduce wound size and alleviate pain in patients with chronic venous or mixed etiology wounds.[14] However, there are limited studies exploring the use of HA in injectable forms.

Aim

This study aimed to investigate the effects of hyaluronic acid injections at suture wound sites to enhance wound healing in BALB/c mice.

Materials and Methods

Preparation of experimental animals

For this experimental study, 18 male laboratory mice of the BALB/c strain aged 8-10 weeks were used. The animals were obtained from the Experimental Medical Research Centre of Birjand University of Medical Sciences, Birjand, Iran. Ethical standards for work with laboratory animals were applied throughout the work. The study protocol was approved by the ethics committee of Birjand University of Medical Sciences (IR.BUMS.REC.1400.364). The mice were housed in clean individual cages with free access to food and water. A 12-hour light-dark cycle was maintained, and the temperature was set at 22-23 degrees Celsius.

Indicators measuring method

Initially, 18 healthy male BALB/c mice were kept in an appropriate environment. To induce a skin wound, the animals were anesthetized by intraperitoneal injection of a combination of ketamine and xylazine (100:10 mg/kg). The hair on the dorsal neck was shaved, and a full-thickness skin wound, encompassing the dermis and hypodermis, was created with a scalpel under sterile conditions and in accordance with surgical principles. The mice were divided into experimental and control groups, each containing 9 mice. A one-centimeter incision was made on the back of all mice and subsequently sutured. In the experimental group, a single dose of 0.05 ml (equivalent to 1150 μg) of hyaluronic acid (REVOFIL Plus, Korea) was injected on both sides of the wound immediately after suturing. The control group was kept without the injection of any substances at the site of the suture. Finally, photographs were taken from the wound site along with length measurements. On days 7, 14, and 21, three mice from each group were anesthetized, and skin samples were collected for histological evaluation.

Histological analysis

Tissue samples were fixed in a 10% formaldehyde solution, dehydrated with ascending concentrations of alcohol, and cleared in xylene during tissue processing stages. Subsequently, they were embedded and blocked in paraffin, and 5 μm sections were cut using a microtome. Finally, the sections were stained with hematoxylin and eosin (H&E) and subjected to the trichrome Mason technique to evaluate collagen synthesis and arrangement. After staining, slides were prepared for interpretation, and ultimately, 12 slides from each group were randomly examined by a pathologist to assess tissue changes, which included evaluating the number of fibroblasts, collagen density, epithelial thickness, and granulation tissue extension. The findings were then reported accordingly. Slides were imaged using a Euromex-CMEX-10 camera at magnifications of 10, 40, and 100. For histological analysis, the images were processed with Image J analysis software.

Statistical studies

Quantitative parameters, including the number of fibroblasts, collagen fiber thickness, epithelialization, and the area of granulation, were assessed using Image J software. The quantitative data were expressed as mean ± standard deviation (SD). One-way repeated-measure analysis of variance, Bonferroni test, and independent samples t-test were applied for data analysis. Statistical analysis was performed using SPSS version 26. A statistical significance level of 0.05 or lower was considered significant.

Results

In this study, 18 adult male BALB/c mice were divided into two groups: the experimental group, which received hyaluronic acid injections at the suture site, and the control group, which did not receive any injections at the suture site. Histological samples were collected on days 7, 14, and 21, followed by the preparation of slides for microscopic analysis. The images obtained were assessed for various parameters, including the number of fibroblasts, collagen fiber density, epithelialization, hair regrowth at the wound site, and the area of granulation tissue in both the experimental and control groups.

Number of fibroblasts

The results of this study revealed that on days 7 and 14 following the injection of artificial hyaluronic acid, the fibroblast count in the experimental group was significantly higher than in the control group **(Table 1) (Fig. 1)**. However, by day 21 post-injection, the mean fibroblast count did not differ significantly between the two groups.

