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Role of Hyaluronic Acid in Modulating Wound Healing Progression

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Abstract

Background: Wound is the damage of the cellular and functional continuity of any living tissues. The wound healing process is a complex, multistage, and involve many changes and development steps. Management of wounds is frequently encountered with different problems related to their phases and conditions.

Objective: The aim of this study was to evaluate the role of hyaluronic acid in modulating the wound healing process through the evaluation of cytokines levels and histological changes.

Methodology: This experimental study was carried on animal models using 24 male mice aged 8-16 weeks with a weight of (22 – 32 g). 12 male mice taking placebo treatment, and 12 male mice treated with hyaluronic acid. Blood and tissue samples were collected and wound lengths were measured after 3,7,14, and 21 days of treatment. IL 2, VEGF, TNFα, bFGF were evaluated from serum and tissue using the ELISA technique.

Results: There was an improvement in healing quality in HA treated group with complete, scarless healing after 14 days of treatment. Cytokines and growth factors measured in this study showed wide variation and modulation between HA treated and untreated wounds.

Conclusion

Hyaluronic acid enhances wound healing via multiple processes. It modulates inflammation, promotes angiogenesis and re-epithelization, and reduce scar tissue formation.

Keywords: hyaluronic acid, wound healing, IL 2, VEGF, TNFα, bFGF.

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Introduction

The skin is the largest human organ [1] . Its main function is the protection against damage to internal tissues from pressure, radiation, chemicals and other threats [2] ' [3]. It also regulates the temperature and the amount of water released into the environment [4]. The skin is a three layers structure, which are the epidermis, the dermis, and the subcutaneous tissues [3] .

Due to its position and role as a protective layer, the skin is the most common site of injury or wounds. Wound is the damage of the cellular and functional continuity of any living tissues [5]. Wound healing is a multipart process that occurs in a known order and time frame [6].

It is an extremely dynamic process, involves the interaction of extracellular matrix molecules, resident cells, immune cells, and many soluble mediators [7].

Hyaluronic acid (HA) is a naturally produced polysaccharide, consists of glucuronic acid and N-acetyl-glucosamine subunits. The highest amounts of HA are found in the skin, eyes, synovial fluid surrounding the joints, and in cartilage. The main function of HA is to keep the tissues moist [8].

The synthesis of HA rises during tissue damage and the healing process. In addition to its role in providing the moisture to the tissue, HA regulates several features of tissue repair, including inflammatory cells activation to enhance immune response and provides the framework for cell migration and blood vessel formation [9].

In this study, we tried to elucidate the effect of exogenously injected HA on wound healing progress from the immunological and histological points of view.

Materials and methods

BALB/c albino male mice (n=24), aged 8-10 weeks with an average weight of (26±5 g) were used as a model in this study. All animals were housed in a conventional cage at the animal facility of the college of medicine/ University of Babylon before the experiment. The animals were randomly divided into two groups (12 mice in each group):

1. Negative control group: Each animal in this group received normal saline (0.1 ml) at a distance of 0.5 cm of the wound edge.
2. Hyaluronic acid (HA) group: Each animal in this group received a hyaluronic acid injection (1000 mcg) at a distance of 0.5 cm of the wound edge.
3. Normal control group: This group of animals received nothing and no wounds were performed. Blood and skin

samples from these animals were used to determine the baseline levels of cytokines.

Induction of wound model

The hair at the dorsal skin was removed using a commercial depilation machine 24 hours before the beginning of the experiment. Chloroform was used for anesthetization. A 10% povidone-iodine swabs were used for disinfecting the depilated dorsal skin of the mice then incisions of 1 cm length were made through the skin at the dorsum of the mice using surgical blades. A spray of lidocaine was used to reduce postoperative pain.

Hyaluronic acid injection

One ml of the Hyaluronic acid (Hyamira, Italy) was diluted one ml of distilled water to produce (10000 mcg/ ml) stock solution. Each animal was injected with 0.1 ml (1000 mcg) at a distance of about 0.5 cm from one side of the wound site. Each animal in the control group was injected with 0.1 ml of normal saline.

Wounds follow-up

Wound healing progress was followed up daily and images were captured to evaluate and documents morphological features of the wound healing progress.

