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Evaluation of Chitosan as Dressing for Skin Wound. Histopathological Experimental Study in Rabbits

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الخلاصة

الأهداف: تقييم التأثيرات الطبية للجايتوسان على التئام الجروح. **المواد وطرائق العمل:** خمسة عشر من الأرانب المحلية استخدمت في هذه الدراسة. كل أرنب خدر باستخدام مادة الكيتامين هايدروكلورايد ومادة الزايلازين كمخدر ومرخي للعضلات ، زرقت هذه الأدوية في عضلة الفخذ . بعد التأكد من التخدير بفقدان انعكاس قرص الأذن، اجري(٤)قطوع بطول ١ سم لكل منها في جلد الأرنب في منطقة الظهر بعد حلقها من الشعر ثم قسمت هذه القطوع حسب معالجتها إلى أربعة مجاميع، قطوع سيطرة من دون أية معالجة أو خياط، قطوع سيطرة تمت خياطتها فقط، قطوع تم وضع مادة الفحص فيها فقط، قطوع تم وضع مادة الفحص فيها مع خياطتها. كل أرنب أعطي عضليا جرعة واحدة من اوكسي تتراسايكلين مباشرة بعد العملية. تم تقسيم العينات عشوائيا إلى ثلاثة مجاميع (G1, G2, G3) وذلك حسب وقت تضحية الأرنب (٣,٧,١٤)يوم بعد العملية الجراحية على التوالي والحصول على خزعة من القطوع الأربعة لفحصها نسيجيا تحت المجهر. تمت قراءة الشرائح النسيجية من قبل أربعة مختصين بعلم الأنسجة المرضية. معدل القراءات الأربعة عولج إحصائيا باستخدام برنامج SPSS الإصدار ١٨ . **النتائج:** اظهرت النتائج ان هناك فرق معنوي في عينات البحث جميعها بالنسبة للاستجابة للالتهابات لمادة الجايتوسان عند استخدام مسحوق هذه المادة كضماد . حيث كانت قيم P (0.005, 0.022, 0.002) للقطوع الأربعة عند (3 ,7,14) يوم على التوالي في حين لا يوجد أي فرق معنوي في أعادة تكوين النسيج ألطلائي بين المجموعات الأربع في كل فترات الالتئام وفقا لقيم (P) في اختبار فريدمان. **الاستنتاجات:** يعتبر الجايتوسان كمسرع جيد لالتئام الجروح من خلال خفض معدل الالتهابات.

ABSTRACT

Aims: To evaluate the effect of medical chitosan on wound healing. **Materials and Methods**: An experimental open skin wounds were made on the dorsal side of fifteen domestic rabbits. A 0.04gm of medical chitosan (degree of acetylation=90.0%) was applied. Each individual animal given intramuscular dose of kitamin hydrochloride (anesthetic and analgesic) 4mg/kg and xylazin base (anesthetic and muscle relaxant) 5mg/kg injected into rabbit's thigh muscle. After 10-15 minutes, anesthetic integrity was checked by testing loss of ear pinch reflex. Four skin incisions were made in the dorsal site of the animal skin. Each incision was about 1cm in length. These incisions were divided into 4 groups according to placement of the material and suturing (Ca: control incision left without suturing or placement of material, Cs: control suturing of the incision only, Cha: chitosan placement only, Chs: chitosan placement and suturing of the incision). Post operatively single dose of 5mg/kg oxytetracycline antibiotic intramuscular injection was given immediately. The animals were randomly subdivided into three groups (G1, G2, G3) with five rabbits in each group and specimen obtained from each rabbit for histopathological study according to the time of sacrifices 3, 7, 14 days after surgery respectively. Biopsies took from each site of operation and examined by four histopathologists and the overall readings taken and processed statistically using SPSS version 18.0. **Results**: There was a significant variation in inflammatory response to chitosan when applied as powder dressing at all periods of healing. *P*-values were found to be 0.005, 0.022, 0.002 between four groups at 3, 7, 14 days respectively. While there was no significance in reepithelializeation between four groups at all periods of healing according to *P*-values of Friedman test. **Conclusion**: Medical chitosan appeared good healing accelerator by decreasing rate of inflammation and prevent infection.

