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# In vivo Evaluation of Gelatin/Hyaluronic Acid Nanofiber as Burn-wound Healing and Its Comparison with ChitoHeal Gel

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**Abstract:** The study aims at performing a comparative assessment of two types of burn wound treatment. The present study was designed to prepare crosslinked and blended two natural polymers nanofiber scaffolds using gelatin (GE) and hyaluronic acid (HA). The GE/HA composite nanofibrous membranes with varied GE/HA weight ratio have also been successfully fabricated by an electrospinning method. The average diameter of GE/HA fibers was in the range of 20 to 150 nm. In vivo efficacy was also investigated based on a deep second degree burns model for Wistar rats. At 14 days post-operation, the dermal defect basically recovered its normal condition. A percentage of wound closure of GE/HA composite nanofibrous membranes and ChitoHeal gel reached up to 81.9 % and 77.8 % respectively, compared with 65 % of the untreated control ($p<0.05$). Also, histological parameters were assessed on postoperative day 7 and 14. The results of in vivo experiments showed that more epidermis was formed in the gel and scaffold groups compared to the control group. The numbers of inflammatory cells in these two groups were also smaller as compared with the control group, which could well be the reason for the delayed healing in the control group.

**Keywords:** Burn wound healing, Gelatin/HA, Electrospining, ChitoHeal gel, In vivo test

## Introduction

Skin is an important organ in human body. It can protect and maintain the environmental stability in vivo. If the skin is destroyed, wound healing is completed through four precisely and highly programmed phases: hemostasis, inflammation, proliferation and remodeling [1-3]. In the treatment of bedsores or serious burn wounds, wound dressings are often used to enhance healing. Developments in many aspects of burn healing have led to the increased survival of victims from serious burns. As a result, deaths caused by burns have been reduced more than half during the past 40 years [4,5]. A wound dressing is a protective barrier used to support many features of the healing process. The main functions of wound dressings are to facilitate wound healing and minimize scarring [6].

Traditional wound healing agents have been largely replaced for chronic wounds and burns by the more recent and advanced dressings. The modern dressings are mainly classified according to the materials from which they are produced including hydrocolloids, alginates and hydrogels, and generally occur in the form of gels, thin films and foam sheets [7]. The selection of materials is very important from wound healing application point of views. A wide range of passive, interactive, and bioactive wound dressing materials with different clinical merits have been developed [7-9].

A wound dressing material, particularly for burns, should be permeable to moisture and oxygen and the texture should be adhesive to dry skin. Thus, electrospinning is very useful method for wound dressing materials which accelerate wound healing [2,4,8,10-13]. For a perfect wound dressing, materials should have the capability of absorbing wound exudate and protecting the ulcer from dehydration. Wound dressing materials should also be comfortable, stable, non-antigenic, non-toxic and cost-effective. Among the various materials that can be used to produce electrospun fibers, natural polymers are limited to silk collagen, DNA, alginate, chitosan and more recently fibrinogen, gelatin and hyaluronic acid [14,15].

Gelatin is a natural biopolymer derived from collagen with strong polarity. Hyaluronic acid (HA), the main component of the extracellular matrix of connective tissues, has excellent properties of biocompatibility and biodegradation. The addition of HA was expected to improve the spinnability of aqueous gelatin solution and prepare blend fibrous products for biomedical applications. The electrospinning of GE/HA blends had previously been studied [16,17]. Gelatin, a biocompatible and degradable substance, has been widely

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used to develop wound dressings of certain types of open wounds (e.g., traumatic, thermal, or chronic wounds) [18]. In previous studies, gelatin/polyurethane nanofiber containing silver-sulfadiazine [4] and silk fibroin/gelatin electrospun nanofibrous dressing loaded with astragaloside IV (AS) [19] were prepared as burn wound dressings. It is generally accepted that the incorporation of HA is beneficial in tissue repair products [20,21]. When HA physiologically degraded into smaller-sized fragments, it facilitates wound healing by promoting angiogenesis. Therefore, HA helps cell migration and cell proliferation and keeps the skin moist [22,23]. Therefore, HA with gelatin may be advantageous to prepare scaffolds that can mimic the ECM in terms of topography and chemical composition because of the inherent properties of gelatin and the unique characteristics of the electrospun fibers. Moreover, chitosan is an attractive natural polymer for biomedical use due to its reported desirable bioactive properties scar reduction and wound healing [15,24]. Chitosan can be processed into various forms, including films, hydrogels, nanoparticles, scaffolds, and sponges leading to the proposed applications for wound dressing [25,26].

