

EFFECT OF CHITOSAN SHEET ON INFLAMMATION IN RABBITS

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ABSTRACT

This study was conducted to investigate the effect of chitosan sheets on inflammation. Accordingly chitosan sheet was isolated and prepared from the exoskeleton of the native shrimps in Basrah Province. The inflammation was induced and evaluated by polyvinyl sponge to determine the events which occur during the inflammatory process during different intervals in the presence or absence of chitosan sheets. For this purpose 24 male rabbits were used and two linear skin incisions (2 cm) were made on the dorsal aspect in both sides of each rabbit. The inflammatory process at 1, 3, 7 & 15 day post wounding was evaluated macroscopically in terms of hyperemia, suppuration, dryness, and adhesion of margins and microscopically in terms of infiltration of inflammatory cells specially neutrophils and macrophages, fibrovascular granulation tissue.

The results of macroscopic evaluation revealed that at first post wounding day the test wound was significantly ($p < 0.05$) more hyperemic than control wounds followed by a significant decrease in the severity of hyperemia in test wounds at 1st, 3rd, 7th, and 15th post wounding days ($p < 0.05$). The test wounds were more dry with less suppuration towards the end of the experiment than control wounds ($p < 0.05$). The adhesive contact between the margin of the wounds was significantly higher in test wounds than in control wounds throughout the period of experiment ($p < 0.05$).

The microscopic evaluation was showed significant elevation of the infiltration of neutrophils in the sponge of test wounds compared to that control wounds. From the 3rd day the severity of neutrophils infiltration became significantly lesser in test wounds and disappeared completely at 15th day ($p<0.05$). The infiltration of macrophages was significantly more in the sponge of test wounds than in control wounds through the period of experiment. Fibrovascular granulation tissue significantly more obvious in test wounds than in control wounds through out the period of experiment ($p<0.05$).

INTRODUCTION

Inflammation is a defense reaction induced by damage or injury, characterized by redness, swelling, hotness, pain and loss of function .The primary objective of inflammation is to localize and eradicate tissue injury and repair the surrounding tissue. In fact for the survival of the host, an inflammation is necessary and it is beneficial process (1).The sequel of acute inflammation depend upon the type of tissue involved and the amount of tissue destruction, which depend in turn upon the nature of the injurious agent. The possible out comes of acute inflammation are resolution, suppuration, organization and progression to chronic inflammation. (1,2).

Chitosan is used for making medicinal preparation of prolonged effect, production of switch material which is homocompatible, biodegradable and highly stable in wet condition, production of burn and wound healing dressing, making artificial skin graft, artificial kidneys and lowering the acidity of juices and extracts (Anti-ulcer) (3,4).

Chitosan exhibits myriad biological action namely: hypocholesterolemic, anti-microbial, anti-inflammatory, and wound healing properties (5).

Chitosan, as a polysaccharide, has been considered to be advantageous in their applications as a wound dressing material. Both chitin and chitosan have many useful and advantage biological properties in wound dressing namely their biocompatibility, biodegradability, haemostatic activity, anti-infectious activity and the property to accelerate wound healing (6,7,8). The presence of non-biodegradable foreign material in soft tissue often causes foreign body reaction elicited by the body's immune system that can result in sever inflammation and soreness around the implant site. However, biocompatible, biodegradable polymers may elicit an initial immune response, it is often

possible to control their degradation rate to decrease the length of time during which foreign material is in contact with living tissue (9).

Howling *et al.* (10) studied the effect of chitosan on the proliferation of dermal fibroblast and Keratinocytes *in vitro*, their results demonstrated that highly deacetylated chitosan can modulate human skin cell mitogenesis *In vitro*. Analysis of chitosan effect on cells in culture may be useful as a screen for its potential activity *In vivo* as wound healing agent.

Ueno *et al.* (11) concluded that chitosan is beneficial for large open wound in animals by enhancing the functions of inflammatory cells and promoting the organization and granulation tissue formation.

From the mentioned above, this study is designed to answer the following questions: Does Chitosan has effect on an inflammatory response, *in vivo*? And what are the in inflammatory components affected with Chitosan?

MATERIALS AND METHODS

Isolation and Purification of Chitosan Sheet: The process of isolation and preparation of Chitosan sheet includes three stages; isolation and purification of chitin, isolation of chitosan, and preparation of chitosan sheet.

