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EFFECT OF CHITOSAN SHEETS ON WOUND HEALING

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ABSTRACT

The study was aimed to evaluate the effect of chitosan sheets on wound healing process and its activity as a wound dressing materials. Accordingly chitosan sheet was isolated and prepared from the exoskeleton of the native shrimps in Basrah Province. Twenty-four male rabbits were used and two full-thickness circular cuts (2cm in diameter) were made on the dorsal aspect of each rabbit. The healing process was evaluated macroscopically by evaluation of the properties of chitosan sheets on wound in terms of (adherence, absorption, and fluid accumulation) on different intervals (i.e.1^{st},3^{rd},7^{th},and 15^{th} post wounding day). On the other hand the contraction rate in tested and control wounds were evaluated during different intervals (i.e. at 1^{st},3^{r} ,7^{l} ,and 15^{th} post wounding day).The healing process was evaluated microscopically in terms of (infiltration of neutrophils and macrophage infiltration, new blood vessels and fibroblast proliferation and Re-epithelialization).

The result of macroscopic evaluation showed that chitosan sheets were firmly adherent to the wound with underlying mild fluid accumulation during the first three post wounding day. At the same time the sheets started to disappear and completely absorbed at 7^{l} day after wound creation. The result of the effect of the chitosan sheet on wound contraction demonstrated that the contraction rate of tested wounds was significantly higher than in control wounds through the period of experiment. In the treated group complete wound closure with contraction rate of 100% was reached at 10^{th} post wounding day while the control wounds failed to close completely till the end of experiment at 15^{l} day. The results of microscopic evaluation of wound healing process were demonstrated significant increase ($p<0.05$) infiltration of neutrophils in test wound at the first post wounding day then decrease and completely disappear while persist in control wound. Infiltration of macrophages significantly increased ($p<0.05$) in tested wound during the period of experiment. The fibrovascular granulation tissue and Re-epithelialization significantly more obvious in tested wound than in control wound through the period of experiment ($p<0.050$).

INTRODUCTION

Wound healing is an active area of interest for many researchers on account for it's importance at the dynamic pathway that optimally lead to restoration of tissue integrity and function. Wound healing process may be divided into four continuous phases namely **haemostasis, inflammation, proliferation** and **maturation or remodeling (1).**

Polymers are widely used in synthesis of biomaterials that used to be in direct contact with body tissue and used mainly in medical and medicinal techniques, drug delivery system, artificial vulvas, surgical structures, articular prosthesis, production of contact lenses in ophthalmology (2,3).The polymer that used insynthetic biomaterial must be biocompatible, i.e. non-toxic, easily excreted from the body, there is no side effect, non-immunogenic and non-carcinogenic (4).

Chitin and chitosan are non toxic, non allergenic and free of side effect (5).

Stone *et al.* (6) studied the use of chitosan to treat burns and other severe skin tensions. The research teams have found that areas treated with substrate incorporating chitosan demonstrated an early return to normal skin color, facilitated rapid wound re-epithelialization and regeneration of nerves with the vascular dermis.

Howling *et al.* (7) 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. An accelerated healing of incisional wound was obtained by Wang *et al.* (8) using flexible, thin, transparent novel chitosan alginate membrance.

Application of the photo-cross linkable chitosan hydrogel on full-thickness skin incision made on the backs of mice significantly induce wound contraction and accelerated wound closure and healing compared with the untreated controls **(6,9,10).**

MATERIALS AND METHODS

Isolation and Purification of Chitosan Sheet;The chitin was extracted from exoskeleton of shrimp available in local market using the Hirano method **(11).** The percentage of chitosan obtained material was 13% from the original exoskeleton of shrimp that used as the first step. 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 filtered, 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 **(12)**.

Animals and Husbandry: Twenty four adults male, domestic rabbits, weighing 1-1.5 kg, were brought 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.

Full-Thickness Skin Wounds: Under aseptic technique and general anesthesia, two full-thickness circular cuts, each of 2cm in diameter, one on the right shoulder and the other on the left shoulder were done. The right shoulder cut was considered as (Test), and the left-sided cut considered as (control). The test wounds were covered completely with chitosan sheet. The control wounds were dressed with conventional gauze band. Then both wound were dressed with non-adherent occlusive gauze bandage to hold the chitosan sheet and the gauze band in their place and to keep the wounds clean.

