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RESEARCH ARTICLE

Application of hydrogel chitosan extract from the house fly (*Musca domestica vicina*) on burn wound healing of mice

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Abstract

Burn injury is first and foremost an injury to the skin. The burn wound healing process of mice skin was histologically described during the first seven and fourteen days. To evaluate the effects of chitosan on early extension of burn wound, deep partial-thickness burns were performed on the dorsum of mice. Mice were subjected to burn injury on their shaved dorsum, and they were subjected to topical hydrogel chitosan, euthanized and specimens of skin were sectioned and stained.The results showed marked increase in wound contractions areas of mice treated with 7 and 14 days with hydrogel chitosan. An increased re-epithelialization in chitosan-treated animals at day 14^{th} of treatment, relative to the untreated burn control mice. The hydrogel-treated burns were so perfectly healed with intact re-grown epidermis, which was difficult to distinguish from normal skin. On day 7, a continuous incomplete epidermal layer was observed under the crust, and the number of inflammatory cells had decreased slightly. On day 14, a complete epidermal layer (comprising all the layers from the corneal to the basal layer). Also, rapid epithelialization was found in photomicrographs of wounds treated with the hydrogel indicated the wounds treated for 14 days shown improvement in wound healing activity compared to wounds treated for 14 days**.** These results indicate that chitosan induces could accelerate burn wound healing associated with diminished inflammation.

INTRODUCTION

Wounds are physical injuries that result in an opening or breaking of the skin. The ability of the skin to repair itself after a minor wound is remarkable, but when the damage is severe or occurs in large amounts of skin area, proper and immediate coverage of wound surface with an adequate dressing is needed to protect the wound and accelerate wound healing **(Chathopadhyay *et al*., 2002).**

Burns remain a public health issue, especially in terms of morbidity and long-term disability, throughout the world, but particularly in developing countries **(Koide, 1998).** Burn wounds have a complex healing process, cause severe discomfort, and are prone to infection and other complications. Burns are tissue lesions from thermal origin for exposure to flames, hot surfaces and liquids, extreme cold, chemicals, radiation, or friction **(Jorge and Dantes, 2003).** Burn injury damages blood vessels both in the immediate area of the wound to cause restriction or cessation of blood flow at the site of injury and modifications to blood flow in the surrounding area **(Zawacki, 1974).**

Even with improved prognosis **(Barret and Herndon, 2003)** and progress in the use of biological skin substituent **(Silva and de Castro, 2002),** burns are an important cause of mortality **(Sheridan *et al*., 2000).** The final aim of burn management and therapy is wound healing and epithelization as soon as possible in order to prevent infection and to reduce functional and aesthetic after effects (**Campos *et al*., 2005).**

Burns are classified depending on the lesion severity into superficial or first degree, when lesion is restricted to the epidermis or skin causing redness; partial thickness or second degree that can be superficial when

reaching the epidermis and superficial dermis, showing hypersensitivity and pain, or deep full-thickness (third degree) when it extends to the deepest layer of the dermis and may have reduced sensitivity with red and/or white coloration of the tissue **(Rangel and Pereira, 2007).**

Pereira *et al.* (2012) aimed to establish an experimental protocol for induction of deep second degree thermal lesions in Wister male rats to obtain clinical and histopathologic data that will facilitate understanding of results concerning the evolution of the healing action of topical therapeutic agents.

Insects are a large, unexplored and unexploited source of potentially useful compounds for modern medicine **(Pemberton, 1999).** A great array of insects and their products are used as drugs in the traditional medicines such as traditional Chinese and Korean medicine. The housefly (*Musca domestica*) belonging to the Diptera is common regarded as an important sanitary pest insect. However due to its short life cycle, high fecundity, and efficient digestion of organic waste, it has become a source of protein, chitin and chitosan **(Jing *et al.*, 2007; Hao *et al.*, 2008).**