Table 1. Microscopic data of wounds in the experimental group and control group on different days of study

Parameter	Day	Hyaluronic acid	Control	T (*p* value)
Number of fibroblast cell	Day 7	32.17±6.69	26.33±5.66	**2.31** (0.03)
	Day 14	28.67±6.71	21.00±3.33	**3.55** (0.003)
	Day 21	10.83±4.59	7.42±3.42	2.07 (0.051)
Collagen fiber density (OD)	Day 14	0.20±0.03	0.16±0.03	**3.13** (0.005)
	Day 21*	0.25 (0.24-0.29)	0.21 (0.18-0.25)	**19** (<0.001)
The area of granulation (μm2)	Day 7	12121.77±4583.86	9596.19±2895.20	1.61 (0.12)
	Day 14**	7138.97 (6158.03-9937.14)	5012.97 (2913.77-8425.39)	**42.00** (0.09)
	Day 21	4824.68±815.22	3252.32±789.23	**4.80** (<0.001)
Epithelialization (μm)	Day 7	4.63±0.97	4.86±0.88	−0.62 (0.54)
	Day 14	5.87±1.12	5.38±1.51	0.90 (0.38)
	Day 21	6.27±0.66	5.47±1.12	**2.14** (0.04)

Bold numbers indicate significant relationship at 0.05 significance level; * distribution of data was not normal in hyaluronic acid group; **: distribution of data was not normal in control group; OD: optical density.