Blood and skin Samples Collection

After 3, 7, 14 and 21 days from the single-dose injection of HA and normal saline, the animals were anesthetized with chloroform, about 1 ml of blood was obtained directly from the heart of each mouse using disposable syringe , the skin samples from the site of the wounds (1 cm^2) were collected and divided into two parts ; the first one fixed in formaldehyde (10%) for histological investigation and the other part stored at -20 C^0 for immunological assays .

Preparation of blood and tissue samples for immunological assays

Blood samples were placed in gel tubes and keep at room temperature for 15 minutes, then centrifuged at 4000 rpm for 15 minutes to collect the sera and preserved at -20 C^0 until use. The tissue crushed into small fragments and washed in ice-cold Phosphate Buffer Saline (PBS) (0.01M, pH=7.4) to eliminate any blood. Tissue fragments were weight then homogenized in PBS (1:9 weight to volume) with a glass homogenizer on ice. The suspensions were further fragmentize using an ultrasonic cell disrupter. The homogenates were then centrifuged for 5 min at 5000×g to get the supernatant. Determination of blood and tissue interleukin IL-2, vascular endothelial growth factor VEGF 2, basic fibroblast growth factor b FGF, and Tumor necrosis factor-alpha TNF-α levels were done using ELISA technique and commercially available kits (Elabscience, China) following the instructions of the manufacturing company.

Preparation of histopathological sections

Specimens fixed in 10% formaldehyde were embedded in paraffin, sectioned, and stained with H&E stain. All slides were examined by a pathologist without previous knowledge of the treatment.

As part of the histological evaluation, the following parameters were evaluated: fibrosis, the presence of congested blood vessels, necrosis, ulcer and the presence of inflammatory cells.

Statistics analysis:

Data were analyzed using Sigma plot V12.5. All the results were expressed as Mean ± S.D. "Unpaired t-test" was used to compare the area of wound healing in all the groups on Days 3, 7, 14, and 21 of the experiment. $P \leq 0.05$ was considered as statistically significant.

Results

Morphological features

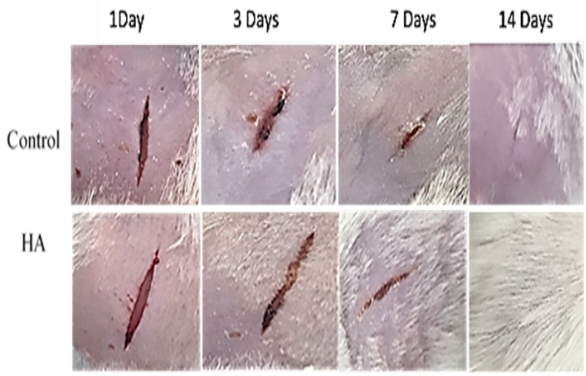

Figure 1. Morphological progress of wounds healing from day 1 to day 14. Wound length ≈ 1 cm, wound width ≈3 mm.

Figure (1) show the healing progress starting from day1 to day 14. The wounds were macroscopically evaluated post-wounding from Day 0 until complete wound closure (re-epithelialization). By macroscopic observation, when the surface of the wound area exhibited a similar appearance as that of the surrounding uninjured normal skin region with no inflammation, it was determined that complete wound closure (re-epithelialization) had occurred.

As shown in figure (1) the HA treated group showed rapid healing progress compared with the control group. No scar tissue is observed in HA treated wound. In addition to HA effect on the wound, it stimulates the growth of hair around the wound.

Immunological effects of hyaluronic acid

Figure (2) show the effect of hyaluronic acid (HA) on the serum and tissue cytokines levels. Cytokines measured in this study include; basic fibroblast growth factor (bFGF), interleukin 2 (IL2), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF).

As shown in figure 2 (G&H), serum basic fibroblast growth factor (bFGF) revealed a significant increase in day 7 and 21 compared to normal and negative control levels, while in tissue, it exhibited local elevation in day 14 and decreasing in day 21.

Tissue Interleukin 2 levels strongly increased on day 14 (figure 2 A&B). Serum TNF α significantly increased on day 7 while tissue levels increased on day 14 (figure 2 C&D). VEGF levels increased only in serum samples on days 7 and 21, while tissue levels remain lower than control levels (figure E&F).