In addition to their value as a livestock feed, other valuable materials, such as protein or peptides, chitosan, phospholipid, and antibiotics have been extracted from the housefly larvae **(Iaboni *et al.*, 1998; Hou *et al.*, 2007; Ai *et al.*, 2008).** **Zhang *et al.* (2011)** indicated that the dried housefly larvae contain approximately 55% protein, 9.1% crude chitin, and 8.8% fat**. Ren and Shi (2002)** found that their larvae are an excellent source of high-quality protein, polyunsaturated fats, vitamins, minerals and other nutrients for human and animal**.** Compared to shrimps or crabs **(Rodde *et al.*, 2008; Youn *et al.*, 2009),** the cuticle of the housefly larvae is much easier to extract chitin from because it contains smaller amounts of crude protein, crude fat, and ash.

Zhang *et al.* (2011) made an experiment to characterize the chitosan extracted from the cuticle of housefly larvae. Commercially, chitin and chitosan are of great importance owing to their relatively 6.89 % of nitrogen (**Dutta *et al.*, 2004**). Chitin is the second most natural abundant carbohydrate bipolymer next to the cellulose, and can be found in the exoskeletal materials of crustaceans and insects, and cell walls of bacteria and fungi. Differently from chitin, chitosan is soluble in acidic solution (pH< 6.4) as a result of the protonation of amino groups on the d-glucosamine residues. It is obtained by partial deacetylation of chitin. Furthermore, chitosan is a white, hard, inelastic, and nitrogenous polysaccharide found in the exoskeleton as well as in the internal structure of invertebrates. Because of its low toxicity, it was found that chitosan appears to have no adverse effects after implantation in tissues and, for this reason; it has been used for a wide range of biomedical applications **(Dutta *et al.*, 2004).**

Chitosan is used in drug delivery, cell delivery systems, orthopaedics, wound healing **(Daniele and Camelia, 2008),** ophthalmology, pharmaceuticals and bone healing **(Senel and Clure, 2004).** Many investigators found that chitosan is able to accelerate the wound healing, re-epithelialization and normal skin regeneration **(Tsai and Su, 1999; Daniela and Camelia, 2008). Alsarra *et al*. (2009)** reported that chitosan may be used to inhibit fibroplasia in wound healing and promotes tissue growth and differentiation in culture. **Shi *et al.* (2009)** found chitosan products to accelerate wound healing, decrease treatment frequency and give comfortable and painless wound surface protection. Therefore, the objective of this study was to evaluate the possible effect of chitosan on burn wound-healing as a gel extract from the adult housefly. The study was done to observe the induction of deep second-degree thermal lesions in the male mice to obtain histopathological data that will facilitate understanding of results concerning the evolution of the healing action of topical therapeutic chitosan agent.

2. Materials and methods

Rearing of *Musca domestica*

Cultures of *M. domestica* were established in the laboratory of Zoology Dep., Faculty of Science, Alexandria University. They were maintained at 27 ^{o}C, and relative humidity of 70 %. The larvae of these Musca were fed on chicken's mash.

Chitin extraction

Chitosan was prepared from the adult housefy according to the method of **Yen *et al.* (2007)**. After 4 four days of the developed adult flies, they were washed with 15% (W/V) aqueous sodium chloride solution and freeze-dried. The dried flies were grind using a mill to obtain crude powder, and 10 g of the dried was treated with 100 ml of 1 mol/L aqueous sodium hydroxide solution at 95 ^{o}C for 6 h to remove protein. The mixture was then filtered and washed with deionized water to obtain a neutral and crude chitin precipitate. Chitin was neutralized by washing with deionized water and freeze-dried. The crude chitin was N-deacetylated with 400 mg/mL sodium hydroxide solution

at 70 ºC for 8 h. After filtration, washing to neutralize with deionized water and freeze drying. For removing the impurities from raw chitosan, chitosan was then dissolved in acetic acid solution (1%) and the impurities were removed by filtration through nylon net with a mesh diameter of 0.050 mm, chitosan was obtained and stored at -20 ºC.