In a previous study, a sponge prepared from hyaluronic acid collagen sponge was used for skin healing in rats. It was also concluded that this sponge succeeded in creating a suitable environment for wound healing (a conclusion in agreement with results of our research). However, it was shown in previous research that collagen alone was not able to create a moist environment for wound healing [27]. It must be mentioned that collagen is a very expensive polymer and gelatin has an extremely low price compared with collagen. This is a very important factor in introducing commercial products.

The main aim of this work was to develop a nanofibrous wound dressing prepared from gelatin/ HA material by electrospinning with various blending ratios. GTA vapor was used to improve the stability of the fiber mats in a moist environment. The researchers also sought to compare the wound healing rate in a burn rat model treated with either a bioactive dressing (gelatin/HA nanofibrous membranes and a commercial product, ChitoHeal gel) or conservative treatment (gauze).

## Experimental

### Materials

Hyaluronic acid (HA, MW=1200000) was provided by Shandong Freda Biochem Co., Ltd. (Jinan, China). Formic acid (FA) and N,N-dimethylformamide (DMF) were supplied by Merck, Germany. Gelatin (bovine skin, type B) was purchased from Sigma-Aldrich (St Louis, MO, USA). The commercial product, ChitoHeal gel, was obtained from ChitoTech Company (Tehran, Iran). All the solvents were used without further purification.

### Preparation of Spinning Solution

20 % (w/v) GE solutions with DMF was prepared at 40 $^{o}$C under gentle stirring for 20 min. Dissolve certain amounts of HA powder in DMF under gentle stirring for 15 min, and then add a specific amount of distilled water into the HA solution according to the volume ratio of DMF to water (2:3) and continue to stir the solution for 8 h until the solution became transparent. The spinning solution of 1.5 w/v% HA in DMF/water system was prepared. Then, add the GE solution into the HA solution with specific volumes to obtain the GE-HA solutions (GE/HA=93/7 and 97/3, weight ratio) [28].

### Electrospinning

The fibers were prepared by the use of electrospinning technique. A counter electrode was located at 15 cm apart from the capillary tip. The applied voltage was 20 kV and a syringe pump was used to feed the polymer solution and the feeding rate was fixed at 0.1 m*l* $h^{-1}$. All experiments were conducted at room temperature and the fibers were collected on an aluminum foil.

### Chemical Cross-linking of Nanofiber Mats

Electrospun mats were crosslinked to enhance the mechanical properties and pH stability. After the electrospun nanofiber was dried in vacuum for 24 h, glutaraldehyde (GA) vapour cross-linking was carried out by placing the membrane above the GA solution (25 wt%) in a sealed desiccator at room temperature for 48 h. Then, the GA cross-linked GE/HA scaffolds were washed three times with distilled water and dried in vacuum.

### Characterization of the GE/HA Nanofibrous Dressing

#### *Scanning Electron Microscopy (SEM)*

The morphology of the electrospun nanofibres was studied by scanning electron microscopy KYKY SEM (China; EM-3200) equipped with image analyzer software at an accelerating voltage of 20 kV. Prior to observation, mat samples were attached onto the stubs using a double-sided tape and then coated with gold. The diameter, diameter distribution and uniformity were measured with image analyzer software (Sigma Scan Pro 4.0). For each experiment, average fiber diameter and distribution were determined from about 100 measurements of the random fibers.

#### *FTIR Analysis*

The infrared (FT-IR) spectra were recorded on photo acoustic mode at the frequency range of 5000-4000 $cm^{-1}$ with 256 consecutive scans at 8 $cm^{-1}$ resolution on a Thermo-Nicolet 6700 P FTIR Spectrometer (Germany).

