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Original Article

Evaluation of Chitosan Gel on Burn Wound Healing and Keratinocytes Function

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Abstract

Skin burns are common skin injuries that can result from exposure to various sources of heat by which, depending on the cause, different degrees of burns are generated. In restoration of superficial burns by re-epithelialization, the skin is rebuilt, but the skin color is different than the healthy tissue. Keratinocytes represent the major cells of the epidermis. These cells are sourced from ectoderm and are continuously being formed from the basal level. The main task of these cells is to make the protein keratin. During differentiation, keratinocytes move toward the superficial layers of the epidermis and generate more keratin. These cells are colorless, but by manipulating melanocytes (the melanocyte cells produce pigment in the skin), one can control the darkness degree of the skin. Keratinocytes, in particular, produce chemical signals which, in turn, are responsible for regulating the distribution and amount of melanin pigment in melanocytes. In this study, using an aluminum stamp weighing 85 grams, at a temperature of 80 $^{\circ}$C, superficial burns, of 2nd degree, were generated on six rabbits. The rabbits were divided into two groups; a control group and the second one in which a chitosan (CHI) gel was used as a dressing. After treatment, histopathology tests and the TEM results confirmed that, in the CHI treatment group, the restoration led to acceptable results. It was shown that if the repair was performed with a CHI based gel, the keratinocyte appeared with their original structure after restoration, but if the treatment was done without the CHI dressing, the number of these cells increased, more stimulation of melanocytes occurred and, ultimately, it led to an unwanted color change of the burnt area.

Keywords: Chitosan gel, Keratinocytes, Superficial burns, Epidermis, Cytokine, Skin color variation, Epidermis cell structure

1. Introduction

Skin is the largest, heaviest, and one of the most complex and effective organs. The skin is a living organ that breathes, rebuilds itself, and performs a variety of physiological actions [1]. Fully differentiated cells of the skin reside in one of the three layers: the epidermis, dermis, and hypodermis tissue. The outer layer of the

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skin, the epidermis, which is thin, is made of squamous epithelium. This external tissue plays a significant role in the protection of the body. The epidermis is formed mainly by keratinocytes cells with other cell types, melanocytes, Merkel cells, and Langerhans cells also contributing [2]. The dermis contains blood vessels, nerves, and glands (sebaceous and sweat). This layer is a protective and nutritive tissue with its main components being collagen, elastin, and hyaluronic acid; it provides the flexibility and elasticity to the skin and is able to protect the skin against water loss [3, 4]. The hypoderm is the deepest layer of the skin and is attached to the muscles and underlying tissues [5]. The hypoderm includes fat cells that are known as adipocytes. They work to protect the internal organs and isolate inner structures against sudden changes in temperature [6].

Keratinocyte cells, originating from the ectoderm, are the most abundant epidermal cells [7]. These cells are produced continuously from the basal layer, and their main task is synthesis of the protein called keratin [8, 9]. Melanocytes are cells with an oval nucleus, and a clear cytoplasm around the nucleus [10]. The main work of melanocytes is producing the pigment called melanin. Melanin plays a role in determining skin color and protects the inner cells from the harmful effects of ultraviolet radiation [11]. Melanin production activity in melanocytes is influenced by solar ultraviolet radiation which results in the dark color of the skin after exposure to sunlight [7, 12].

A skin burn is common skin damage that can result from exposure to various sources of heat, including hot water, steam, flames, hot objects, chemicals, electricity and even excessive exposure to sunlight [13, 14]. Skin burns can be of either partial skin thickness or full thickness [15]. In partial-thickness burns, the epidermis or epidermis and upper dermis are involved but the underlying tissues are not harmed [16]. In full-thickness burns, the epidermis, dermis, and the hypodermis layers are damaged [17]. First degree burns are superficial burns; only the outer layer of skin, the epidermis, is involved [18]. Second-degree burns are burns of moderate-intensity in which the epidermis and a portion of the dermis but not all of it, suffer damage [19]. In third-degree burns, all three layers of the skin are damaged. Skin substitutes and dressings have become one of the most attractive areas of research in biomaterials science in the last decade [20]. Application of a dressing on a burn wound can temporarily block infection and encourage healing [21, 22]. Technology's role in burn wound coverage is not only protect against infection but promotion of more efficient burn healing and reduction of patient suffering [23, 24]. It is known that polysaccharides, like CHI, chitin and hyaluronic acid, can accelerate wound