Preparation of hydrogel chitosan

Hydrogel chitosan was prepared by dissolving 2% (w/v) of chitosan in 1% (v/v) aqueous acetic acid. Methylparaben sodium salt (0.1%, w/w) as a preservative was added to the tested samples. The samples were stirred and the resulting gel solutions were sonicated to remove air bubbles **(Alsarra, 2009)**.

Experimental animals

Fifty mice were used in the evaluation of the wound healing properties of the tested agents. Healthy male albino Swiss mice, 12 weeks old and weighing 30 ± 5 g were obtained from the Faculty of Agriculture, Alexandria University, Alexandria, Egypt. They were housed in polypropylene cages (maximum of 4 mice per cage), had free access to food and clean drinking water. Also, they were acclimatized for two weeks under the controlled environmental conditions (12-h light/dark cycle, temperature 23 ± 2 ºC, humidity 55 ± 10%). This was in accordance with the recommendations in the University Guide for the Care and Use of Laboratory Animals, approved by Animal Care and Use Committee.

Establishment of skin burn wounds

Forty mice were subjected to 15–20% total body surface area burn injury after the hair on the dorsal area of each was carefully shaved. Next, each mouse was anesthetized, and the burn was induced by immersing skin surface for 5 min through a hole in the boiling water (reached 100 ^{o}C measured with a thermometer), where the second degree burn wound was established **(Liu *et al.*, 2009).** The burned mice were randomly divided into two experimental groups (20 mice/each). One group of the burned mice was treated with the hydrogel chitosan, and mice of the other one were no treated and considered as burned control mice. The treatment applied on the wound was carried out shortly after burn wound was produced, and 50 µl of the gel was applied to the burn wound bed, and the experiment was lasted for 14 days.

Visual observation of the burn wound

The burn wound size measurements for all burned mice and the treated were used to calculate the percent wound contraction, using the following equation **(Alsarra, 2009): %** wound contraction = A_0 - A_t/ A_0, where: A_0 is the original burn wound area, and A_t is the burn wound area at the time of biopsy.

Histological study

Mice were sacrificed by ether inhalation, and the skin, including the entire wound with adjacent normal skin was excised out. Specimens of the control untreated mice skin, the burned control skin as well as specimens of the treated burned skin were excised out on the 7th day and 14th day of experiment. Skin tissues were fixed in 10% neutral formalin, followed by routine histological processing (dehydration, clearing with xylol, and processed for embedding in paraffin wax) **(Bancroft and Gamble, 2007).** Thick skin sections of 5µ were stained with hematoxylin and eosin (H&E), examined under the light microscope, and photographed.

Statistical analysis

Statistical analysis for all data was done using the SPSS software package version 17.0, and the results were expressed as the mean ± Standard Deviation (SD).

3. Results

3.1. Macroscopic evaluation and gross appearance of skin wound of the burned mice

During the duration of experiment, no mouse was died, and most of the animals were remained healthy without evidence of infection. Furthermore, the results revealed that thermal burns are white in color, painful, and with no bubbles or edema until the 3rd day after burning. Gross changes and a general appearance as well as size of burn wounds were observed daily. The results revealed large wound burned areas in mice during the first three days after burn. However, on day 7th and 14th after treatment with hydrogel chitosan, mice showed a gradual observable increase in wound coverage. Furthermore, the results revealed significant increase in wound contraction areas in the 14th day (2.05 ± 0.21) after treatment with the hydrogel chitosan, compared with the 7th day (1.22 ± 0.12) of that treatment.