### In-vivo Wound Healing

#### *Burn-wound Model*

Thermal skin burn was modeled in 24 Wistar male rats

weighing 200-250 g. The rats were randomly divided into three groups each composed of 8 rats for the study to compare healing of wounds. After hair removal, contact thermal burn was infected at the middle of the paravertebral region by applying a copper plate heated to 90 °C at a force of 1.3 N for 10 sec under ether anesthesia, which produced grade IIb (second-degree burn). After generating burn wound regions (2×3 $cm^2$), 70 % ethanol was used for disinfection. Every wound was enclosed with one of antiseptic gauze (control), ChitoHeal gel (a commercial wound dressing), and sterilized GE/HA nanofiber wound dressing. Formulations were repeatedly applied on the burned areas once daily for 14 days. For the control wounds, they were just cleaned with cotton wetted with normal saline each day. The diameter of the wounds formed were monitored and measured periodically.

***Wound-healing Rate***

Images of the wound area were taken with a digital camera. The wound was traced, and the traced area was calculated using the Image J (National Institutes of Health) software program. Wounds were digitally photographed on 1, 7, and 14 days post wounding while maintaining a constant optical zoom. The percentage of wound closure was calculated by the initial and final area using metric ruler during observation as follows:

$$\text{Wound size reduction (\%)} = [(A_0 - A_t)/A_0] \times 100$$

where $A_0$ is the initial wound area and $A_t$ represents the open area of wound at the time of biopsy on day 1, 7, and 14 accordingly [29].

***Histopathologic Examination***

On day 7 and 14, all animals were scarified under anesthesia, and wounds were removed from animal for histopathological evaluation. The removed skin specimens from each group were collected from wound treatment. Histological assessment was performed only for formulations with highest improvement in wound healing to further compare them. Specimens were fixed in 10 % buffered formalin, processed, embedded in paraffin, cut into 5 mm pieces and stained with hematoxyl in and eosin (H&E). Sections were visualized under a light microscope at 400× magnification. For histopathological analysis (8 rats per group), all animals were scarified under anesthesia on day 7 after surgery because the maximal changes occurred during the first week after wounding [16].

**Statistical Analysis**

Each experiment was repeated for at least three times and the data were obtained by calculating the mean of experiments. Statistical analyses were performed using One-way ANOVA (SPSS 16) and $p<0.05$ was considered as p-value indicating the statistical significance of differences.