healing and prevent leakage from inflammatory cells. Previously, the role of numerous chitosan based research has been investigated in wound healing including modifications of chitosan, antimicrobial and anti-inflammatory properties, chitosan with synthetic /natural polymer blend, chitosan based composite scaffolds, chitosan based sponges, chitosan based oil immobilized, chitosan based extract immobilized scaffolds and chitosan based drug loaded scaffolds. CHI gel also acts as an ideal wound coverage [25, 26]. This material is biocompatible, biodegradable, hemostatic, anti-infection and, most importantly, can accelerate the healing. CHI based gel is a strong tissue adhesive. In this study, a CHI gel was prepared and its effectiveness was studied on the healing rate and scar formation of superficial burns, taking into consideration the keratinocyte's function.

2. Materials and Methods

2.1. Materials

CHI (MW 100–150 kDa, degree of deacetylation-80%) was purchased from Yunzhou Biochemical Corp. (China) and analytical grade acetic acid was obtained from Merck (Merck KGaA, Germany). All other reagents were also of analytical grade and used as supplied.

2.2. Chitosan Gel Preparation

CHI gels were fabricated, as formerly reported, with similar amendments [27]. Concisely, the CHI powder was sterilized in an autoclave at 120 ˚C for 15 min under 2 atm. An acetic acid solution was filtered through 0.22 microM membrane filters before utilization. Then, 0.1 M acetic acid solution was gradually added under continuous stirring to 2% CHI powder until a clear solution was obtained. The samples creation was done in a laminar flow hood, nonstop in instantaneous sterile packaging. To remove the solvent by evaporation, the gels were enclosed in cells with 0.22 μM filter caps and placed in an oven for 48 h at 37 ˚C under vacuum; the filter caps were then detached and the samples were vacuum-packed with hermetic caps. Each sample, with dimension of 2×2 cm, was sterile and stored at 4 ◦C.

2.3. Fourier-Transform Infrared Spectroscopy (FT-IR)

The CHI gel samples were tested by ATR-FTIR spectroscopy utilizing a Tensor 27 spectrophotometer with a diamond ATR attachment (Bruker, Germany)

2.4. Swelling Capacity of the Chitosan Gel

The dried chitosan hydrogels were cut into small squares with about 1 cm height. The swelling properties of the chitosan gel samples were investigated by placing the samples in a solution at room temperature and removing at 10, 20, 30, 60, and 120 minutes after soaking. At these programmed periods of times, the samples were removed from the solution, weighed and returned to the same container until equilibrium.

The swollen weight was obtained and the swelling ratio (SR) of the samples calculated by means of Equation 1:

$$\text{Swelling ratio (\%)} = [(W_s - W_d)/W_d] \times 100 \qquad (1)$$

where W_s is the weight of the swollen chitosan hydrogel at various swelling times and W_d is the weight of dry gel.

2.5. Preparation of the Skin of the Animal Burn Subjects

In this project, adult female rabbits (New Zealand White rabbits) weighing 1788 ± 368 g were used. The rabbits were kept in cages under standard laboratory conditions. During the experiment, they were under veterinary supervision and given adequate food. The experiments were performed in accordance with established guidelines for experimental research with animals, published by the Austrian Government in 1989. For adapting to the new environment conditions, they were kept in the laboratory cages for a week prior to testing on them began. Before infliction of the burn, the rabbit's hair was removed using scissors and then razors until the surface was perfectly smooth and free of hair. It was a dorsal area on the neck behind the animal's head because the rabbit skin thickness from the top of the neck to tail is almost identical, with no difference between them.

2.6. Infliction of the Burn Injury

Infliction of the burns was conducted using an aluminum stamp at 80 °C. The surface was a circle with a 4 cm diameter. To ensure consistency of the experiments, we used a thermometer to measure its temperature. About 5 minutes was necessary using a heater to increase stamp temperature up to 80 °C [28]. The burn was inflicted on the dorsal skin of the rabbits which were positioned in sternal recumbence. The modified region was initially cleaned with chlorhexidine and rinsed with sterile saline solution. The aluminum stamp was applied vertically with its weight (85 gr) without external pressure for 14 seconds on the desired location. The site was immediately sterilized with gauze soaked with saline at 22 °C and cooled for 5 minutes. One group of the three rabbits then had the chitosan gel applied immediately after the sterilization while the other group of the three rabbits had their wounds covered with sterile gauze. For the first group of rabbits an amount of gel with the thickness of about 1 mm was placed on the wounds, spread gently by hand and a non-adhesive pad was placed on it to hold it in place. The dressing, including the gel was changed every day, with the treatment continuing for twenty days.