3. 2. Microscopic observations

3. 2. a. Histological appearance of skin sections of the control unburned mice

The skin is composed of two main layers, the epidermis and dermis, and a variable third layer, the hypodermis **(Figs. 1 & 2).** The epidermis is the external surface of the skin, where it is composed of a keratinized stratified squamous epithelium that covering the surface. The main cell populations of epidermis consists of many cellular layers: stratum basal (malpighian) which is the germinal layer of epidermis; stratum spinosum contains cells which are in continuous of growth and early keratin synthesis (cells without nuclei); stratum granulose which is characterized by intracellular granules, contribute to the process of keratinization, and the cornified layer consists of flattened cell remnants composed of the fibrous protein, keratin **(Figs. 1 & 2).**

The basal layer is the deepest layer of the epidermis and is responsible for the constant production of keratinocytes. The cells of this layer are cuboidal or low columnar in shape and are attached to the basement membrane, which separates it from the underlying dermis **(Fig. 2).** Further, the epidermis is supported by a thick layer of dense fibro-elastic tissue called the dermis. The fibroblasts that are found in the dermis act to form and maintain the structure of the collagen which gives the skin its tensile strength **(Figs. 2 & 3).** The junctions between the epidermis and dermis are characterized by downward folds of the epidermis called epidermal or ret ridges which interdigitate with upward projections of the dermis called dermal papillae. This dermis is attached to underlying tissues by a tissue of adipose connective tissue, called the hypodermis or subcutaneous layer which contains variable amounts of adipose tissue **(Fig. 4)**.

3. 2. b. Histological observation of skin wound of the burned mice

After initial burning and elimination of necrotic tissue, a deep second degree burn was noticed. The epidermis was completely destroyed and interrupted **(Fig. 5)** and the wound gap was filled by necrotic material and many inflammatory cells could be observed at the burn site **(Fig. 6).** The burned mice exhibited wounds covered with a thick layer of eschar **(Fig. 7).** The burned region is covered with blood clots, and the layers consisting of necrotic tissues and infiltrated cells are seen under the blood clots **(Fig. 8).** In addition, many hair follicles were basically disappeared**.** The dermal layer was half the thickness of the adjacent unburned region and the striated muscle showed necrotic myofibers in the deepest part of the wound.

3. 2. c. Histological observation of 7 days of skin wound of the burned mice after treatment with hydrogel chitosan

The results revealed that 7 days of the burned wound mice treated with hydrogel chitosan showed that the burn degree was less severe than the untreated burned mice, indicating that chitosan is greatly prevented the extension of burns. Although, the wounded region is covered with a crust, the epidermal layer is incomplete under the crust. Tissue still presented a moderate destruction of the epidermis and dermis, and incomplete re-epithelialization of the epidermis was observed **(Figs. 9 & 10).**

3. 2. d. Histological examination of 14 days of skin wound of the burned mice after treatment with hydrogel chitosan

Microscopic evaluation demonstrated that hydrogel chitosan-treated burns after 14 days were so perfectly healed with intact re-grown epidermis, and further rapid epithelialization was found, indicating an observable improvement in wound healing activity. Re-epithelization showed a higher number of newly formed multilayered epithelial layers **(Fig. 11)**. The epidermis was thickened at its cut edges as a result of mitotic activity of the basal cells **(Fig. 12).** In addition, the differentiation of nuclear keratinocytes to keratinized cells was continued. This was confirmed by the appearance of keratin layer above the epithelial layers with nuclear cells **(Fig. 12).** The density of hair follicles was more than those treated for 7 days with hydrogel chitosan. It was of an interesting to note marked reduction in the quantity of adipose tissue and occasional inflammatory cells. an increase in the thickness of the underlying skeletal muscle compartment, as well as the presence of many sweat and sebaceous glands are also observed in most skin sections **(Fig. 13).**