## Results and Discussion

**Electrospinning and Morphology of Fibers**

In this study, GE-HA/DMF-water solutions were prepared

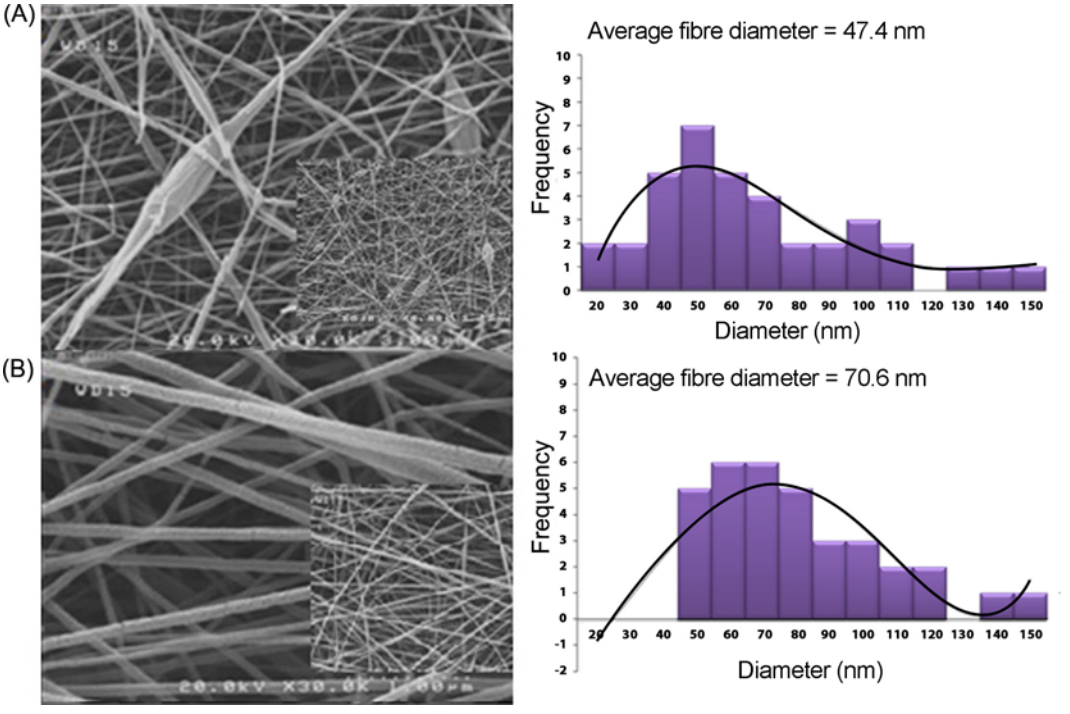


**Figure 1.** SEM micrographs (magnification=3000× and 30000×, scale bar=5 μm) and histogram distribution of fiber diameter of electrospun gelatin/hyaluronic acid fibres; (A) 93:7 and (B) 97:3.

to investigate the electrospinning process of two natural biopolymer blends. The concentration of HA was fixed at 1.5 w/v% and the volume ratio of DMF to water was 1.5. The SEM morphologies of the GE/HA electrospun which contain various concentrations of biopolymer (GE/HA: 93/7 and 97/3) are shown in Figure 1. SEM images at both low and high magnifications indicated that uniform GE/HA nanofibrous membranes could be fabricated. Membranes at different GE/HA compositions and different average diameters ranging from 20 to 150 nm could also be produced by electrospinning. Additionally, nanofibrous GE/HA was successfully generated without the formation of beads when the proportion of gelatin increased (Figure 1(B)). In the present procedure, the overall polymer concentration increased with increasing GE content. Also the higher polymer concentration led to an increase in the average diameter of GE/HA nanofibers. Furthermore, gelatin concentration had a significant effect on the average fiber diameter. The average fiber diameter increased from 47.4 to 70.6 nm as the gelatin concentration increased. The reason for the positive correlation is that the higher viscosity resisted the extension of the jet.

## FTIR Analysis

Chemical analysis was performed to prove the existence of hyaluronic acid and gelatin in scaffolds and the removal of solvent from electrospun mats. Intensity of peaks in two scaffolds is very different due to the difference in the concentration of polymers in scaffolds. As shown in Figure 2, the peak of 1647.15 $cm^{-1}$ is related to amide bond in the net gelatin. Additionally, the $NH_2$ existing in gelatin attacks the carboxylic functional groups of hyaluronic acid and enhances this peak. The peak 3100-3400 $cm^{-1}$ is related to the absorption of hydroxyl group. The peaks absorbed at 1541.25 $cm^{-1}$ and 1450.40 $cm^{-1}$ are related to symmetric and asymmetric stretching vibrational bonds of carboxyl groups. Also, the peaks in the range of 1000-1100 $cm^{-1}$ are assigned to the absorption of the bond between carbon and oxygen which is the specific peak of hyaluronic acid. All specific peaks of hyaluronic acid and gelatin were also identified in their hybrid which suggested the existence of these two polymers in the above-mentioned scaffold.