Key words: Chitin, Chitosan, Wound healing, polymorphonuclear cells**.**

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INTRODUCTION

Health care professionals and the United States health care system have found that chronic wounds affect 5.7 million patients and cost an estimated 20 billion dollars annually. [1,2] Understanding wound healing through biochemical, physiological, cellular, and molecular levels provides the surgeon with a sound framework for making clinical decisions aimed at optimizing the healing response.[3] Wound healing is a complex process in which the organ such as skin or tissue repairs itself after injury. It includes a complex physiological and overlapping sequence of cellular and biochemical responses directed toward restoring integrity of physical barrier and functional capacity following injury.[4-6] Several studies were directed towards utilizing natural substances available in lands and seas for producing biological material that accelerate wound healing and control infection. Among the novel families of biological macromolecules, whose relevance is becoming increasingly evident, are chitin and its main derivative, chitosan. Chitin is considered the second most abundant natural polysaccharide after cellulose on earth.[7] It was first described in 1811 by Henri Braconnot that laid the foundations of the carbohydrate polymer science, it is found in nature as the skeletal materials of crustaceans and insects, and as a component of the cell wall of bacteria and fungi. It is formed from rigid crystalline with intra- and inter-molecular hydrogen bonds which make it insoluble in water. Chitosan is a natural macromolecular polysaccharide,polymer of D-glucosamine that was discovered by Rouget in 1859. It makes up the cell wall of *Mucor rouxii* and is simply obtained by deacetylation of chitin.[8, 9] Chitosan is a versatile biopolymer obtained from the full or partial deacetylation reaction of chitin. Its main structure is β (1-4) linked 2- acetomido-2-deoxy-β-D-glucose (*N*-acetylgucosamine). It has been documented to possess a variety of amazing biological properties such as biocompatibility, biodegradability, have the ability for accelerating wound healing process, controlled release of drugs and wound dressing. [10- 14] Chitosan possesses other biological properties such as non-antigenicity, nontoxicity, antimicrobial activity and hemostasis. It is considered as a hygienic candidate in a broad spectrum of applications, as a suitable food packing material and also applied in drug delivery systems and tissue-engineering scaffolds.[12, 15-18]

MATERIALS AND METHODS

Medical chitosan (MC) Package of Medical Chitosan powder (MC), 500gm (degree of acetylation=90.0%) from shaanxitop pharmachemicalco, ltd. China is used as a wound dressing material. Small glass container is used to carry the chitosan for sterilization by U.V light prior to place it in the surgical wound using small streilizable plastic scope which holds 0.04 gm. of chitosan power to be applied on the surgical wound.

- Animals Fifteen, domestic, 6-8 months old rabbits were used to conduct the study. The weight of each was about 2.3 $\pm$ 0.5 kg. The animals were housed in an animal house in college, made for that purpose (room temperature about 18-22° C with 12:12 hrs. light-dark cycle),[19] fed a normal food and water *ad libitum.*
- Creation of Skin Wounds General anesthesia was performed by given each individual animal intramuscular dose of ketamin hydrochloride, panther united kingdom (anesthetic and analgesic) 4mg/kg in 50mg/ml and xylazin base, interchemi co. Holland (anesthetic and muscle relaxant) 5mg/kg in 20mg/ml, [20] injected into rabbit's thigh muscle, after 10-15 minutes, anesthetic integrity was checked by testing loss of ear pinch reflex. [19] Each rabbit was then placed in prone position on the surgical board covered by sterile towel exposing the dorsal side only of the animal and prepared for aseptic surgery. The surgical area was shaved using lotion hair removal, washed by distal water and sterilized by scrapping using a piece of gauze with hexatane 4% by al-rahma pharmaceutical co. Jordan. The surgical area was divided into four quarter; each quarter represented a group of study, then a 1cm longitudinal full thickness line incision was done on each quarter. The study material (MC) was applied on two incisions using small a spoon, its weight 0.04gm, one spoon for each incision and the two incisions were sutured, one incision with MC

and the second incision without MC. The other two incisions (one contains MC and the second one without MC) were left without suture. Finally, wound area was covered by sterile gauze and rapped by plaster. Post-operatively, single dose of 5mg/kg oxytetracycline antibiotic (razak, tehran, iran) intramuscular injection was given. [21]

- *Classification of groups:*

According to the time of sacrifice, the animals were randomly subdivided into three groups with five rabbits in each group, for histopathological study

- G1: The animals were sacrificed 3 days after surgery for specimen's collection.
- G2: The animals were sacrificed 7 days after surgery.
- G3: The animals were sacrificed 14 days after surgery.

Each group was subdivided into four groups according to material (medical chitosan) application:

- Cs: Control with suture.
- Ca: Control alone (without suturing).
- Chs: Chitosan with suture
- Cha: Chitosan alone (without suturing).

- Histological Observation

Specimens were preserved in 10% formaldehyde (Turkey) and stained with hematoxylin and eosin reagent (China) and examined under light microscope [22] with degree of magnification was 100X.

Inflammation Scoring:

- Score 1: Predominance of acute inflammation.
- Score 2: Predominance of granulation tissue.
- Score 3: Predominance of chronic inflammation (fibroblasts beginning to proliferate).
- Score 4: Resolution and cicatrization (reduction or disappearance of chronic inflammation). [23- 25]

Reepithelialization Scoring:

- Score 0: Reepithelialization at the edge of the wound.
- Score 1: Reepithelialization covering less than half of the wound.
- Score 2: Reepithelialization covering more than half of the wound.
- Score 3: Reepithelialization covering the entire wound, irregular thickness.
- Score 4: Reepithelialization covering the entire wound, normal thickness. [23- 25]

RESULTS

Three histological sections were prepared from each specimen. Each was examined, under light microscope by four histopathologists for inflammation and reepithelializeation and scored separately. The means of four readings were calculated and settled as a final score for statistical analysis.