2.7. Preparation of Samples

Sampling was done in the tenth, twentieth, and thirtieth days. Before sampling the animals were anesthetized. Using a surgical blade, surgical scissors and forceps, tissue was removed from the desired location. Samples from the wound center along with some healthy tissue around the wound were removed. The tissue removed from each rabbit included the dermis and epidermis. Each tissue was divided

into two parts. One part was placed in 10% formalin for histopathology and the other was placed in 2.5% glutaraldehyde to be used for TEM (Philips CM200 FEG transmission electron microscope).

2.8. Degree of Injury

Some of the tissue samples that had been placed in the 10% formalin were delivered to a pathologist. After suitable sample preparation and staining, the pathologist used light microscopy to confirm the burns were second degree.

2.9. Statistical Analysis

All data obtained as the mean with the standard deviation (mean ± SD) from three experiments. Statistical calculations were performed using parametric study of variance ANOVA (Bonferroni) by SPSS 22.0 software. P value were measured significant at $p < 0.05$ in comparison with control.

3. Results and Discussion

3.1. Characterization of Chitosan Gel by FTIR

An FTIR spectrum of the chitosan hydrogel is shown in Fig. 1. Broad peaks at 3367 cm^{-1} and 2943 cm^{-1} are attributed to –OH and –C–H stretching in the chitosan, respectively. A peak located near 1562 cm^{-1} is attributed to the N–H groups. A specific peak of the chitosan hydrogel is at 1651 cm^{-1}. This relates to the formation of imine bonds (C=N). The peak at 1408 cm^{-1} is attributed to the CH_3 symmetrical deformation mode. An additional two peaks around 1154 and 1075 cm^{-1}, correspond to the C–O stretching vibrations in the chitosan gel.

Figure 1 shows The FTIR spectra of chitosan hydrogel. A specific peak of the chitosan hydrogel is at 1651 cm^{-1} attributed to the formation of imine bonds (C=N). Broad peaks at 1154 and 1075 cm^{-1} are related to the C–O stretching vibrations in the chitosan gel.

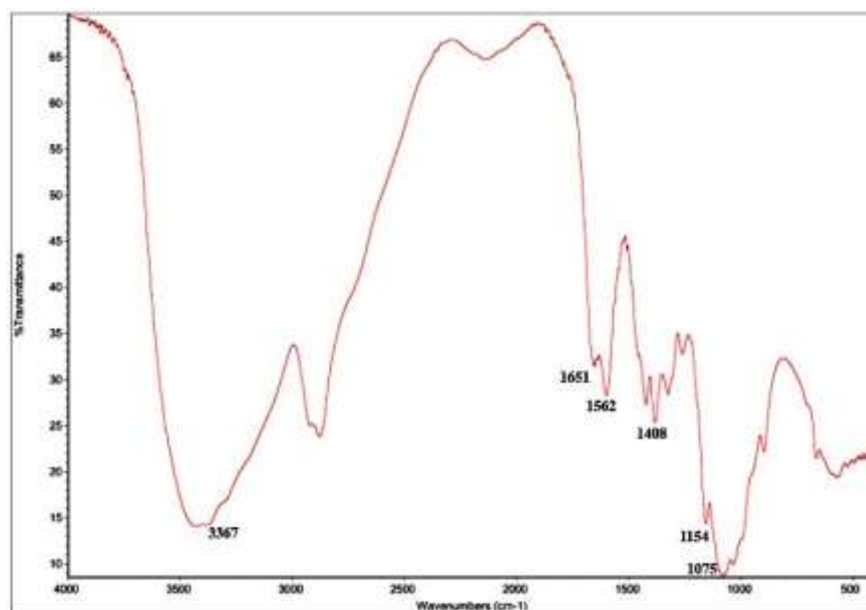

Figure 1. The FTIR spectra of chitosan hydrogel.