Macroscopic Examination of Wound Healing: All rabbits were examined daily for general health conditions and any abnormal changes were recorded. Wounds were examined at 1st, 3rd, 7th, and 15th post wounding day. Before wound evaluation, the bandages were removed and photographs were taken. The sheets were examined in terms of adherence, absorption, and fluid accumulation, using the following score: ***Score (1)*** Represented poor sheet adhesion, poor sheet absorption and fluid accumulation. ***Score (2)*** Represented moderate adhesion, moderate absorption, with a little fluid accumulation. ***Score (3)*** Represented good adhesion, good absorption (complete or near complete), and no fluid accumulation.

The margin of each wound was examined and determined with marker, the degree of wound contraction (expressed as percentage) was calculated using the following equation **(13)**.

$$[A_{Day\,0} - A_{Day\,X} / A_{Day0}] \times 100$$

A_{Day0} = The surface area of the wound at the operative day.

A_{DayX} = The surface area of the wound at a given post wounding day.

The period of wound closure was also estimated and expressed as the number of days required for complete closure with no raw wound area left **(14)**.

Biopsy Collection and Microscopic Evaluation: To evaluate the effect of chitosan sheet on full-thickness wound, both test and control wounds were excised at intervals of 1st, 3rd, 7th and 15th post wounding day (6 animals at each interval). That was done by an elliptical incision around the ulcer with a suitable margin under general anaesthesia and aseptic technique. The residual wounds were sutured and dressed. The excised wound put in sufficient amount of 10% phosphate buffered thickness piece was taken by formalin fixative for at least 48 hours. Then 3 transverse cutting through the center of each wound and submitted for paraffin embedding, hematoxylin-Eosin (H-E) stained histological sections. The histological sections at each interval examined by light microscope to evaluate the severity of inflammatory cell infiltration, new blood vessels formations, and fibroblast proliferation, collagen deposition, and re-epithelialization. Those parameters were scored as follows: ***Score (0)*** Represents (non), ***Score (1)*** Represents (mild), ***Score (2)*** Represents (moderate) ***and Score (3)*** Represents (severe).

Statistical Analysis: Results were expressed as means ± SD. The differences between groups were tested by one –way analysis of variance and LSD multiple comparison test was then performed by using SPSS (sigma-state statistical package). The results were considered significant when ($P<0.05$) with 95%confidence interval

RESULTS

Properties of Chitosan Sheet as a Wound Dressing: The proprieties of chitosan sheet at different intervals of experiment are shown in Table (1). The sheet was firmly adherent to the wound with mild fluid accumulation during the first three days post wounding. At the same time the sheet started to disappear and absorbed completely at 7th day after wound creation.

Table (1): Adhesion, Absorption and Fluid accumulation for chitosan sheet at different intervals (1st , 3rd , 7th and 15th) wounding

Duration of wound	Adhesions	Absorption	Fluid accumulation
1st day	3.0 ± 0000 [a]	2.0 ± 0000 [a]	2.0 ± 0000 [a]
3rd day	2.0 ± 0000 [b]	3.0 ± 0000 [b]	3.0 ± 0000 [b]
7th day	-	-	-
15th day	-	-	-

-Number of animals =24 rabbit, means ± SD

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

Effect of Chitosan Sheet on Wound Contraction Rate: Table (2) showed the wound contraction rate in test and control groups at different intervals of experiment. The contraction rate of tested wounds were significantly higher than that of control wounds throughout the period of experiment fig. (1). In test group, complete wound closure with a contraction rate of 100% was reached at 10th post wounding day. While control wound failed to close completely till the end of the experiment at 15th day.

Table (2): The effect of chitosan sheet on wound contraction rate in treated and control group at 1,3, 7 and 15 day post wounding.