Isolation and Purification of Chitin: The chitin was extracted from exoskeleton of shrimp available in local market using the Hirano (12) method with simple modification. Briefly the exoskeleton was washed with Water, left at room temperature till full dryness, chopped with mortar and hammer then grounded. One hundred fifty grams of the powder treated with diluted solution of 3-5% hydrochloric acid (HCl) for 24 hours then filtered, the precipitate washed with distilled water until neutralization and dried. The precipitate was treated with one liter of 3-5% sodium hydroxide (NaOH) at 90°C with continuous mixing for 3 hours, filtered and the precipitate was washed until neutralization, then dried. The resulting brown granules 24% from the original exoskeleton of shrimp, that was used as first extraction as chitin.

Isolation of Chitosan: Twenty four grams of the isolated chitin was treated with 750ml solution of (40-50%) sodium hydroxide (NaOH) at 90°C with continuous mixing by magnetic stirrer for 2 hours, left to cold, flittered and washed with water, then neutralized 3 times with 10% HCl till reaching a pH of 8, then filtered and dried, the resulting product is chitosan, as a white powder. The percentage of chitosan obtained material was 13% from the original exoskeleton of shrimp that used as the first step (12).

Preparation of Chitosan Sheet: Two grams from the prepared chitosan was dissolved in 100ml of 0.1N of glacial acetic acid at room temperature for 24 hour to ensure complete dissolving. The resulting solution was flittered, put in a Petri dish and left to dry at room temperature, a transparent sheet was obtained. The sheet was carefully peeled off from Petri dish and stored in an air tight glass container maintained at room temperature until further investigation (13). **Fabrication of Discs:** Polyvinyl sponge (Iraq, local industries) of standard size (2x1x1cm) and 100mg dry weight. The cleansed sponge was sandwiched between 2 discs of cut silicon rubber (Arther H. Thomas and Co.) each disc was 0.2 cm thick and was trimmed to fit the dimensions of the sponge. The three discs were secured together with a centrally located silk stitch. The polyvinyl sponge put in glass container and left for 30 minute in autoclave for sterilization before used (14). Polyvinyl sponge model, was designed to induce inflammation and to study the significance of the inflammatory process in wound repair, the two silicor rubber discs were used in order to eliminate cellular infiltration from the top and bottom surface of the discs. **Animals and Husbandry:** Twenty four adults' male, domestic rabbits, weighing 1-1.5 kg, were bought from the local market. The animals were examined physically to ensure a good health then housed in separated cages (25-28°C), left in the laboratory for 7 days for acclimation, fed a commercial rabbit food and allowed unlimited water.

Surgical Operation: The surgical operation was carried out under general anesthesia by i.m. administration of 10mg/kg xylazine hydrochloride (Romepun, Bayer) and 500mg/kg ketamine hydrochloride (Ketanes, Alke). The rabbits were shaved at the back, near the neck, followed by rubbing the skin with 70% ethanol. Two linear skin incisions were made on both sides of the dorsal aspects, near the neck of each rabbit. The incisions were made by sterile scalpel with aseptic technique through the epidermis, dermis, and subcutaneous fat; each incision was 2 cm long. The right-sided wound was used as test and left-sided wounds as control. One polyvinyl sponge was implanted in each wound, both test (right) and control (left) wounds, then two chitosan sheets 0.3gr with suitable size, were inserted in the test wounds on either sides of the sponge in the wound. The chitosan sheets were not used in control wounds. The wounds were closed by interrupted suturing, the wounds covered with gauze band to prevent detachment, self infection or cutting suture.

Macroscopic Examination: Postoperatively, all rabbits were examined daily for general health condition. The Wounds were examined at intervals of 1, 3, 7 and 15 days after

wound creation. The score of 1-3 was used as mentioned in Table (1) to evaluate the dryness of wound, suppuration, hyperemia, and epithelialization (adhesive contact between wound margins). (15).

Table (1): Scoring the Hyperemic, Dryness, Suppuration and Epithelialization of Chitosan Sheet Tested Wounds

Score	Hyperemia	Dryness of wound	Suppuration	Epithelialization
1	++	-	++	-
2	+	+	+	+
3	-	++	-	++

Microscope Examination: The wounds (control and test) were excised (under general anesthesia) at intervals of 1st, 3rd, 7th and 15th post wounding days, 6 animals at each intervals including the sponge by an elliptical incision around the wound. The biopsy specimen put in 10% phosphate buffered formalin fixative for at least 48 hours. Three millimeter thickness slice taken across the wound margins and the underlying sponge. After proper fixation the biopsies were dehydrated with alcohol, followed by dealcoholization by xylene, and then impregnated with paraffin wax and blocked. Using the rotary microtome, representative 3-5µm thick sections were obtained and stained with haematoxyline – eosin (H-E) stains Histopathological examination to evaluate of inflammatory reaction was made according to the severity of neutrophils and macrophages infiltration, new blood vessels and fibroblast proliferation and collagen deposition, those were scored as follows: Score (0) represents (non) Score (1) represents (mild), Score (2) represents (moderate) Score (3) represents (severe).