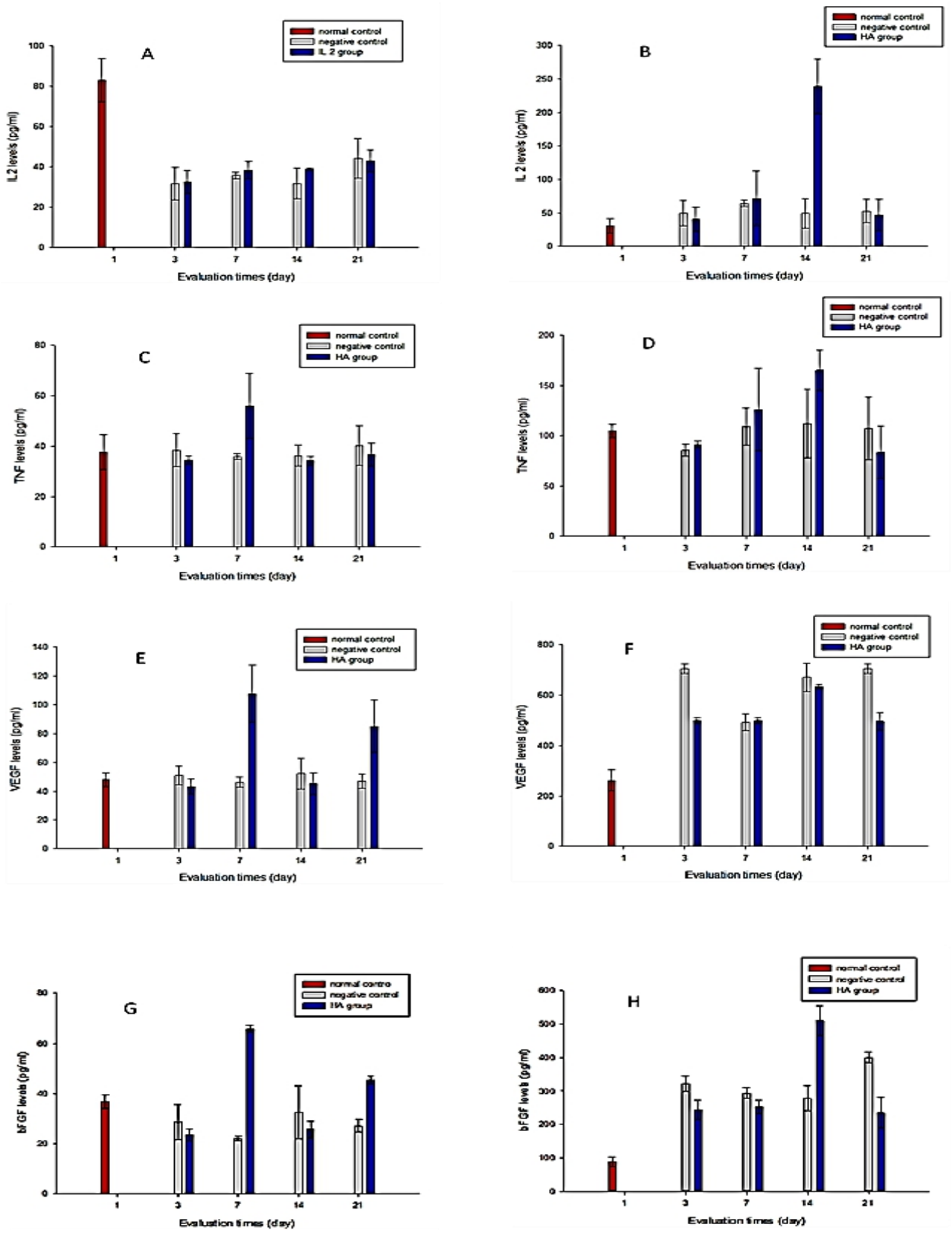

Figure 2. Cytokines and growth factors levels during the Hyaluronic acid (HA) application. Figures (A, C, E, and G) illustrate serum levels, while figures (B, D, F, and H) illustrate tissue levels. In Normal control, blood and tissue samples were taken from unwounded animals. Negative control represents samples taken from wounded animals without HA application.

Histopathological changes

Wounds treated with HA showed amazing histopathological changes started from the first days of the treatment. Treated tissue showed no fibrosis, necrosis, ulceration, or infiltration of inflammatory cells as shown in table (1).

Table 1. summary of histological changes in wound tissues.