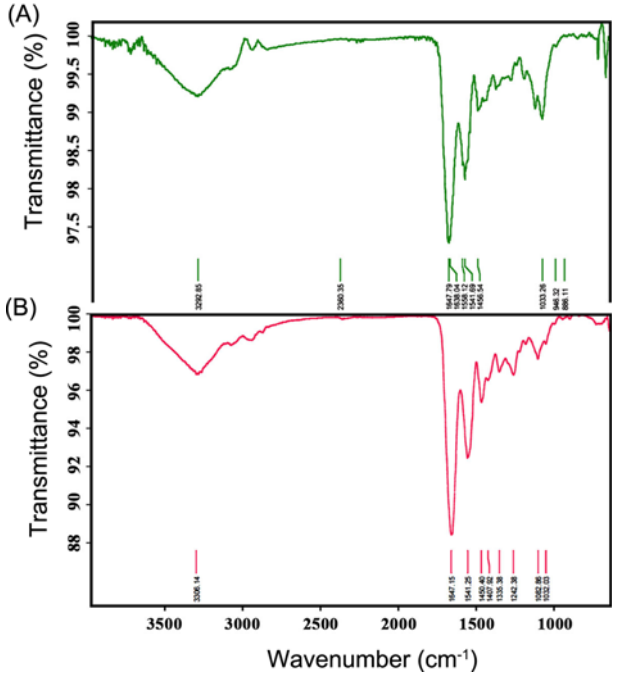


**Figure 2.** FTIR spectra of electrospun gelatin/hyaluronic acid fibres; (A) 93:7 and (B) 97:3.

## In-vivo Study

Although all mammals have essentially similar skin structures, there are interspecies differences in their various body regions. This research was conducted on Wistar strain rats. The macroscopic presence of wounds treated with sterile gauze, ChitoHeal gel (a commercial wound dressing), and sterilized GE/HA nanofiber wound dressing on several post-operative days are illustrated in Figure 3. Each wound was studied for a time period of 1, 7 and 14 days post-operation. All rats remained alive in the post-operative period until expiry. They showed no indication of necrosis. As Figures 4 and 5 show the comparative size decrease of the wounds was treated with several substances. The results of morphometric showed that the wound area in the group of gel and scaffold has been reduced with higher intensity than that in the control group on the seventh day, while there was no significant difference between them on the fourteenth day. The difference in the healed areas in these two groups was significant. Thus it can be concluded that the gel and the scaffold have been effective in reducing wound area on the seventh day. On post-operative day 7, the scaffold of GE/HA and commercial product considerably reduced the wound size in comparison with the antiseptic gauze. In the present study, the trend of wound healing was evaluated on the first, seventh, and fourteenth days.

However, histological studies showed that the results were apparent, the epidermis were formed incompletely and there were excessive inflammatory cells in the wound although the reduction of wound areas was satisfactory in the control group on the fourteenth day. The results of previous studies on the effect of gelatin on wound healing indicated that this difference was no longer significant on the tenth and fifteenth days although the healed area in the experimental group showed a significant increase in comparison with the control group on the fifth day. This result is consistent with the findings of the present study [17].

Samples were taken on the 7th and 14th days for pathological studies. These studies showed that there were evident differences in many of the wound healing-related variables. Figure 6 shows samples of the 7th and the 14th

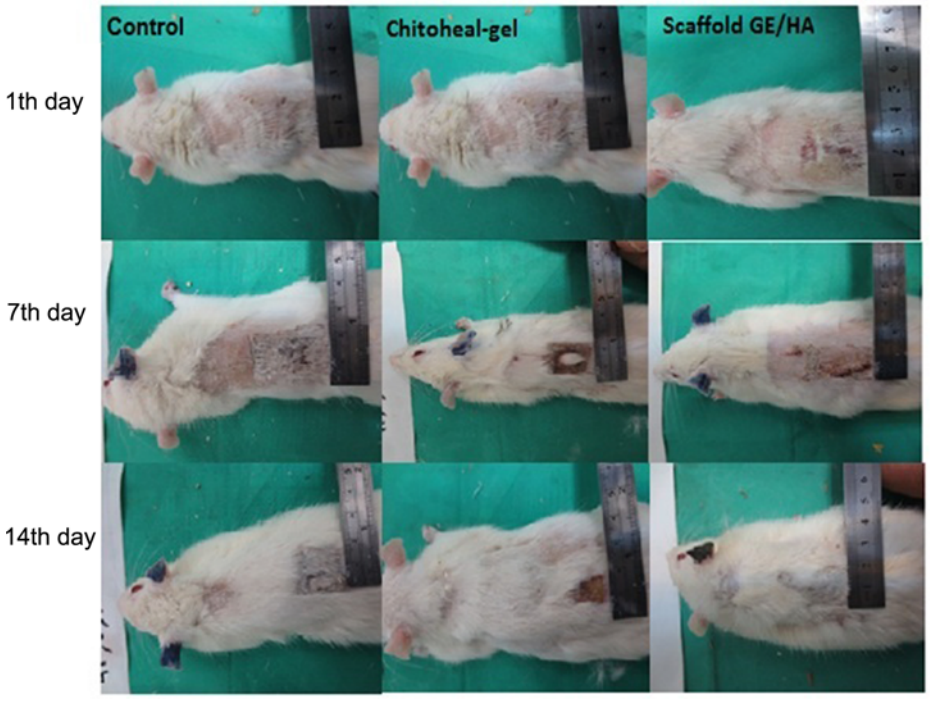


**Figure 3.** Macroscopic appearances of skin wounds treated with gauze as control, ChitoHeal gel (containing chitosan) and GE/HA nanofibrous at day 1, 7 and 14 in excision wound model.