Wound condition	Degree of contraction			
	Day-3	Day – 7	Day –10	Day –15
Chitosan sheet	42 ± 9.7 [a]	93.2 ± 4.7 [a]	100 ± 000 [a]	100 ± 000 [a]
Control	15.8 ± 7.3 [b]	76.1 ± 7.7 [b]	80.1 ± 6.5 [b]	88.2 ± 4.4 [b]

-Number of animals =24 rabbit, means ± SD

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

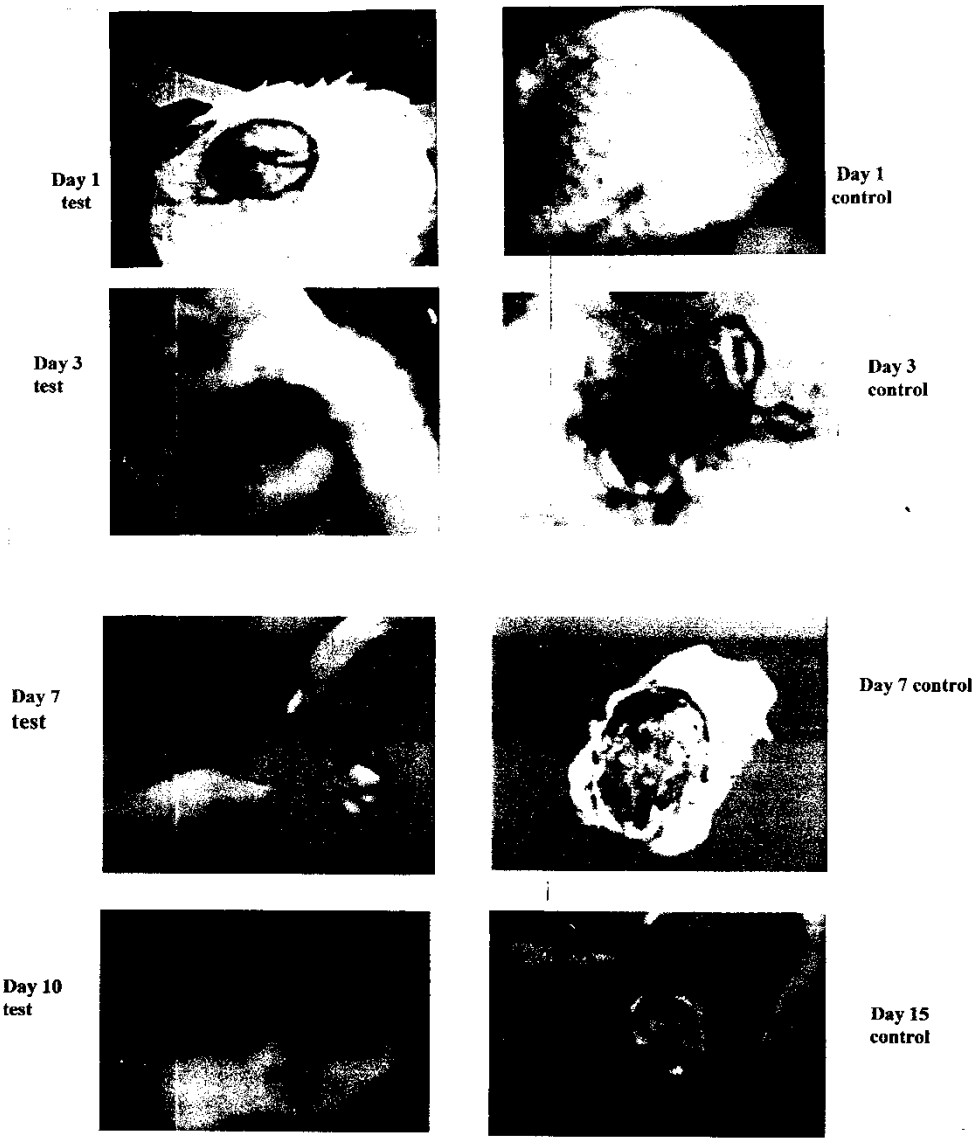


Fig. (1): Wound contraction rate in test and control wound 3rd, 7th, 10th, and 15th post wounding day with original wounds at 1st day.

Microscopic Evaluation of Wound Healing: The results of microscopic evaluation of full-thickness wounds in both test and control groups were showed in Table (3). On first post wounding day the infiltration of neutrophils was significantly increased in test wound fig. (2) than in control wounds fig. (3). From the 3rd day the neutrophils infiltration became significantly less severe in test wounds and disappear completely at 15th day ($P < 0.05$).

The infiltration of macrophages were significantly more severe in test wounds than in control wound through out the first week of experiment and disappeared at the 15th day. The fibrovascular granulation tissue and Re-epithelialization started to appear at 3rd day in both test and control groups, it was significantly more obvious in test wounds than in control wounds through out the period of experiment ($P<0.05$) fig.(4),(5),(6),(7),(8), (9).