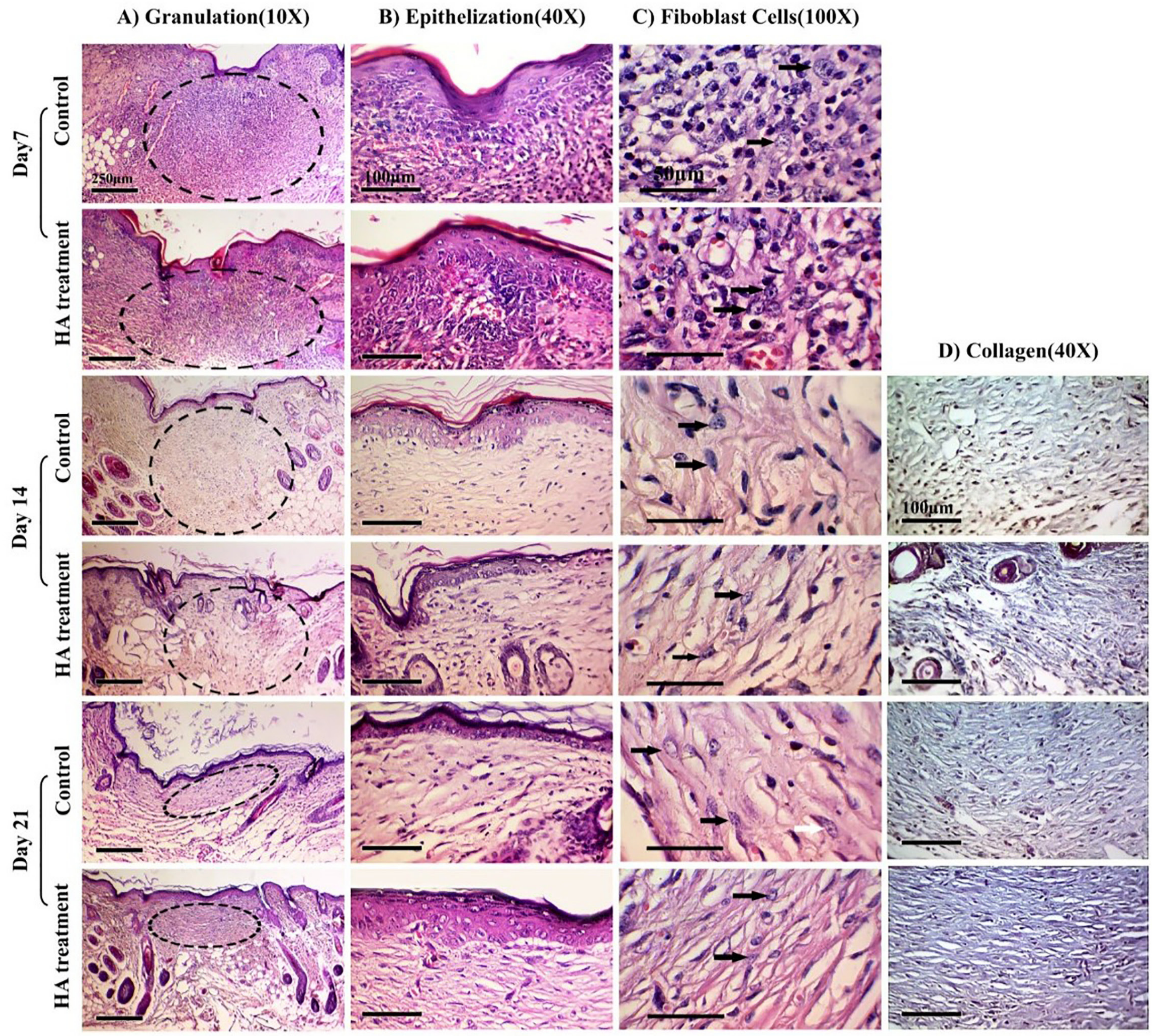

Figure 1. Comparison of the granulation area (column **A**), thickness of epithelialization (column **B**), number of fibroblast cells (column **C**), and amount of synthesis and arrangement of collagen fibers (column **D**) in the control and treatment groups at 7, 14, and 21 days.

Density of collagen fibers

The results of this study showed a significantly higher collagen fiber density in the intervention group compared to the control group on days 14 and 21 post-injection. Furthermore, the collagen fibers in the experimental group demonstrated improved organization and arrangement at the wound site in the experimental group on both days 14 and 21 than in the control group **(Table 1) (Fig. 1)**.

Granulation tissue

The results of this study indicated no significant difference in area of granulation tissue on day 7 after injection. However, on days 14 and 21 after injection, the granulation tissue area in the experimental group was significantly greater **(Table 1) (Fig. 1)**.

Epithelialization

The results of this study showed no significant difference in epithelial thickness between the two groups on days 7 and 14 post-injection. However, on day 21 post-injection, the epithelial thickness in the experimental group was significantly greater than that in the control group **(Table 1) (Fig. 1)**. Furthermore, microscopic examination revealed a higher number of hair follicles at the wound site in the experimental group compared to the control group on days 14 and 21.

Discussion

Our study demonstrated the positive effects of hyaluronic acid injections on wound healing in suture incisions. The hyaluronic acid-treated group exhibited significantly superior outcomes compared to controls, including enhanced reconstruction and organization of collagen fibers, increased fibroblast proliferation, larger areas of granulation tissue, and a higher rate of epithelialization. These findings underscore the multifaceted role of hyaluronic acid in facilitating various stages of the wound healing cascade.

Fibroblast accumulation and collagen fiber proliferation

In our study, the number of fibroblasts was significantly higher in the experimental group compared to the control group on days 7 and 14 after injection. This finding aligns with previous research demonstrating beneficial effect of hyaluronic acid on fibroblast proliferation. For instance, Hameed et al. investigated the role of hyaluronic acid in the progression of wound healing; their study reported fibroblast growth factor levels in the tissue increased locally at day 21.[15] In a systematic review conducted by Price et al., the effects of hyaluronic acid on skin regeneration and wound healing were examined. The study concluded that HA, due to its long-chain molecular structure, stimulates fibroblast proliferation both in vivo and in vitro conditions. [16] Recent studies have also confirmed the beneficial effect of hyaluronic acid on fibroblast phenotype and proliferation.[17,18]

For instance, research has shown that HA facilitates transforming growth factor-β1 (TGF-β1)-dependent fibroblast proliferation, highlighting its role in wound healing processes.[19,20] Additionally, in vitro studies have demonstrated that the addition of HA to human dermal fibroblast cultures increases proliferation and synthesis of collagen type I (COL1A1), indicating its potential in enhancing skin regeneration.[21]

A recent study published in *Scientific Reports* examined the combined effects of green tea and HA gel on fibroblast activation and wound healing. The research demonstrated that HA plays a significant role in tissue repair mechanisms by enhancing fibroblast proliferation and migration. The study concluded that the synergistic application of green tea and HA gel can effectively promote wound healing, highlighting HA's involvement in tissue repair processes.[22]

These findings underscore the multifaceted role of HA in modulating fibroblast activity, thereby contributing to improved wound healing and tissue regeneration.