3.2. Result of Swelling Capacity Measurement

Figure 2 characterizes the chitosan hydrogel swelling kinetics in water. At first, the amount of water uptake progressively increased and then tended to even out. The near equilibrium swelling was attained after 35 min.

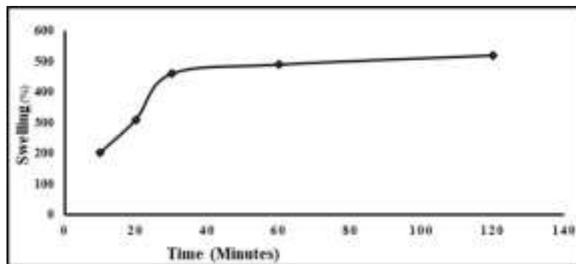

Figure 2. Swelling manner of the chitosan gel in water as a function of time.

3.3. Lee Index (Body Mass Index)

The Lee Body Mass Index (BMI) was calculated from the formula. BMI = $BW^{1/3}$(g) / nasoanal length (cm). As Fig 3 shows, it is obvious that by the tenth day the Lee index had decreased in both groups. The stress generated by the anesthesia and the burn caused a weight loss, as expected. After the tenth day wound healing progressed slowly with time and the Lee index for all groups increased slowly due to the animal gaining weight with reducing stress and developing wound healing. Increasing weight gains between the tenth and thirtieth days, after the initial drop, in the control group was higher than in the treatment group with chitosan gel due to the overwhelming response between chitosan and the repairing tissue. It is obvious that in the repair process, body forces focused on the healing process and resulted in weight loss.

Figure 3 shows Lee index for the control group with a group wound covering (a) and Lee index for the group treated with the chitosan gel (b). By the tenth day the Lee index had decreased in both groups. After the tenth day the Lee index for all groups increased slowly**.** Increasing weight gains between the tenth and thirtieth days, in the control group was higher than in the treatment group with chitosan gel. The values are the average of three replicates (n=3).

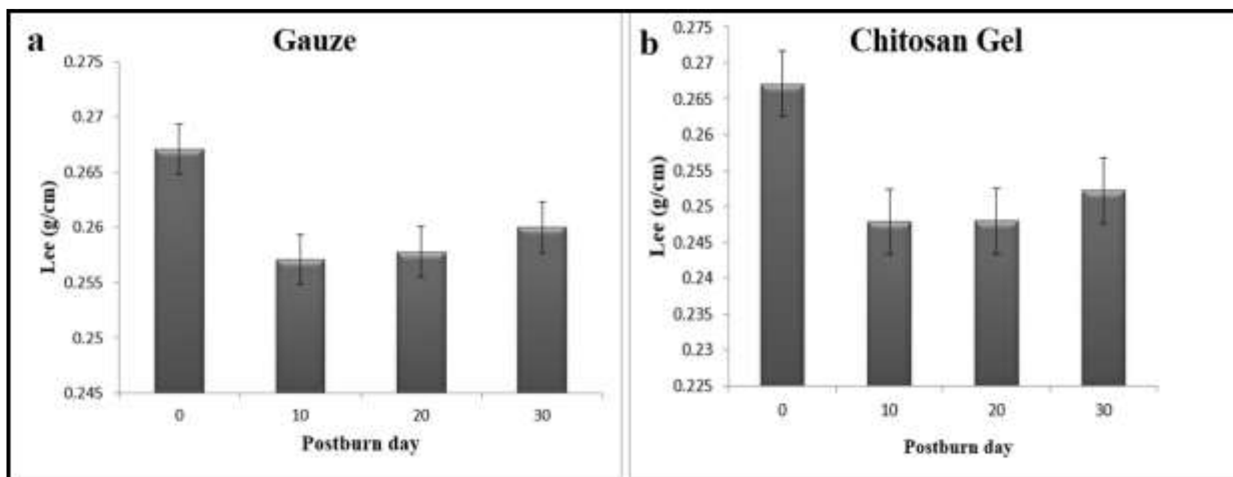

Figure 3. Lee index for the control group with a group wound covering (a) and Lee index for the group treated with the chitosan gel (b).

3.4. Migration Rate of Wound Edge (WEMR)

Using the revised Gilman equation (below), the migration rates were calculated from the changes in the position of the wound edge.