Table (3): Neutrophils infiltration, Macrophages infiltration, New blood vessel and fibroblast proliferation, Collagen deposition and Re -epithelialization

Duration of wound	Neutrophils infiltration	Macrophages infiltration	New blood vessel and fibroblast proliferation	Collagen deposition	e- epithelialization
1st day (T)	3.0±0000 [a]	1.0±0000 [a]	0.0± 0000 [a]	0.0 ± 0000 [a]	0.0 ± 0000 [a]
1st day (C)	2.0±0000 [b]	0.0 ±0000 [b]	0.0± 0000 [a]	0.0 ± 0000 [a]	0.0 ± 0000 [a]
3rd day (T)	2.0±0000 [c]	3.0±0000 [c]	2.0±0000 [b]	2.0±0000 [b]	2.1± 0.40 [b]
3rd day (C)	3.0 ±0000 [d]	1.0 ±0000 [d]	0.3 ± 0.51 [c]	0.1 ± 0.40 [c]	0.5 ± 0.54 [c]
7th day (T)	1.0 ±0000 [e]	1.6 ± 0.51 [e]	3.0 ±0000 [d]	3.0 ±0000 [d]	3.0 ±0000 [d]
7th day (C)	3.0 ±0000 [f]	1.0 ±0000 [f]	1.0 ±0000 [e]	0.6 ± 0.51 [e]	1.0 ±0000 [e]
15th day(T)	0.0 ±0000 [g]	00 ±0000 [g]	3.0 ±0000 [f]	3.0 ±0000 [f]	3.0 ±0000 [f]
15th day(C)	2.0 ±0000 [h]	1.6 ± 0.51 [h]	2.0 ±0000 [g]	2.0 ±0000 [g]	2.0 ±0000 [g]

-Number of animals =24 rabbit, means ± SD

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

T = test wounds

C = control wound

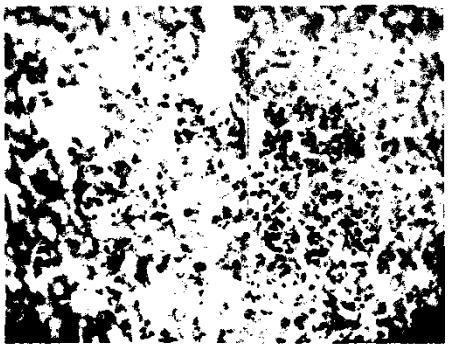


Fig.(2): Severe infiltration of neutrophils in test wounds on1st post wounding day

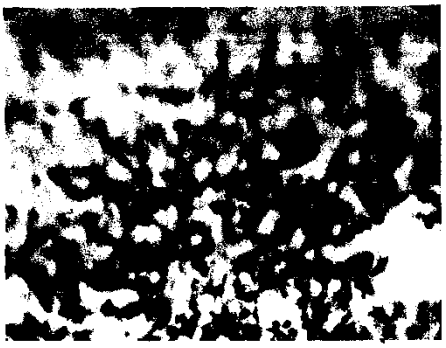


Fig.(3):Less Severe infiltration of neutrophils in control wounds on 1st post wounding day

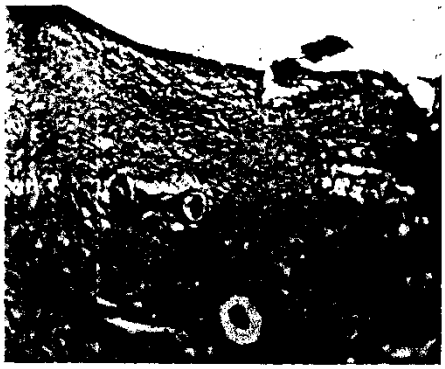
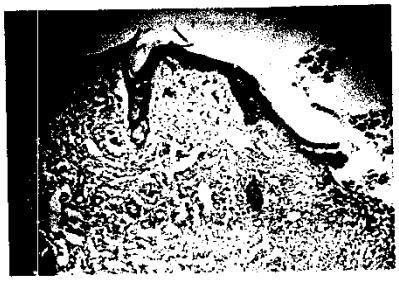


Fig.(4):The test wound showed infiltration of macrophages and few neutrophils with start epithelialization on 3rd post wounding day

