

Application of chitosan for wound repair in dogs.

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Abstract: This experimental work was applied to study the effect of topical application of chitosan powder on the stimulation of healing of full thickness skin wounds. Experimental surgical wounds were done in 12 apparently healthy male Mongrel dogs of nearly the same age and weight. Experimental animals were classified into 4 groups each consisted of 3 dogs according to time of euthanasia. Full thickness equal longitudinal skin incision wounds were created on each dog's both side at the dorsal aspect of the animal. A comparative study was applied between wounds treated with chitosan powder and control wounds washed only by saline solution. Wound healing was clinically evaluated during the period of the experiment. Euthanasia was done at different period intervals, one week, two weeks, three weeks and four weeks after incisions. Specimen were taken for histopathological investigations. Results proved rapid regeneration and reepithelization of the wounds treated with chitosan powder compared with those of the control group. Clinically, complete healing was seen after 3 weeks in the chitosan treated wounds which delayed to 4 weeks in the control ones. Histopathological investigations proved presence of more pronounced granulation tissues in the chitosan treated wounds than in the control ones. Healing started at 3 weeks post- incision in the treated group and complete repair was achieved at four weeks. Complete regeneration of epidermal cells with keratin layer occurred which was similar to the normal skin associated with dermal connective tissue proliferation. The fibroblast cells laid down a network of collagen fibers which appeared as wavy collagen bundles surrounding the neovasculature of the wounds, whereas, in control group, hyalinosis of subcutaneous granulation tissue and haphazardly arranged collagen fibers were observed. In conclusion, chitosan proved to be a suitable biomedical agent used for the acceleration of wound repair due to its biocompatibility, easy application and high effectiveness.

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1. Introduction

The skin is considered the largest organ of the body and it has many different functions. Cells on the surface of the skin are constantly being replaced by regeneration from below with the top layers sloughing off. The repair of an epithelial wound is merely a normal physiological process (Willi and Sharma, 2004). Wound healing depends on elimination of any source of infection and regeneration of the lost layers (Ishihara, Ono, Sato, Nakanishi, Saito, Yura, Matsui, Hattori, Fujita, Kikuchi and Kurita, 2001).

The majority of wounds heal without any complications, other types are chronic and resist the normal repair. Chronic non-healing wounds involving progressively more tissue loss giving rise to the biggest challenge to wound-care product researchers (Ueno, Yamada, Tanaka, Kaba, Matsuura^{c-}, Okumura, Kadosawand Fujinaga , 1999)

One of those new products widely used in the medical field is chitosan , a water soluble and a bioadhesive product extracted primarily from shellfish sources. More than 40 years have elapsed since this biopolymer had aroused the interest of the scientific

community around the world for its potential biomedical applications (Takashi and Toru, 2001 and Stoker, Reber, Waltzman, 2008). Its role in wound healing has been studied by many researchers (Ueno, Yamada, Tanaka, Kaba, Matsuura, Okumura, Kadosawa & Fujinaga, 1999).

Chitosan is also one of the bioactive dressing which delivers substances active in wound healing (Jayasree , Rathinam and Sharma, 1995). In dogs satisfactory results were obtained regarding healing with fast hemostasis, rapid regeneration of the full thickness wounds that involved the muscle layers (Ueno Yamada, Tanaka Kaba, Mitsunobu Matsuura , Okumura, Kadosawa & Fujinaga, 1999).

Biochemical and histological changes of chitosan in wound healing has been reviewed by (Azad, Sermsintham, Chandkrachang, and Stevens, 2004) as chitosan provides a non-protein matrix for three dimensions tissue growth and activates macrophages for tumoricidal activity. It stimulates cell proliferation and histoarchitectural tissue organization. It will gradually depolymerize to release N-acetyl-b-D-glucosamine, which initiates fibroblast proliferation

and helps in ordered collagen deposition, stimulating increased level of natural hyaluronic acid synthesis at the wound site. **Okamoto, Shibasaki, Minami, Matsuhashi, Tanioka, Shigemasa (1995)** and **Jarmila & Vavrikov (2001)** mentioned that, chitosan helps in faster wound healing and scar prevention, chitosan derivatives have also an antimicrobial, antitumour and antioxidant activities. The introduction of azido functions in chitosan has provided photo-sensitive hydrogels that crosslink in a matter of seconds, thus paving the way to cytocompatible hydrogels for surgical use in wounds as coatings, scaffolds, drug carriers and implants capable to deliver cells and growth factors (**Minami, Okamoto, Matsuhashi, 1992**).