Group	Day	Fibrosis	Necrosis	Ulcer	Congested vessels	Inflammatory cells		
						Lymphocyte	Neutrophils	Macrophage
Positive control	3	-	+	+	-	+	+	+
	7	-	-	-	+	-	-	-
	14	-	-	+	-	+	+	-
	21	-	+	-	-	+	+	+
HA	3	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-
	14	-	-	-	-	-	-	-
	21	-	-	-	-	-	-	-
Negative control	0	-	-	-	-	-	-	-

Table 2. Morphological healing progress of wounds from day 1 to day 21. Initial wound length 10 mm

Groups	Day 0	Day 3	Day 7	Day 14	Day 21
Control group	10	5.66±1.5	1.33±0.57	0	0
HA group	10	7±2	1.6±0.5	0	0

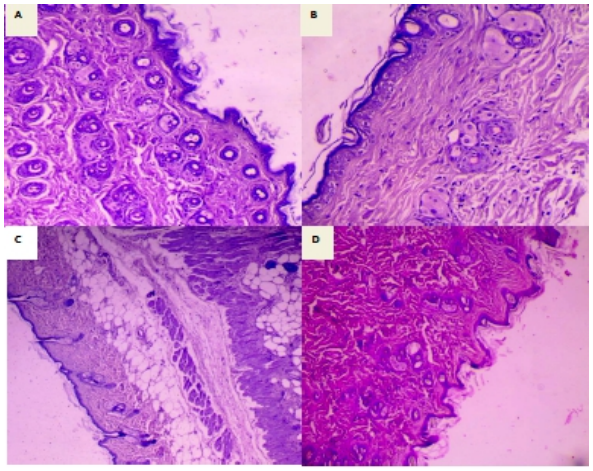

Figure 3. Microscopic appearance of skin wound tissues treated with HA. (A) at day 3 (x40), (B) at day 7 (x20), (C) at day 14 (x4) and (D) at day 21 (x10). No signs of inflammation or scar formation with normal tissue integrity recovery during the second week.

Figure (3) shows the recovery time layout of the wounds treated with HA. It is clear that HA reduces inflammation significantly but does not affect the healing progress. In fact, it helps to recall normal texture without passing through the normal healing stages literally.

Discussion

Wound healing is a complex, sequential biological process that aims to replace damaged tissue by a new living one and restoring the tissue integrity [10]. This process involves crosstalk between fibroblasts, keratinocytes, and immune cells during the early phase of wound healing. Soon after the injury, the hemostasis stage is initiated by the formation of a temporary matrix, activation of immune cells to secrete cytokines and growth factors[11].

The inflammatory phase develops during the first 24 hours of injury and lasts for up to 48 hours. The key cells of this phase are neutrophils and monocytes/macrophages[10].

Hyaluronic acid (HA) is a huge glycosaminoglycan involved in most cellular activities like proliferation, migration, and tissue repair. It is vital for activating keratinocyte and for re-epithelialization process [11]. As depicted in Figure 1, it is clear that HA treated wounds healed faster than untreated wounds. HA improved the healing outcomes by decreasing the time required for complete wound enclose and re-epithelialization.

Normally, intrinsic Hyaluronic acid has vital functions in normal epidermis. It is a free-radical scavenger, is a fundamental part of the extracellular matrix of basal keratinocytes, and has a role in keratinocyte proliferation and migration. Thus, it has an important role in the re-epithelization process. Many studies have confirmed the effects of exogenous HA in improving the wound healing process in rats and hamsters. Previous studies have shown that Hyaluronic acid content remains high for longer periods in fetal wounds than in adult wounds, thus reduce collagen deposition and scarless healing [12]. HA protects cells from damages via the prevention of apoptosis, this is mediated by decrease inflammation and fibrosis [8]. These anti-inflammatory and other histopathologic effects are apparently seen in the table (1). The wound enclose is not accelerated by the injection of external HA (table 2).

Cytokines and soluble growth factors are critical in regulating cell functions such as proliferation, migration, degradation and synthesis of extracellular matrix components during the process of wound healing [13] [14].

From figure 2 A&B, HA had no effect on IL-2 levels during the inflammatory phase. This will improve wound quality since the persistent inflammation in the wound generates a delay in wound healing, and lead to wound chronicity [15]. Chronic inflammation, a hallmark of the non-healing wound, predisposes tissue to cancer development [16]. Interleukin-2 has various, sometimes contradictory, functions during an inflammatory response. IL-2 contributes to both the initiation and the termination of inflammatory responses. The timing of IL-2 releasing may be critically important to its influence on wound healing. Locally released IL-2 could impact the rate and quality of the closure by enhancing immune and skin cell proliferation and differentiation at the wound site as well as skin and blood vessel cell proliferation. Later, decreasing IL-2 may help encourage the resolution of inflammation by attracting and increasing regulatory immune cells [17].