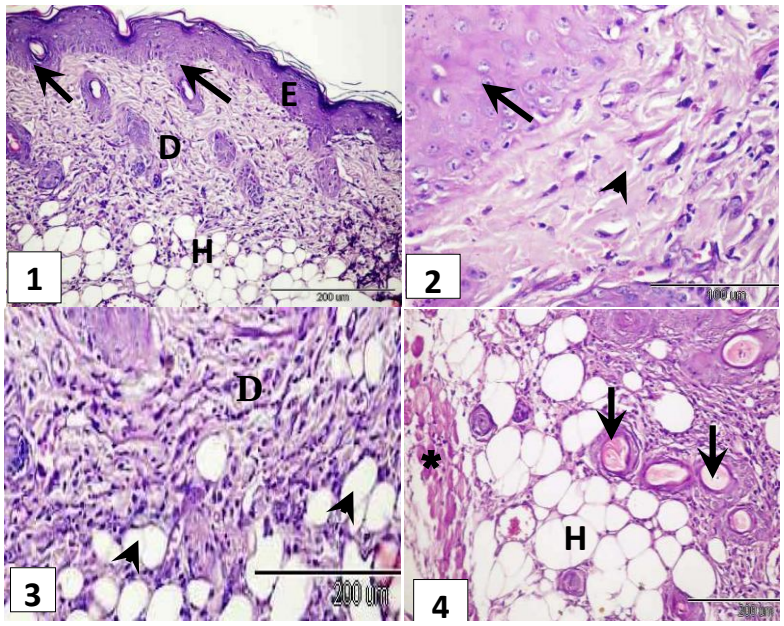
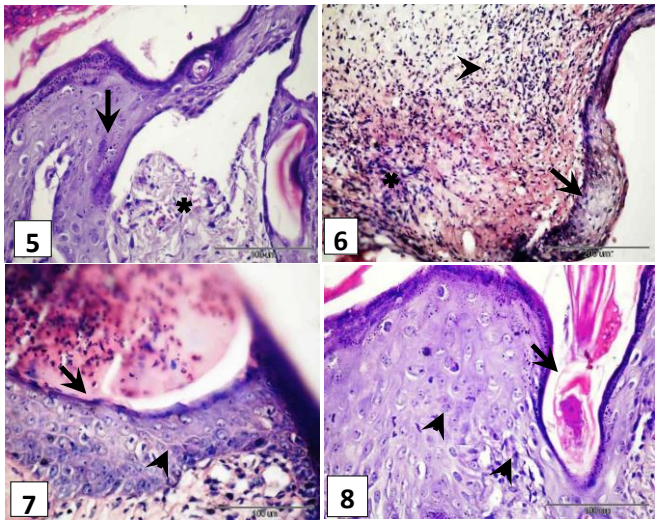


Fig. 1. Light micrograph of skin of control mice showing the keratinizedsratified squamous epithelium of epidermis (E) and dermis (D); Note: the dermal papillae (arrows) and hypodermis (H). H&E stain.

Fig. 2. Light micrograph of skin of control mice showing the cellular layers of epidermis: stratum basal (malpighian) and stratum spinosum (arrow); dermis consists of fibroblasts (arrowhead). H&E stain.

Fig. 3. Light micrograph of skin of control mice showing the layer of dermis (D) consists of fibroblasts; Note: the fat cells (arrowheads) of adipose tissue of hypodermis. H&E stain.

Fig. 4. Light micrograph of skin of control mice showing the adipose tissue of hypodermis (H); Note: the muscular layer (*) underneath the skin layers, and presence of hair follicles (arrows). H&E, stain.



Figs. 5 & 6. Light micrographs of the burned skin mice showing the deep second degree of burn; Note that the epidermis was completely destroyed and interrupted (arrows); the wound gap was filled by necrotic material (*); inflammatory cells (arrowhead) at the burn site. H &E stain.

Fig. 7. Light micrograph of the burned skin mice showing the infiltration of numerous inflammatory cells (arrow) at the site of burn; Note: the marked destruction of the epidermal layer (arrowhead). H & E, stain.

Fig. 8. Light micrograph of the burned skin mice showing that the burned region is covered with blood clots (arrow) and many inflammatory cells; Note the presence of necrotic tissue (arrowheads) under the blood clots. H&E stain.