The aim of the present work was to study the effect of topical application of chitosan on full thickness open skin wound regeneration and to evaluate its efficacy as an accelerator in wound repair.

2. Material and Methods

This experimental work was carried out on 12 experimental apparently healthy male Mongrel dogs of nearly the same age and weight. The animals were divided into 4 groups each group consisted of 3 animals. Aseptic preparation for the intended surgical fields was done and two full-thickness equal 10 cm longitudinal skin incisions were created on each dog's both side at the dorsal aspect of the animal. In each dog, one wound (left side) was treated with chitosan powder and the other wound (right side) was washed by saline solution and considered as a control. The 4 groups were divided according to time of euthanasia as follows: group I intended for euthanasia 1 week postwounding, group II 2 weeks, group III 3 weeks and group IV at 4 weeks post- incision. .

Chitosan powder was repeatedly applied on the wounded areas (one application per day) and the control wounds were washed only by saline solution (fig.1). Clinical observations were done at 3 days and 1,2,3,4 weeks post-incision. Examined clinical parameters included measuring the wound length, examination of the wound for the presence or absence of infection and progress of healing process.

All animals were euthanatized at each intended previously mentioned period and full thickness specimen were taken from each wound site including the neighboring tissue for histopathological examination to evaluate the process of wound

healing. Collected samples were preserved in formalin solution 10 % concentration, sectioned and processed, then stained with Hematoxylin & Eosin as well as Trichrome stain (**Bancroft, Stevens, and Turner, 1996**) and subjected for histopathological examination.



Fig (1): An experimentally incised wound in a dog, showing topical application of chitosan powder.

3. Results

Clinical observation of the treated and control wounds indicated that hemostasis was clear directly after the topical application of chitosan. After 3 days, treated wounds showed fresh well defined straight lips diminished from 10 cm length to 8 cm and the muscle layers showed coaptation and healing in the treated wounds faster than in the control ones. One week postwounding the chitosan-dressed wounds had been healed more promptly as compared with the control wounds manifested by regular wound lips and the wound's depth diminished with minimal gap than in the control group (fig. 2). Two weeks post- incision, the chitosan treated wounds showed clean sharp apposed wound lips with complete muscle and subcutaneous tissue healing compared with the control wounds which showed larger apertures and delay in muscle healing (figs.3,4). Three weeks post- incision, nearly complete regeneration of all treated wounds occurred which delayed in the control wounds. The incisions of the chitosan treated wounds occluded with growth of hair tufts covering the healed lips. The control wounds showed partial occlusion with little apertures indicating delayed healing (fig. 5). Four weeks post-incision, the control group showed complete healing and the skin was covered with hair.



Fig (2): One week postwounding. A- represents the chitosan treated wounds B- represents the control wounds



Fig (3): Two weeks postwounding , notice, A- treated wounds diminished in size and healed faster and in a regular manner than the control ones, B.



Fig. (4): Two weeks postwounding, notice the muscle layer healed in the chitosan group (A) faster than in the control group (B).



Fig (5): A- Three weeks postwounding representing treated wounds completely healed and covered by hair, B- Control wounds. Notice , healing and closure of the wounds was delayed with the presence of small aperture.

Histopathological results:

Results correlated well to gross findings. One week post-incision, the chitosan treated wounds showed a hyperactive fibroblastic proliferation (fig.6 a), whereas, in control group, the pronounced picture was massive infiltration with neutrophils associated with haemorrhage (fig.6 b).