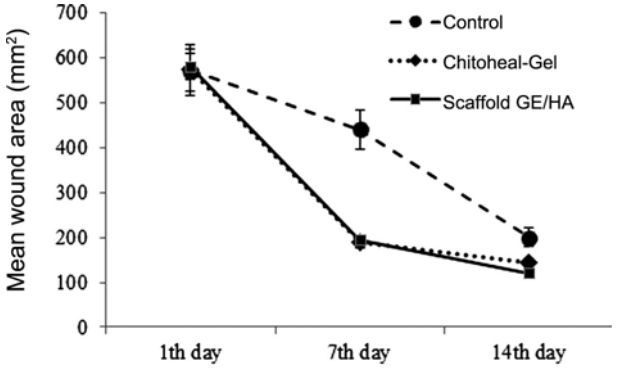


**Figure 4.** The assessment of burn wound healing.

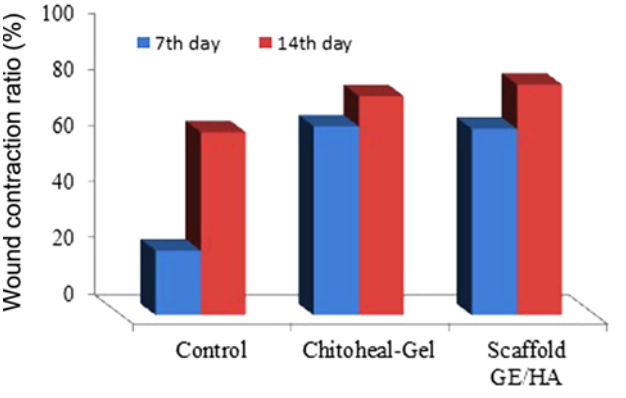


**Figure 5.** Wound contraction ratio of the wounds treated with control, ChitoHeal gel and GE/HA nanofibrous membrane.

days in the control, the gel and the scaffold groups. The superficial and deep parts of the dermis were microscopically studied due to differences in the quantity and arrangement of collagen fibers, resident cells, and the manner blood supplied. Regardless of the anatomical and histopathological difference, histopathological studies of the whole thickness of the dermis will lead to inferring results. It should be added that a part of this result was related to the mentioned differences.

On the 7th day, the dermis was fibrotized and repaired, whereas one-third of the epidermis healing process was incomplete in the control group. The number of defensive cells had been minimized but 4 or 5 spherical infectious foci had formed at the bottom of the dermis. Also, there were still signs of coagulation on the surface of the epidermis. Results related to the gel group indicated that the epidermis had been formed completely, the keratin level was normal and, in the wounded area under the epidermis, systematic healing fibrosis had taken place. A small amount of granulation tissue remained in the wounded area and the rest indicated advanced fibrosis with the number of defensive cells at a minimum (or no defensive cells in some spots) and some epidermal hyperplasia. The epidermis had been formed in the samples of the scaffold group. Inflammatory cells were