WEMR (mm/day) = $\{(A_b\text{-}A_a)/ ([P_a+P_b]/2)\}/ (b\text{-}a)$

In this equation, A represents the area, P represents the perimeter length and a and b indicate the beginning and end of the day's observation. As can be seen in the Fig 4 on all days the wound area and perimeter of the wound treated with chitosan gel were slightly less than the control group. This shows that chitosan increased wound healing and closure, and the wound's area was reduced slightly.

Figure 4 shows the wound areas 10 and 20 days after the burn (a), wound perimeters 10 and 20 days after the burn (b), and Rates of wound edge migration toward closure (c). The rate of wound edge migration in the chitosan gel was greater than that of the control group. Besides, the wound area and perimeter of the wound treated with chitosan gel were slightly less than the control group. Values followed by the same letter are not significantly different based on Duncan's Multiple Range Test (DMRT, at $P < 0.05$).

As figure 4 (c) shows, the rate of wound edge migration in the chitosan gel was greater than that of the control group.

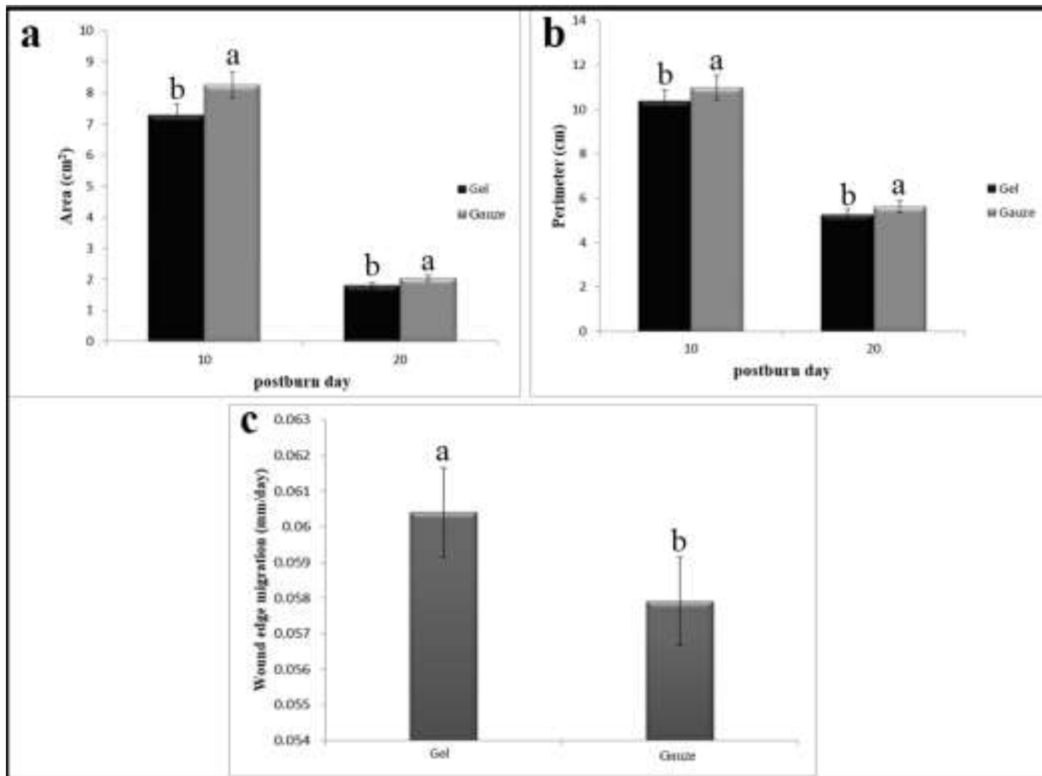

Figure 4. The wound areas 10 and 20 days after the burn (a), wound perimeters 10 and 20 days after the burn (b), and Rates of wound edge migration toward closure (c).