At 2 weeks post- wounding, angiogenesis was early in the treated group with formation of new blood vessels, fibroblasts proliferation associated with infiltration with polymorphnuclear cells and macrophages (fig. 6 c). Examined control sections revealed epidermal and dermal necrosis associated with infiltration with neutrophils and macrophages with fibroblasts proliferation (fig. 6 d).

3 weeks post-wounding granulation tissue was more pronounced by the chitosan treatment than in the control group, proliferation of epidermal cells with different mitotic figures (fig. 6 e) as well as highly vascularized and organized subcutaneous granulation tissue was noticed (fig. 6 f).

In the chitosan treated group, 4 weeks post-wounding, complete regeneration of epidermal cells with keratin layer similar to the normal skin associated with dermal connective tissue proliferation (fig. 7 a). The fibroblast cells laid down a network of collagen fibers appearing as wavy collagen bundles surrounding the neovasculature of the wound (figs. 7 b & c), whereas, in control group, examined sections revealed massive infiltration with polymorphnuclear cells, hyalinosis of subcutaneous granulation tissue (fig. 7 d) and haphazardly arranged collagen fibers (fig. 7 e).

4. Discussion

Restoration of tissue continuity after injury is a natural phenomenon, The process of wound healing begins immediately following surface lesions or just

after exposure to radiation, chemical agents or extreme temperatures (Cohen, 1989). Some biomaterials were found to accelerate wound regeneration and increase the quality of the wound healing (Gomma, 2010). One of the new biomaterials is chitosan which exhibits a variety of physicochemical and biological properties resulting in numerous applications in fields such as water treatment, agriculture, fabric and textiles, cosmetics, nutritional enhancement, and food processing (Willi, Paul and Sharma, 2004). In addition to its lack of toxicity and allergenicity, and its biocompatibility, biodegradability and bioactivity make it a very attractive substance for diverse applications as a biomaterial in pharmaceutical and medical fields (Balassa & Prudden, 1984 and Sevda & McClure, 2004).

The present study investigated its use for wound repair and in promoting skin wound epithelisation where it has been tried as a wound-healing accelerator in veterinary medicine (Gomma, 2010).In the present study, after the application of chitosan topically, local hemostasis was directly observed in all treated animals which is considered beneficial in accidental bleeding wounds and could act as a hemostatic dressing as well as for wound healing.This result agreed with (Pusateri, McCarthy, Gregory, Harris, Cardenas, McManus and Goodwin, 2004) who mentioned that hemostasis is immediately obtained after application of most of the commercial chitin-based dressings to traumatic and surgical wounds. Our clinical observations indicated that chitosan showed effective results in treatment of open wounds as the surgical wounds treated with chitosan powder had accelerated and early stages of healing compared with the control wounds applied in the same animal under the same conditions, this was attributed to its action as chitosan

is considered a film forming and protective polysaccharide.