RESULTS

Macroscopic Evaluation: Table (2) showed the macroscopic appearance of inflammation tested with chitosan sheet compared with the control wounds at different intervals. At the first post wounding day the test wounds were significantly ($P < 0.05$) more hyperemic than control wounds. This is followed by a significant decrease in

severity of hyperemia in test wounds at 3rd, 7th, and 15th post wounding days ($P < 0.05$) than control wounds.

The test wounds became drier with less suppuration towards the end of the experiment than control wounds ($P < 0.05$). The adhesive contact between the margins of wound was significantly higher in test wound than in control wounds through out the period of experiment ($P < 0.05$).

Microscopic Evaluation: The results of microscopic evaluation of inflammation in both test and control groups are showed in Table (3). At first post wounding day the infiltration of neutrophils was significantly more severe in the sponge of test wound than the sponge of control wounds Fig.(1&2). From the 3rd day, the severity of neutrophils infiltration became significantly less in test wounds and disappear completely at 15th day ($P < 0.05$) compared with control wounds. Fig (3&4). The infiltration of macrophages was significantly more in the test wound, fig. (3) than in the control wound, fig. (4) through out the period of experiment with the absence of macrophage in treated wound at (15th) day compared with control wound which showed mild-moderate presence of macrophages. The fibrovascular granulation tissues started to appear at 3rd day in both test and control groups, it is significantly more obvious in test wound fig. (5),(6) than in control wounds fig. (7). through out the period of experiment ($p < 0.05$).

Table (2): Hyperemia, Suppuration, Dryness and Adhesion of Margin of wound for chitosan sheet (T) and control (C)

Duration of wound	Hyperemic	Suppuration	Dryness of Wounds	Adhesion of margin
1 st day (T)	1.00 ± 0.000 ^a	1.1 ± 0.40 ^a	1.00 ± 0.000 ^a	1.0 ± 0.000 ^a
1 st day (C)	1.5 ± 0.54 ^b	2.0 ± 0.000 ^b	1.00 ± 0.000 ^a	1.00 ± 0.000 ^a
3 rd day (T)	2.3 ± 0.51 ^c	2.3 ± 0.51 ^c	2.0 ± 0.000 ^b	1.6 ± 0.51 ^b
3 rd day (C)	1.1 ± 0.40 ^d	1.6 ± 0.51 ^d	1.3 ± 0.51 ^c	1.0 ± 0.000 ^c
7 th day (T)	2.8 ± 0.40 ^e	2.6 ± 0.51 ^e	2.6 ± 0.51 ^d	2.3 ± 0.51 ^d
7 th day (C)	1.8 ± 0.40 ^f	1.6 ± 0.51 ^f	1.5 ± 0.51 ^c	1.0 ± 0.000 ^c
15 th day (T)	3.0 ± 0.000 ^g	3.0 ± 0.000 ^g	3.0 ± 0.000 ^f	3.0 ± 0.000 ^f
15 th day (C)	3.0 ± 0.000 ^g	2.0 ± 0.000 ^h	2.3 ± 0.51 ^g	2.0 ± 0.000 ^g

-Number of animals =24 rabbit, means ± SD., T = test wounds, C = control wound.

-The difference in the letters means significant at (p<0.05).

Table (3): Neutrophils, macrophages infiltration, New blood capillary & fibroblast proliferation and collagen fibers of wounds for the sponge of chitosan sheet test wound and the sponge of control wounds