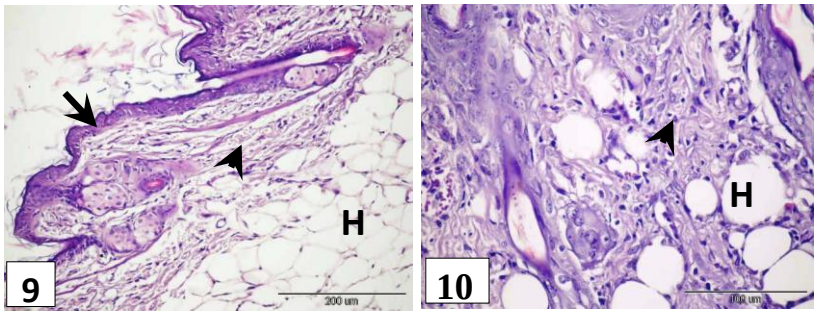


Fig. 9. Light micrograph of the burned skin mice treated with hydrogel chitosan for 7 days, showing the less degree of burning with an incomplete re-epithelization of the epidermal layer (arrow); Note: the moderate destruction of the dermal tissue(*) and the appearance of adipose tissue of hypodermis (H). H&E stain.

Fig. 10. Light micrograph of the burned skin mice treated with hydrogel chitosan for 7 days, showing the appearance of the dermal tissue(*) and the presence of many fat cells (adipose tissue) of hypodermis (H). H&E stain.

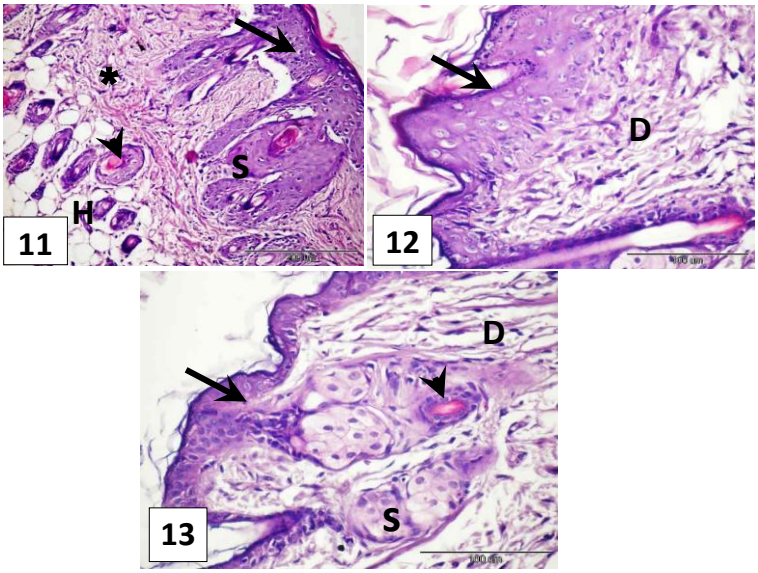


Fig. 11. Light micrograph of the burned skin mice treated with hydrogel chitosan for 14 days, showing the intact re-grown of epidermis (arrow); the moderate destruction of the dermal tissue(*)and the appearance of adipose tissue of hypodermis (H); the presence of many hair follicles (arrowheads) and sebaceous gland (S). H & E stain.

Fig. 12. Light micrograph of the burned skin mice treated with hydrogel chitosan for 14 days, showing the nuclear keratinocytes in the keratin layer above the epithelial layer(arrow); the appearance of dermal tissue (D). H&E stain.

Fig. 13. Light micrograph of the burned skin mice treated with hydrogel chitosan for 14 days, showing the re-epithelization of epidermis (arrow); the appearance of dermis (D); Note: the presence of hair follicle (arrowhead) and the sebaceous gland (S). H&E stain.

4. Discussion

The scope of this study was to determine the therapeutic effect of hydrogel chitosan on the extent of the burned wound healing acceleration. In the current work, the healing period of fourteen days of treatment with hydrogel chitosan was characterized by almost total regression of the inflammatory process, and the results revealed formation of epithelialization in wounds treated with chitosan. **Nagahama *et al*. (2007)** stated that chitosan can be prepared in the form of film or hydro-gel to be used in burn and wound dressing, and also for fabricating suturing threads.