On the other hand, some findings align with existing literature indicating that HA plays a crucial role in ECM remodeling and collagen fiber organization. For instance, studies have shown that HA can modulate the deposition of fibronectin and collagen, essential components of the ECM, during myofibroblast formation.[14]

Furthermore, the study demonstrated an increase in the synthesis of collagen type I as a result of fibroblast proliferation.[17] Existing literature highlights a notable impediment in the accumulation of a disorganized collagen matrix within fetal wounds, resulting in a distinctive scarless healing process. This favorable outcome is attributed to the heightened and prolonged presence of HA in fetal wounds compared to adult wounds. Consequently, interventions aimed at sustaining a matrix rich in HA represent promising strategies for achieving scarless repair and regenerative healing.[23]

Our study further demonstrated that direct injection of HA in the experimental group resulted in a significant increase in collagen fiber density and improved organization compared to the control group.

Granulation tissue

Our findings demonstrated a significant increase in the granulation tissue area in the experimental group on days 14 and 21 post-injection. Similarly, Hameed et al. reported that the application of HA exosomes in wound models stimulated fibroblasts to produce higher levels of angiogenic growth factors, such as VEGF, thereby enhancing wound healing.[15] Additionally, the hygroscopic properties of HA were found to play a crucial role in maintaining hydration and supporting the structural integrity of the extracellular matrix (ECM) during the wound healing process. This support, in turn, enhanced cell migration, adhesion, and proliferation within the granulation tissue.[24] Moreover, evidence indicates that HA possesses the ability to modulate the inflammatory response by inhibiting inflammatory proteases, thereby promoting improved tissue granulation and stabilization.[25]

Epithelialization and hair growth

The results of our study revealed a significantly greater epithelial thickness in the experimental group compared to the control group on day 21 post-injection. Similarly, Kamdem et al., in their investigation of using HA dressings for second-degree burns in children, demonstrated that epithelialization occurred in 96.3% of the burn wounds studied within an average period of 14 days after the procedure.[26] Additionally, Leite et al. demonstrated the efficacy of 0.2% hyaluronic acid in improving skin abrasions in rats. Their study revealed that on days 7 and 14, the HA group had a higher rate of epithelialization compared to other groups, and with all lesions achieving complete re-epithelialization.[25]

Our research findings showed that the experimental group had a significantly greater number of hair follicles on days 14 and 21 post-injection. In a study by Kim et al. on the impact of hyaluronic acid microsponges on minoxidil absorption, it was demonstrated that hyaluronic acid promotes the proliferation, migration, and accumulation of dermal papilla cells, thereby contributing to hair growth. The topical application of hyaluronic acid in mice with chemically induced alopecia led to a reduction of hair loss.[27] Furthermore, the application of hyaluronic acid was found to stimulate hair growth around the wounds.[15]

Conclusion

This investigation highlights the beneficial impact of HA on the wound healing process in the context of suture incisions, significantly accelerating overall recovery. The direct administration of synthetic HA at the wound site resulted in heightened proliferation of fibroblasts and granulation cells. Furthermore, discernible enhancements were observed in epithelialization and hair regrowth at the wound site compared to the control group. The reconstruction and organization of collagen fibers demonstrated marked superiority in the HA injection group as opposed to the control group. These findings collectively emphasize the multifaceted positive influence of HA on the complex dynamics of the wound healing process.

Competing Interests

The authors have declared that no competing interests exist.

Acknowledgments and funding

The authors express their gratitude to the Deputy for Research at Birjand University of Medical Sciences (BUMS) for providing financial support and extend appreciation to the animal house staff in the Medical Faculty for their valuable assistance. Special thanks are extended to Mr. Hamed Shoorei for his insightful discussions and valuable suggestions, which greatly contributed to the development of this research.

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