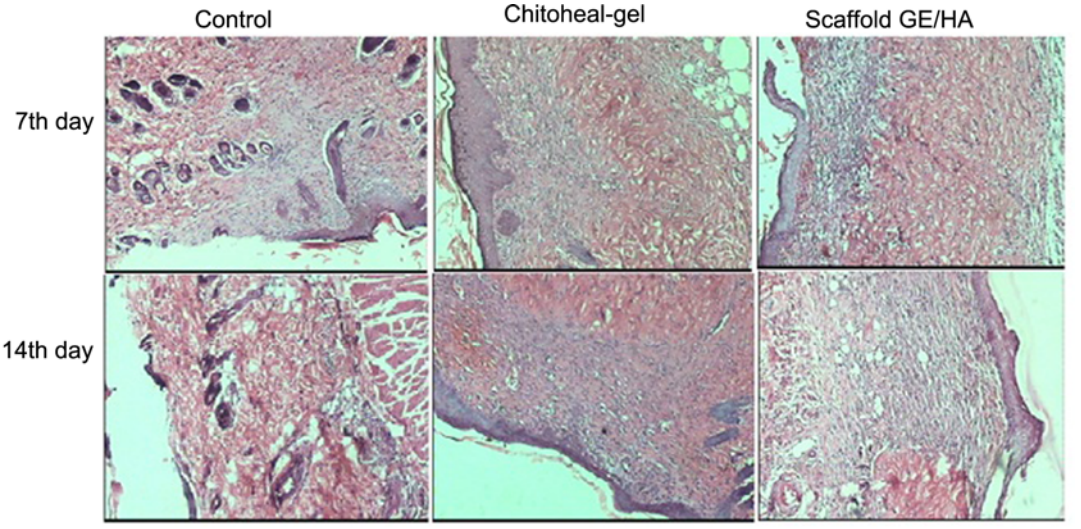


**Figure 6.** The H&E stained sections of the granulation tissue in control, ChitoHeal gel and GE/HA nanofibrous membrane group on the 7th and 14th day of wound healing at 400× magnifications.

also rarely observed, but coagulation had taken place on the surface of the epidermis.

On the 14th day, samples of the control group showed that epidermis had not formed in two-thirds of the wound surface, inflammatory fibrosis had somewhat progressed in the dermis, and relatively large numbers of defensive cells were detected. In the gel group, the epidermis had formed well, the keratin level was normal and, in the wounded area under the epidermis, systematic fibrosis had taken place. Furthermore, samples of the scaffold group revealed that the epidermis had formed throughout the wound. The epidermis was thick enough and no inflammation was observed.

In this research, pictures taken with an optical microscope showed that the wound dressings prepared from gelatin/hyaluronic acid could promote growth and migration of cells. The problem in using bioactive wound dressings is that they can absorb water, which provides a suitable environment for bacterial growth. However, our results indicated that this was an unjustified concern and these polymers were able to inhibit bacterial growth and proliferation due to the presence of acid groups in their structure and creation of an environment with an appropriate pH.

Many developing countries still use traditional dressings such as gauze. However, advanced dressings are more likely to promote healing in chronic wounds. The ChitoHeal gel was based on chitosan and was able to prepare a suitable environment for the wound and prevent inflammatory responses in the wound due to the antimicrobial properties of the chitosan polymer and presence of antimicrobial agents probably used in the preparation of the gel.

One of the important points in wound healing is to reduce scar formation in the wound. In normal skin, delicate bands of collagen in the moist area of the dermis tend to orient themselves parallel to skin surface (epidermis). As shown in Figure 6 taken with an optical microscope, collagen bands in the control group had an irregular shape, which is not desirable in the wound healing process because of its difference from the shape observed in skin under normal conditions.

In normal skin, neutrophils and eosinophils are rarely detected. The eosinophils and neutrophils observed in our research at the wound healing site were considered inflammatory cells which were present because of the wound (since the wound was not caused by traumas or by parasitic agents).

There are lymph vessels on the top layer of the dermis and around skin appendages (hair follicles and glands), but they cannot usually be detected in tissue sections from paraffin blocks. In the dermis region, no connective fibers are usually observed around blood vessels. In this research, lymph vessels were not studied due to the mentioned reasons. The manufacturing cost is the most important issue in moving from fundamental research to commercial application. The price of collagen is very high, being 10 times more than that of GE and HA. Regarding the low manufacturing cost, the GE/HA composite nanofibrous membranes seems to be very promising for commercial application.

## Conclusion

The present study was designed to prepare crosslinked and blended two natural polymers nanofiber scaffolds using gelatin (GE) and hyaluronic acid (HA). The GE/HA composite nanofibrous membranes with varied GE/HA weight ratio have also been successfully fabricated by an electrospinning method. The average diameter of GE/HA

fibers was in the range of 20 to 150 nm. In this research, microscopic study of the wound healing process (two weeks after the wound was inflicted) showed that more epidermis was formed in the gel and scaffold groups in comparison with the control group. The numbers of inflammatory cells in these two groups were also smaller compared with the control group, which could well be the reason for the delayed healing in the control group. In any case, extensive inflammatory cell infiltration, hyperemia, more severe edema in the dermis region, and stability of most growth factors caused infections that were more acute.

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