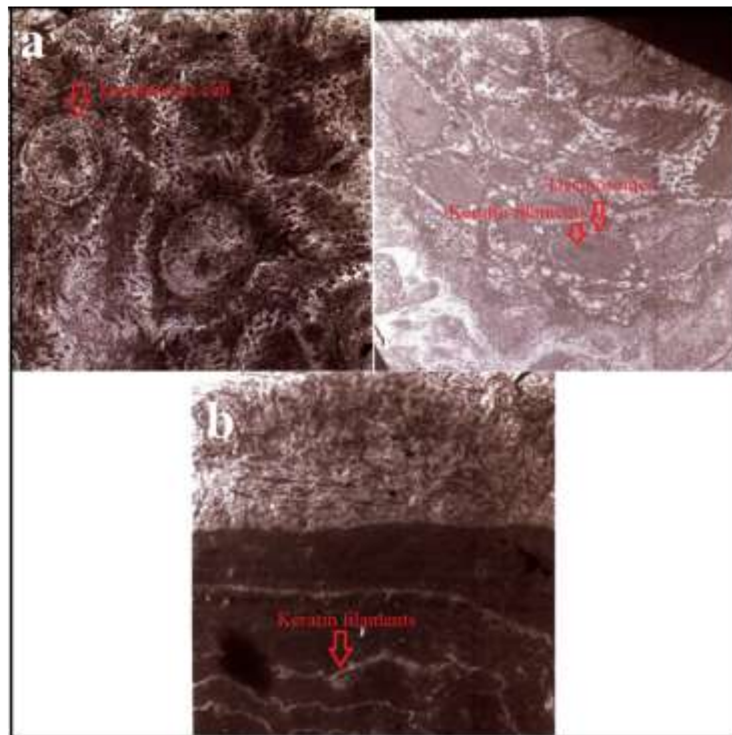

Figure 5. TEM images of samples treated with the chitosan gel. The morphology, shape and structure of these cells were normal (a) and TEM image of the internal structure of keratinocytes cells including desmosomes and keratin filaments (b).

3.5. Microscopic Evaluation

3.5.1. TEM Evaluation

In Figure 5 (a) and 5 (b), keratinocyte cells and the melanocytes are shown. These images show the germ layers of the epidermis contained keratinocytes cells because they were in proximity to the melanocytes. Note that these images are related to the treatment by the chitosan gel which accelerated the re-epithelialization of the epidermis with the keratinocytes is approved. As can be seen in the images the morphology, shape, and structure of these cells were normal; the cytoplasmic membranes and desmosomes of these cells were visible and can be seen. Thus, for the second-degree burns, after the healing process there was no significant change in the structure and morphology of the keratinocytes. Darkening of skin color can be due to increased cytokine and other factors that the keratinocytes produce which have an influence on the melanocytes. This material contains prostaglandins that cause inflammation and increased production by the keratinocytes and increase the melanocytes activity, resulting in their expressing of prostaglandins receptors. Besides, α-MSH and endothelin-1 epithelial cells that possess melanogenic properties for melanocytes, such as the effect of UV on the skin surface in response to burns, are synthesized. The number of keratinocytes in the damaged skin increased due to the stimulation of the proliferation of these cells in wound healing.

Figure 5 shows TEM images of samples treated with the chitosan gel. The morphology, shape, and structure of these cells were normal (a) and

TEM image of the internal structure of keratinocytes cells including desmosomes and keratin filaments (b). The Presence of active and normal keratinocytes confirmed proper tissue regeneration.

Keratin filaments are visible in Fig 5 (b). These filaments verify the healing of the epidermis and the presence of active and normal keratinocytes as a result of tissue regeneration.

It has been demonstrated that chitosan including signaling factors encourages regeneration of tissue components in wound healing process and has progressive application effects on it [29, 30]. In this study, the result demonstrated the effective role of chitosan in wound healing and keratinocytes function. Keratinocytes have a significant function in wound healing, including accelerating deep second-degree burn wound healing, and their proliferation is vital for the complete restoration of the epithelium damaged during injuries. Besides, Chitosan gel improved keratinocyte cell growth by reason of its affinity to adhere to the negatively charged cell membranes its ability to bind serum factors.

4. Conclusion

In second-degree burns, the structure, and morphology of the keratinocytes, after the repair, did not change dramatically. Chitosan gels, as a covering, gently improved and accelerated healing conditions. It accelerated the contraction and re-epithelialization and thereby reduced the duration of healing in particular inflammation phase, reduced the scarring (depending on the depth and extent of the wound), and wound healing after repair regenerated an ideal appearance. The chitosan gel increased the healing more rapidly than a chitosan film, because it kept the wound moist. Chitosan gel easily interacts with skin. Although the rate of wound closure and re-epithelialization in untreated samples was less than in those treated with the chitosan gel, enclosures such as hair follicles in the skin of the control samples underwent faster healing and initiation of the activity.

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