Duration of wound	Neutrophils infiltration	Macrophages infiltration	New blood capillary & fibroblasts proliferation	Collagen fibers
1 st day (T)	3.0 ± 0.000 ^a	1.0 ± 0.000 ^a	0.00 ± 0.000 ^a	0.00 ± 0.000 ^a
1 st day (C)	2.0 ± 0.000 ^b	0.00 ± 0.000 ^b	0.00 ± 0.000 ^a	0.00 ± 0.000 ^a
3 rd day (T)	2.0 ± 0.000 ^c	3.0 ± 0.000 ^c	2.6 ± 0.51 ^b	1.6 ± 0.000 ^b
3 rd day (C)	3.0 ± 0.000 ^d	1.3 ± 0.51 ^d	0.3 ± 0.51 ^c	0.6 ± 0.51 ^c
7 th day (T)	0.50 ± 0.54 ^e	1.8 ± 0.40 ^e	3.0 ± 0.000 ^d	3.0 ± 0.000 ^d
7 th day (C)	2.5 ± 0.83 ^f	1.5 ± 0.54 ^f	1.1 ± 0.40 ^e	1.0 ± 0.000 ^e
15 th day (T)	0.00 ± 0.000 ^g	0.00 ± 0.000 ^g	3.0 ± 0.000 ^f	3.0 ± 0.000 ^f
15 th day (C)	1.0 ± 0.000 ^h	1.5 ± 0.54 ^h	2.0 ± 0.000 ^g	1.6 ± 0.51 ^g

- Means ± SD

-Number of animals =24 rabbit,

T = test wounds, C = control wound

-The difference in the letters means significant at (p<0.05)

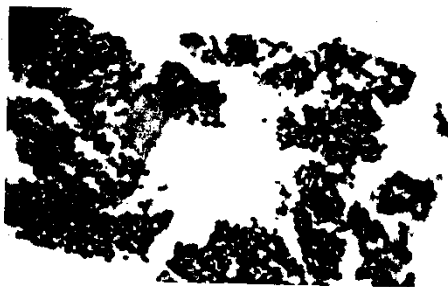


Fig. (1): The sponge of test shows severe infiltration of neutrophils at 1st post wounding day

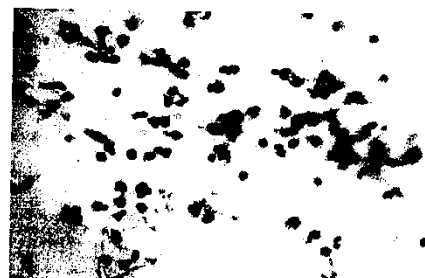


Fig.(2): The sponge of control wound shows less severe infiltration of neutrophils at 1st post wounding day

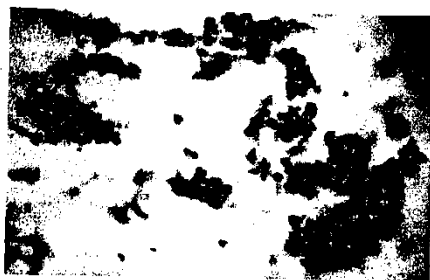


Fig.(3): Infiltration of granular macrophages in the sponge of test wounds at 3rd post wounding day

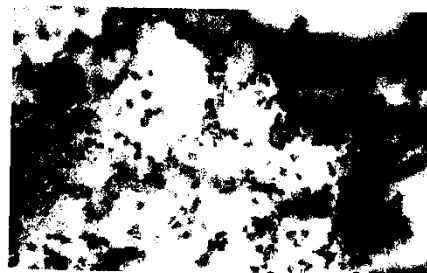


Fig.(4): Severe neutrophils infiltration with less macrophages infiltration in the sponge of control wounds at 3rd post wounding day



Fig.(5): Fibroblast proliferation with collagen deposition and few inflammatory cells in the sponge of test wounds at 7th post wounding day



Fig.(6): Fibrovascular proliferation with in the sponge of test wounds at 15th post wounding day

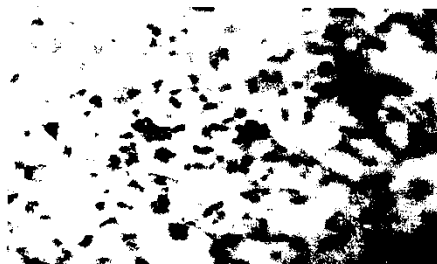


Fig.(7):Less fibroblastic proliferation with persist inflammatory cells in the sponge of control wounds at 15th post wounding day

DISCUSSION

Inflammation should be considered just one factor among series of complex activities causing fast and uncomplicated wound healing (16). In the present study the hyperemia, suppuration, and inflammatory cell infiltration were more severe in test wound than the control wound during the first three days. The result were in agreement with the result of Ueno *et al.* (11) who reported that chitosan accelerate the infiltration of polymorph leukocyte in early phase of wound healing. Chitosan stimulates the polymorph leukocyte to release leukotriene (B_4) and prostaglandin (E_2), stimulate macrophages for phagocytosis and production of Interlukine (IL-1) and growth factors it also stimulate fibroblast for production of Interlukine (IL-8). The accumulation of neutrophils at the site of wound treated with the chitosan that regard as chemoattractant factor because of its chemical composition which contain amino groups that regard a simple polysaccharide positively charged, adsorb some substance involved in cell proliferation and migration such as growth factor and cytokines, from blood plasma or exudat in the wound and the adsorbed substances stimulate the proliferation and migration (17,18,19).