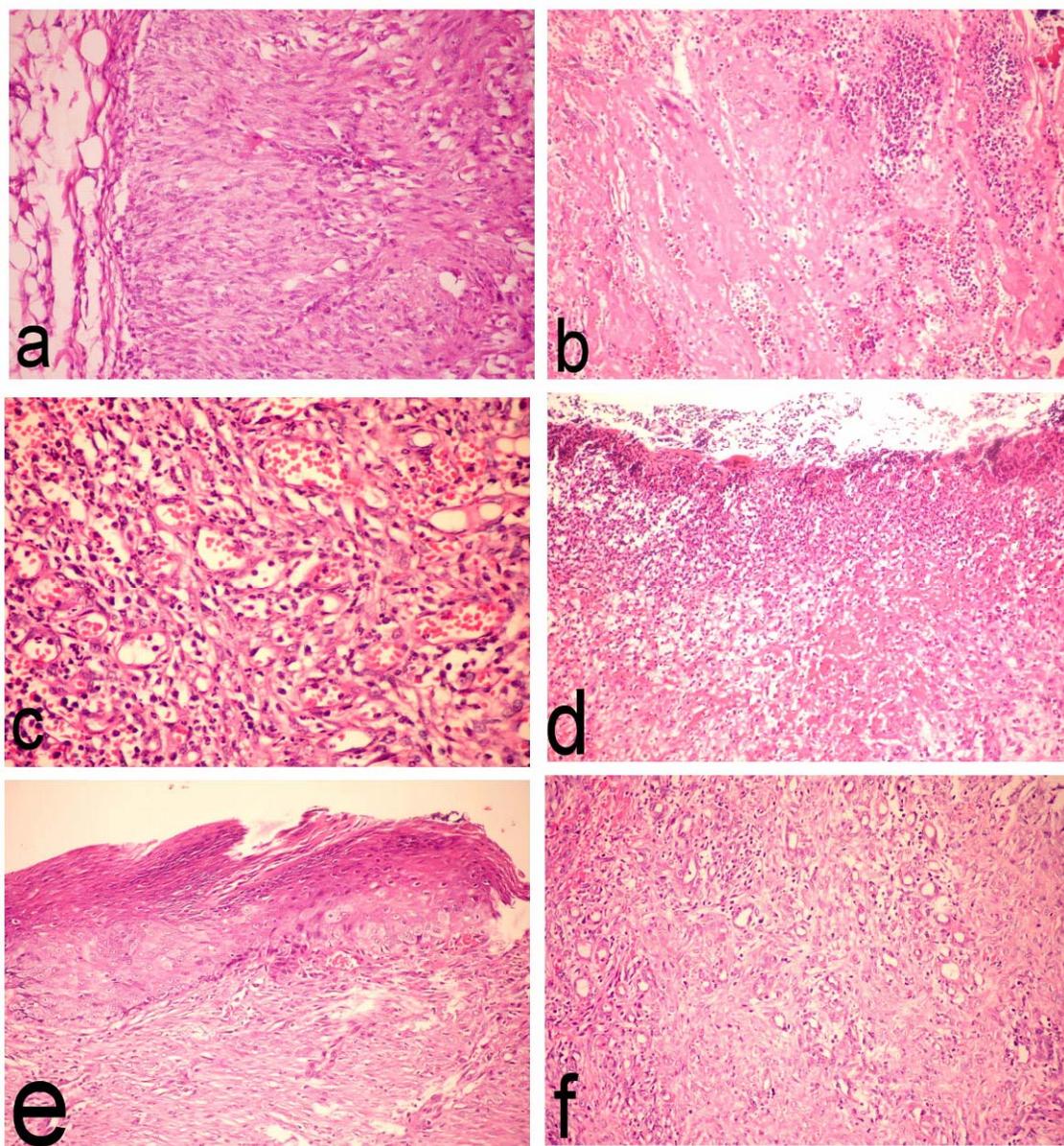


Fig. (6): Photomicrograph of skin wounds in the experimental cases:

- a.** Wounds treated with chitosan 1 week post-wounding showing hyperactive fibroblasts proliferation (H & E stain X 400).
- b.** Control wounds 1 week post-wounding showing massive infiltration with neutrophils associated with haemorrhage (H & E stain X 200).
- c.** Wounds treated with chitosan 2 weeks post-wounding showing angiogenesis with formation of new blood vessels, fibroblasts proliferation associated with infiltration with polymorphnuclear cells and macrophages (H & E stain X 400).
- d.** Control wounds 2 weeks post-incision showing epidermal and dermal necrosis associated with infiltration with neutrophils and macrophages with fibroblasts proliferation (H & E stain X 200).
- e.** Wounds treated with chitosan 3 weeks post-incision showing proliferation of epidermal cells with different mitotic figures and dermal granulation tissue formation (H & E stain X 200).
- f.** Chitosan treated wounds 3 weeks post-wounding showing highly vascularized and organized subcutaneous granulation tissue (H & E stain X 200).