Fig.(5):The control wound showed severe infiltration of neutrophils and less epithelialization on 3rd post wounding day



6): The test wound showed epithelialization with dermal fibrovascular granulation tissue on 7th post wounding day

Fig.(7): The control wound showed less epithelialization on 7th post wounding day

(8):Complete re-epithelialization of test wounds with fibroblast proliferation on 10th post wounding day

Fig.(9):Un complete (failed) re-epithelialization of control wounds on 15th post wounding day

DISCUSSION

The wound healing process involves considerable complex factor **(15)**. Therefore, a detailed evaluation of the curative nature of a healing material in an inflicted skin wound may require a wide range of observation including gross and microscopic examination of wound contraction and complete closure time for evaluation of the wound healing process **(16,17)**.

In the present study the inflammatory phase was longer in control wounds than in test wound with a fibrovascular granulation tissue and re- epithelialization were more obvious and earlier in test wounds. Chitosan accelerate the infiltration of polymorph leukocyte in early phase of wound healing. The migrated polymorph leukocyte synthesize osteopontin which play the novel role in regulating the evolution of wound healing with chitosan treatment at the early phase of healing **(18)**. Wound and burns are susceptible to infection, which lead to delayed wound healing, septicemia and a wide spectrum of other complications. Accumulation and migration of immune cells like macrophage and neutrophils in the wound bed is thus necessary for body's defense against such external infection. Wound treated with chitosan show accumulation of polymorph nuclear cells, angiogensis and healing with minimum scarring **(19)**.

Chitin and its derivatives (chitosan) used as dressing material can increase the activity of wound enzyme mainly the lysozyme which is in turn responsible for depolymerization of chitin itself. The products of chitin degradation are able to enhance granulation tissue formation and help in faster wound healing and scar prevention **(18,20,21,22)**.

The chitosan film treatment might have beneficial influence on the various phases of wound healing such as fibroplasia, collagen synthesize and wound contraction. It was possible that the enhanced healing of wounds in rats by chitosan film was a result of its stimulating activity and/or its capacity to stimulate fibroblast proliferation resulting in the progression of wound healing **(23)**. It was also suggested that chitosan might induce fibroblast to release interleukin-8, which is involved in migration and proliferation of fibroblasts and vascular endothelial cells **(24)**.

There are other important factors that influence wound healing: wet environment, availability of oxygen, antibacterial protection, and stimulation of the cells to proliferate, which in turn result in faster regeneration of the damage tissues **(20,25)**.

Chitosan wound dressing showed excellent oxygen permeability, controlled water vapor transmission rate and water uptake capability with excellent antibacterial activity *In vitro* culture for 1 week **(26)**.

Properties of Chitosan Sheet: The result of the present study revealed that the chitosan sheet has good properties to be as a wound dressing. The wound healing was accelerated, the sheet was tightly adherent to the wound. This result is in agreement with those of Sathirakul *et al.* **(27)** and Khan *et al.* **(28)** who reported that good adherence, reduce contamination and promote tissue bonding.

In this study the chitosan sheet dressing was absorbed and disappear completely on the 7th post wounding day. Ishihara *et al.* **(9,10)** reported that chitosan hyderogel spontaneously disappeared at 8th day. Other studies **(29,30)** demonstrated that the chitin and its derivatives (chitosan) are biodegradable.

In the present study, mild fluid accumulation appear at the 1st day and disappear on the 3rd day. This fluid accumulation may be related to the adhesive propriety of the chitosan sheet dressing. Similar result was obtained by Sathirakul *et al.* **(27)** and Khan *et al.* **(28)**, who reported a positive correlation between adherence and fluid accumulation.

The Effect of Chitosan Sheet on Wound Contraction: Wound contraction is a biological important process in wound healing, especially in the healing of chronic wound, although excessive contraction may finally lead to scar formation **(31)**. The present study showed a significantly higher contraction rate in the chitosan sheet treated wounds with decrease in the period of re-epithelialization. These result are in agreement with Ishihara *et al.* **(9)** who reported that higher contraction rate in chitosan hydrogel-treated wound although significantly increased infiltration of inflammatory cells were not observed in comparison with control wound. Chitosan hydrogel is regarded as a chemoattractive factor that stimulates the migration and proliferation of dermal fibroblast cell **(10)**. Fibroblast secrete IL-8 and other cytokines which in turn induce angiogenisis, fibrosis, and re-epithelialization **(31)**.