In the current study the test wounds became dry after the first three days and signs of healing in form of wound closure start to appear. There is a negative correlation between the exudation and dryness of wound. The dryness of wound increased with the time of wound healing, the wound start wet to finally dry when healing complete. This result is in agreement with that of Peh and Khan (15) who reported that chitosan film

treated wounds dried at a faster rate than those of the control, the presence of exudates on the wound is critical as it could delay wound healing. Sheet dressing, characteristically, have hydrophilic sites which enable them to absorb excess exudates while producing a moist wound environment (20). Moist wound environment reduce loss of protein, electrolytes and fluid, they reduce wound pain and tenderness, exclude bacteria from wound (21,22). On the other hand Mi *et. al.* (23) emphasized that chitosan membrane controlled water loss, oxygen permeability and promote fluid drainage ability when use as wound dressing.

In the present study the appearance of vascular granulation tissue was more obvious in test wounds than in control wounds. This is in agreement with the results of Okamoto *et al.* (24) and Ueno *et al.* (25). They reported that chitosan stimulate the migration and proliferation of fibroblasts and production of collagen. It has also been reported that fibroblasts are stimulated by chitosan molecules to secrete IL-8 (26). Interleukin 8 (IL-8) is known to be angiogenic and chemoattractive to both endothelial and epidermal cells (27).

تأثير رقائق الكيتوسان على العملية الالتهابية في الأرانب

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الخلاصة

تمت دراسة تأثير رقائق الكيتوسان على عملية الالتهاب، ولأجل ذلك تم عزل وتحضير رقائق الكيتوسان من الهياكل الخارجية للروبيان المحلي في محافظة البصرة. حيث تم استحداث وتقييم العملية الالتهابية بواسطة الأسفنج المتعددة الغيبل لمعرفة الأحداث التي تجري خلال العملية الالتهابية بوجود وعدم وجود رقائق الكيتوسان خلال فترات مختلفة (اليوم الأول، اليوم الثالث، اليوم السابع، واليوم الخامس عشر) واستخدم لهذا الغرض ٢٤ أرنباً وتم عمل جرحين خطيين بطول (٢سم) على الجانب الظهري لكل حيوان.

أما تقييم العملية الالتهابية فكان من خلال المشاهدات العيانية للجروح خلال فترة التجربة والتي شملت (فرط الدم، التقيح، جفاف الجروح، انغلاق جافات الجروح) أما المشاهدات المجهرية والتي شملت (ارتشاح الخلايا الالتهابية خصوصاً العدلات وخلايا البلاعم الكبيرة وكذلك الأنسجة الحبيبية الوعائية الليفية).

أظهرت نتائج دراسة المشاهدات العيانية لتأثير رقائق الكيتوسان على عملية الالتهاب، ففي اليوم الأول أي بعد ٢٤ ساعة من استحداث الجروح بأن هناك زيادة معنوية ($P < 0.05$) في درجة فرط الدم في الجروح المعالجة في

اليوم الثالث والسابع والخامس عشر من بدء التجربة. أظهرت الجروح المعالجة زيادة معنوية ($P<0.05$) في جفاف الجروح وقلة التقيح خلال فترة التجربة مقارنة مع مجموعة السيطرة، انغلاق جافات الجروح في الجروح المعالجة أظهر زيادة معنوية ($P<0.05$) خلال فترة التجربة مقارنة مع مجموعة السيطرة. أما النتائج المجهرية فأظهرت زيادة معنوية ($P<0.05$) في ارتشاح خلال العدلات في أسفنجة الجروح المعالجة مقارنة مع مجموعة السيطرة، من اليوم الثالث تصبح شدة ارتشاح خلايا العدلات أقل معنوياً في الجروح المعالجة ويختفي تماماً في اليوم الخامس عشر من التجربة مقارنة بمجموعة السيطرة، ارتشاح خلايا البلاعم الكبيرة أظهر زيادة معنوية ($P<0.05$) في أسفنجة جروح مجموعة المعالجة مقارنة مع أسفنجة جروح مجموعة السيطرة خلال فترة التجربة، تكون نسيج الحبيبي الوعائي كان في زيادة معنوية ($P<0.05$) في جروح مجموعة المعالجة مقارنة مع مجموعة السيطرة خلال فترة التجربة.

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