Results illustrated in figure 2 (C&D) revealed that there is a significant increase in the serum and tissue levels of Tumor Necrosis Factor –α (TNF-α) at days 7 and 14 respectively. TNF-α is quickly released during the first several hours and reached a peak level on day 1, initiating inflammation at wound tissues, and then decreased to the basal level [18].

Previous reports indicated that HA stimulates TNF-α production by macrophages. TNF-α is known to downregulate fibroblastic collagen synthesis and thereby limit scarring [19].

Elevation of TNF-α during the process of wound healing leads to a reduction in the formation of granulation tissue whereas decreased levels of TNF-α encourage collagen

disposition. Increased levels of TNF-α also suppress the function of Transforming growth factor -beta (TGF-b). TGF-b induces the production of ECM by stimulating collagen production. In general, it has been reported that the local elevation of TNF-α releasing enhances wound repair while dropping of TNF-α has the impair the process. Interestingly, Local and systematic levels of cytokines are not the same at all the time. It was found that a decline in local TNF-α expression was detected in the wounds, while systemic serum level was elevated [20].

Vascular Endothelial Growth Factor (VEGF) levels shown in figure 2 (E&F) indicated an elevation in its expression in serum levels at day 7 and 21.

From figure 2 (E&F), the result revealed that there is a significant decrease in VEGF tissue levels in the hyaluronic acid group on days 3 and 21. When high levels of proangiogenic factors such as VEGF produced by keratinocytes, and at least a part of the wound angiogenic response lies downstream of inflammation so modulation of specific inflammatory mediators or inflammatory cells, including macrophage-derive mediators, epithelial mediators, and mast cells, has been shown to yield reduced scar formation and improved healing [21]

In recent studies, it was found that the application of exogenous HA on wound models promote wound healing by stimulating fibroblast to synthesize an increased amount of angiogenic growth factors such as Vascular Endothelial Growth Factor (VEGF) [22]. VEGF functions as an endothelial cell mitogen , chemotactic agent , and inducer of vascular [12] VEGF has potent angiogenesis and vaso-permeability, an activity that led to its initial term as a vaso-permeability factor. This factor is produced in large quantities by the epidermis during wound healing. Cell disruption and low oxygen tension (hypoxia) is a major inducer of this growth factor. Recent data suggest that VEGF may be critical for angiogenesis during granulation tissue formation from day 4 through 7 [23]. VEGF levels in wound tissues reach their peak on day 7, then reduced gradually after that [24].

Recent studies reported that HA can regulate angiogenesis independently without the need for VEGF expression [25]. Thus, since granulation is reduced by HA application, the induction of VEGF production is reduced and remains lower than its level in the control group without affecting the angiogenesis activity.

As shown in figure 2 (G), the hyaluronic acid causes a significant increase in serum level of basic fibroblast growth (bFGF) factor on day 7 and 21, and in tissue level in day 14 (figure 2 H). This growth factor has no role in the inflammatory phase of wound healing although it releases by macrophages and other cells, thus its level is normally elevated in the second phase.

bFGF activates Endothelial cells in order to initiate and modulate ECM biosynthesis, epithelialization, and angiogenesis which are of great importance for normal tissue functions [10] [26].

Clinically, bFGF is widely used in accelerating wound healing, thereby improving scar quality. It stimulates the DNA synthesis and proliferation of cells. In addition, it downregulates collagen I and to upregulate hyaluronic acid (HA) synthesis in skin fibroblasts in vitro and efficiently inhibits terminal differentiation to myofibroblast, the most important mechanism in scarring [27].

Endogenous or natural HA has a very high molecular weight of more than 1000 kDa but it is gradually degraded into smaller fragments having properties depend on their molecular weight. Large molecules mostly have immunosuppressive effects, whereas smaller molecules have a proinflammatory effects [11].

Conclusions

Hyaluronic acid enhances wound healing via multiple processes. It modulates inflammation, promotes angiogenesis and re-epithelization, and reduce scar tissue formation.

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