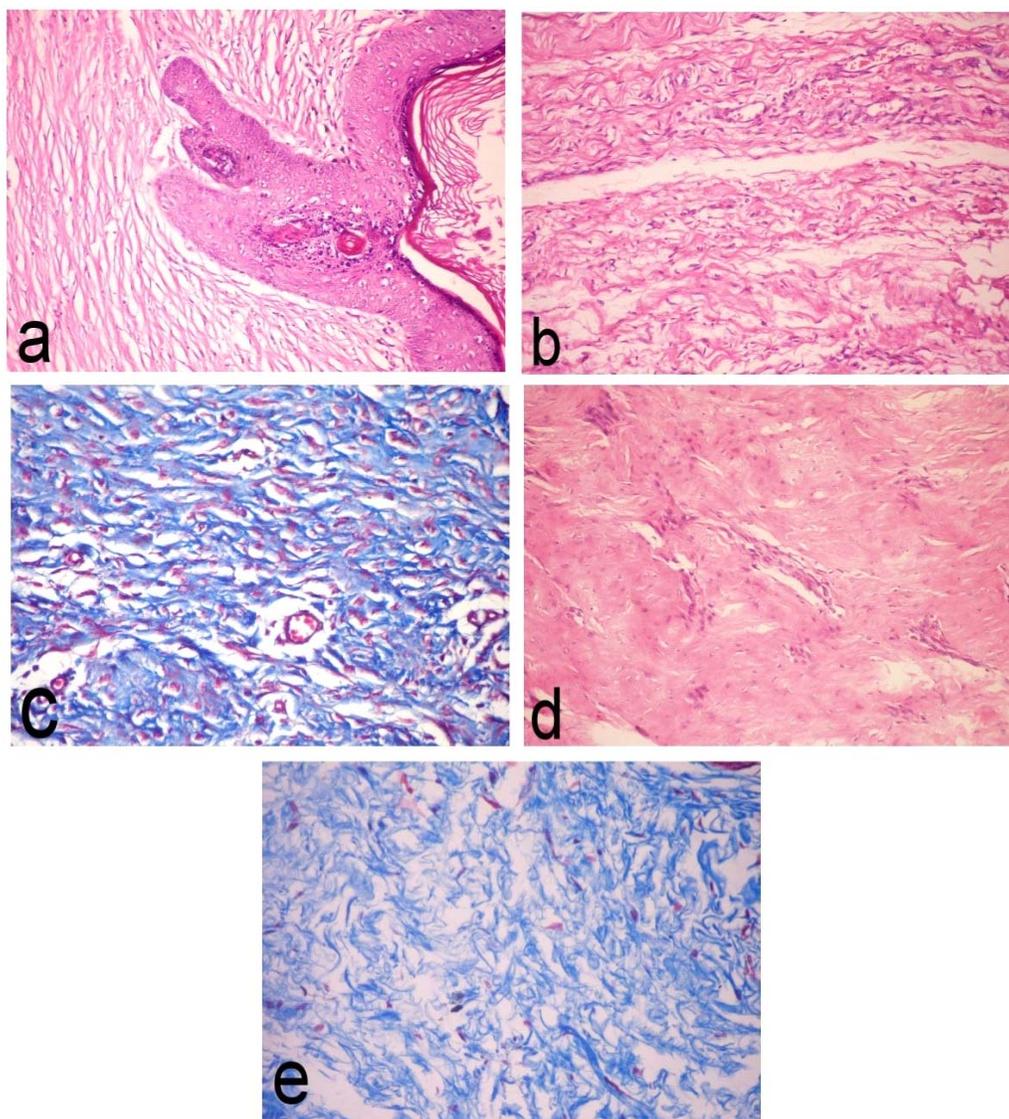


Fig. (7): Photomicrograph of skin wounds in the experimental cases:

- a. Wounds treated with chitosan 4 weeks post-wounding showing complete regeneration of epidermal cells with keratin layer similar to the normal skin associated with dermal connective tissue proliferation (H & E stain X 200).
- b. Wounds treated with chitosan 4 weeks post-wounding showing wavy collagen bundles surrounding the neovasculation of the wound (H & E stain X 200).
- c. Wounds treated with chitosan 4 weeks post-wounding showing wavy collagen bundles surrounding the neovasculation of the wound (Trichrome stain X 200).
- d. Control wound 3 weeks post-wounding showing hyalinosis of subcutaneous granulation tissue (H & E stain X 200).
- e. Control wound 3 weeks post-wounding showing haphazardly arranged collagen fibers (Trichrome stain X 200).