Results of this study revealed thermal burns white in color, painful, with no bubbles, mild edema until the 3rd day after burn. Similar definition is reported by **Johnson and Richard (2003)** that describes the deep second-degree burns and injuries that have pale color with pain in lower intensity compared to superficial second degree burn. In our evaluation variation of the degree of hyperemia in the first three days of experiment that changed from slight to absent was observed.

Histological findings of the burn wounded skin treated with chitosan hydrogel on 14 day showed no inflammatory cells and positive findings were observed. The most significant morphological changes occur during the first seven days of wound healing after seven days of healing, all animals exhibited no complete reepithelization, and low disappearance of acute inflammatory signs. In addition, reorganization of granulation tissue devoid of cutaneous attachments as well as the presence of regenerating myofibers was observed. Moreover, the extent of inflammation at the wound site was decreased markedly in mice treated with chitosan so that the wound area was little and transient compared with the control burned group.

The primary factor in the acceleration of the wound healing process can be attributed to the molecular structure and the quantitative presence of N-acetyl-d-glucosamine **(Choi *et al*., 2001)** as well as the solubility of N-acetyl-dglucosamine and its rapid absorption by the tissues. High molecular weight with high degree of deacetylation chitosan samples demonstrates potential for use as a treatment system for dermal burns.

The primary aim of treatment of burns is to prevent infection, then to promote proliferation of epithelial cells. In the present study, the burn wound healing process of mice skin was histologically described during the first seven and fourteen days**.** Burn wound healing is a complex process, in which residual epithelial cells proliferate in an integrated manner to form an intact epidermis.

No *et al*. (1995) reported that the functional properties of chitosan are reported to be dependent on its molecular weight or viscosity. Many investigators reported that chitosan has been widely used for wound dressings in the form of hydrogel **(Boucard *et al*., 2007; Murakami *et al*., 2010; Ribeiro *et al*., 2009).** Also, **Wang *et al.* (2012)** used the hydrogel sheets of chitosan, honey and gelatin as burn wound dressings. **Kas (1997)** found that chitosan may be used to inhibit fibroplasia in wound healing and to promote tissue growth and differentiation in culture**.**

Alsarra (2009) applied the chitosan topical gel formulation in the management of burn wounds. Wounds treated with high molecular weight chitosan had significantly more epithelial tissue than wounds with any other treatment and the best re-epithelization and fastest wounds closure were found with the high molecular weight chitosan treatment group **(Bindu *et al*., 2010)**. Wound healing is a complex multi-factorial process of the replacement of dead tissue by a vital tissue **(Rubin and Farber, 1994).** This process results in closure of the wound and restoration of a functional barrier. According to **Mandelbaum *et al.* (2003)** the mechanism of tissue repair is the integration of dynamic cellular and molecular processes involving biochemical and physiological phenomena aiming at ensuring tissue restoration.

The healing and repair of the injured dermis occurs as a sequence of events, including three basic phases: inflammation, proliferation, migration, differentiation and maturation of different cell types **(Wang *et al*., 2008).** Wounds that demonstrate delayed healing 12 weeks after the initial insult are termed chronic wound, often as a result of prolonged pathological inflammation. **Shakespeare (2001)** used of keratinocytes as a skin substitute to illustrate some of the problems associated with the development during burn wound healing.

Jawad *et al.* (2007) suggested that hydrogel chitosan treatment has had a benefic influence on the various phases of wound healing. It has been reported that this natural polysaccharide permits regeneration of tissue elements in skin wounds and stimulates activity and/or has capacity to stimulate fibroblastic proliferation which releases interleukin-V and production of type III collagen, stimulation of the macrophages migration, and increase in effusion which forms thick fibrin.

Conclusion: Wounds treated with hydrogel chitosan healed markedly on the 14 day, and histological observations showed that new granulation tissue and epithelialization progressed quickly.

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