Wound contraction in chitosan sheet treated wounds contribute to advanced granulation tissue formation and re-epithelialization. Miller **(32)** reported that the positive correlation between the period of re-epithelialization and wound contraction, indicating, that re-epithelialization promote wound contraction enabling the wound to

heal faster. Experiments using a mouse model have shown that the application of chitosan hydrogel on open wound induces significant wound contraction and accelerates wound closure and healing. Due to its ability to accelerates wound contraction and healing, chitosan hydrogel may become accepted as an occlusive dressing for wound management (9). Wound generally healed by combined process of contraction and epithelial migration. During the proliferative phase of the wound healing process, wound contraction occurred due to the presence of specialized contractile cells, myofibroblast, which pulled the wound edges, together leading to closure of the defect (32).

تأثير رقائق الكيتوسان على شفاء الجروح

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الخلاصة

هدفت الدراسة لتقييم تأثير رقائق الكيتوسان على عملية إصلاح وشفاء الجروح وكذلك تقييم صفاته كمادة مضمدة للجروح، لأجل ذلك الغرض تم عزل وتحضير رقائق الكيتوسان من الهياكل الخارجية للروبيان المحلي في محافظة البصرة. استخدمنا لهذا الغرض ٢٤ أرنباً. وتم عمل جرحين عميقين بقطر (٢ سم) على الجانب الظهري لكل حيوان. تم تقييم عملية إصلاح والتئام الجروح من خلال المشاهدات العيانية والتي شملت تقييم صفات رقائق الكيتوسان لمعرفة مدى صلاحيتها كمادة مضمدة تساعد على سرعة شفاء الجروح من خلال دراسة (صفة التصاقها بالجرح، صفة الامتصاص وتجمع السوائل) في فترات مختلفة من وضع رقائق الكيتوسان (اليوم الأول أي بعد ٢٤ ساعة، اليوم الثالث، اليوم السابع، اليوم الخامس عشر). من الجانب الآخر تم حساب نسبة التقلص للجروح المعالجة ومجموعة السيطرة ومقارنتها خلال فترات مختلفة (اليوم الثالث، اليوم السابع، اليوم الخامس عشر) حتى الوصول إلى الشفاء التام وغلق الجرح. أما تقييم عملية الإصلاح والتئام الجروح من خلال المشاهدات المجهرية والتي شملت ارتشاح خلايا العدلات والبلاعم الكبيرة، تكون الأوعية الدموية، تكاثر الأرومة الليفية وأعادة النسيج الطلائي(الظهارة).

أظهرت نتائج دراسة المشاهدات العيانية لتأثير رقائق الكيتوسان على عملية الإصلاح والتئام الجروح بأن رقائق الكيتوسان تلتصق بقوة في الجرح مع تكون سوائل قليلة جداً. في اليوم الأول من بدء التجربة ثم تبدأ صفة الالتصاق تقل معنوياً($p < 0.05$) ويبدأ اختفاء الرقائق في اليوم الثالث إلى أن يختفي تماماً في اليوم السابع

من بدء التجربة. كذلك أظهرت الدراسة بأن هناك فارق معنوي($p < 0.05$) في نسبة تقلص جروح مجموعة المعالجة مقارنة مع مجموعة السيطرة خلال فترة التجربة حيث أظهرت مجموعة المعالجة انغلاق الجرح مع نسبة تقلص ١٠٠% في اليوم العاشر من بدء التجربة بينما فشلت مجموعة السيطرة في الانغلاق التام للجرح حتى نهاية التجربة في اليوم الخامس عشر. أظهرت نتائج المشاهدات المجهرية بأن هناك فارق معنوي ($p < 0.05$) في ارتشاح خلايا العدلات في مجموعة المعالجة في اليوم الأول من بدء التجربة بعد ذلك يقل إلى أن يختفي تماماً بينما يبقى في مجموعة السيطرة، كذلك هنالك زيادة معنوية ($p < 0.05$) في ارتشاح خلايا البلاعم الكبيرة كذلك هنالك زيادة معنوية ($p < 0.05$) في تكون النسيج الحبيبي الوعائي وكذلك إعادة تكون النسيج الطلائي (الظهارة) مقارنة مع مجموعة السيطرة خلال فترة التجربة.

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