It also promotes the healing of ulcers and burns as was mentioned by (Muzzarelli, 2009). In previous studies, it was mentioned that, the aqueous solution of water soluble chitosan embedded in the wounds was

found to be more efficient as a wound-healing accelerator (Minami, Okamoto, Tanioka, 1992).

Our histopathological results correlated well with the clinical observations as wounds treated with chitosan at 1 week post-wounding showed hyperactive

fibroblasts proliferation which appeared earlier compared with the control wounds that showed massive infiltration with neutrophils associated with haemorrhage.

Two weeks postwounding, advanced granulation tissue formation was more pronounced by the chitosan treatment associated with formation of new blood vessels than in the control group. At that time, there was an increase in the fibroblasts proliferation with infiltration of polymorphonuclear cells which was attributed to the effect of chitosan that enhances the functions of inflammatory cells and phagocytosis to promote healing as was mentioned by (Takashi and Toru, 2001). Promoted angiogenesis was explained by (Muzzarelli, 2009 and Gomma, 2010) as production of the vascular endothelial growth factor is strongly up-regulated in wound healing when macrophages are activated by chitosan. Also blood platelets were activated by chitosan with redundant effects and superior performance which was necessary to support physiologically ordered tissue formation. On the other hand, at that time, control wounds showed epidermal and dermal necrosis associated with infiltration with neutrophils and macrophages with fibroblasts proliferation.

Wounds treated with chitosan 3 weeks post-incision showed early proliferation of epidermal cells and reepithelization with different mitotic figures and dermal granulation tissue formation. On the other hand, in the control wounds, results showed hyalinosis of subcutaneous granulation tissue. Highly vascularized and well organized subcutaneous granulation tissue was seen in the treated wounds, however, the control wounds showed haphazardly arranged collagen fibers.

Four weeks post-incision treated wounds showed complete regeneration of epidermal cells with keratin layer similar to the normal skin associated with dermal connective tissue proliferation. Wavy well oriented mature collagen bundles were seen. On the other hand, control group showed haphazard orientation of the collagen fibers. Those results coincided with Yong-Woo, Yong-Nam, Sang-Hun, Gyeol, Sohk-won, (1999).

Those previous results indicated acceleration of the wound healing compared with the normal physiological repair. Same findings were mentioned by (Muzzarelli, 2009) as chitosan enhances the functions of inflammatory cells and production of leukotriene B₄, macrophages interleukin (IL)-1, transforming growth factor β 1 and platelet derived growth factor and fibroblasts. Moreover (Ishihara et al; 2001) added that, the chitosan mesh membrane showed a positive effect on the re-epithelialization and the regeneration of the granular layer.

In this experimental work, all the chitosan treated wounds were not dressed by any local antiseptic and animals did not receive systemic antibiotics but showed clean and uncomplicated healing this may be attributed to the biological properties of chitosan as it possess bacteriostatic and fungistatic properties particularly useful for wound treatment as was mentioned by (Jarmila and Vavřiková, 2011).

It is important to mention that, in this experiment all the wounds were surgical incisions where there was very little tissue loss, aseptic operations and were easy to heal, meanwhile, accidental or chronic wounds disrupt normal process of healing and is often not sufficient itself to effect repair.

As was mentioned by (Okamoto *et al.*, 1995), complications of chitosan application included the potential for allergy in individuals allergic to shellfish. Clinical trials reported few adverse events, generally limited to flatulence and constipation. Second complication was lethal pneumonia in dogs when given a high dose of chitosan. On the other hand, our results in the application of chitosan powder in dogs did not face any complications, this may be due to the low doses topically applied on treated wounds. Previous researches considered the use of chitosan solution is more suitable for wound-healing than powder due to its easy application and high effectiveness.

In conclusion this study proved that the biomedical product chitosan is effective for tissue regeneration and showed better and faster tissue epithelization. As far as we can see, chitosan seemed to have no known side-effects and safe. Moreover from the different studies reported in the available literatures and our results, chitosan seems to be an excellent candidate dressing material for wound repair.

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