Post-wounding on day 3*:* The wounds of chitosan groups (with and without suture) showed acute inflammation with neutrophils predominance at site of injury, some wounds showed tendency to form granulation tissue with fibroblast proliferation at the site of incision, newly formed blood vessels can be seen. Remnants of the study material can be noticed in some specimens scattered at the top of incision line, reepithelializeation incomplete with irregular thickness and as shown in (Figure 1).

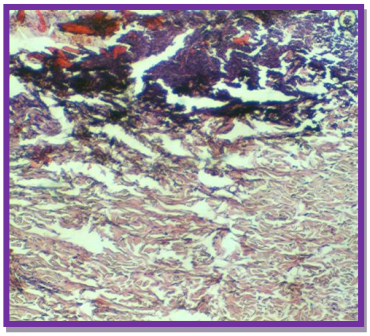

Figure (1): Chitosan with suture showed fibroblast proliferation and formation of granulation tissue. Particles of chitosan could be seen clearly (arrows).H&E, X100

In comparison to control group which showed neutrophil infiltration with few fibroblast cells and as shown in (Figure 2).

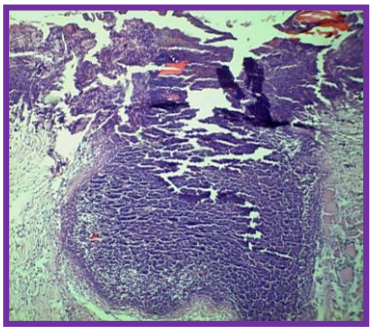

Figure (2): Control without suturing showed acute inflammation with few fibroblasts around the wound (arrow). H&E, X100

Post-wounding on day 7: Regarding histological findings of the chitosan groups (Figure 3) to control groups (Figure 4).

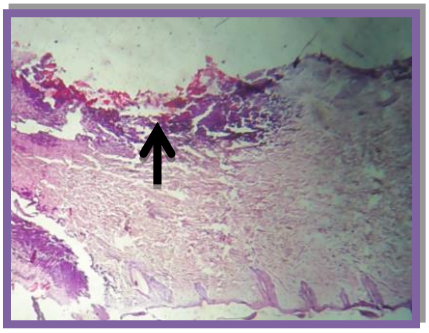

Figure (3): Chitosan with suture showed complete remission of inflammation with irregular epithelium with variable thickness (arrow). H&E, X100

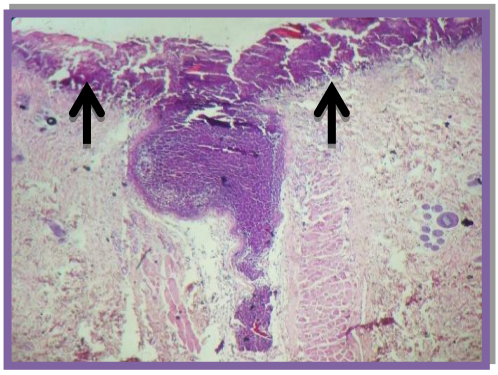

Figure (4): Control without suturing showed irregular epithelium with variable thickness (arrows).H&E, X100.

Inflammation became milder and neovascularization was more remarkable than that of day 3, reepithelializeation covered more than half of the wound, while other covered the entire wound with irregular thickness.

Post-wounding on day 14: The inflammatory reaction of chitosan groups regressed as shown in (Figure 5) with disappearance of fibroblastic activity, reepithelization improved compared to control groups as shown in (Figure 6).

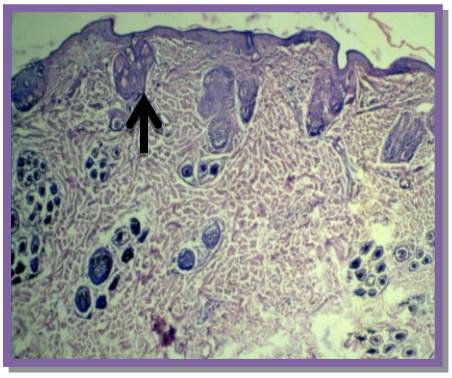

Figure (5): Chitosan without suturing showed complete reepitheliazation with regular thickness (arrow). H&E, X100.

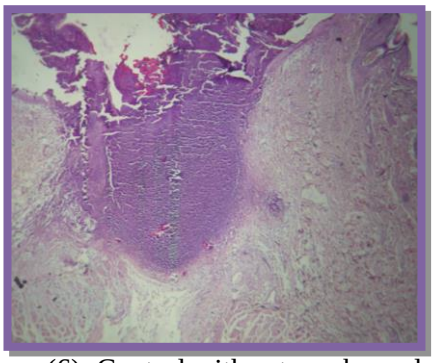

Figure (6): Control with suture showed acute inflammation with PMNs infiltration. H&E, X100.

On Friedman test for 3 days, *p*-value of inflammatory reaction was revealed a significant difference in the inflammation capacity of chitosan groups compared to the control groups at three days period.

While *P*-value of reepithelializeation was revealed no significant difference in reepithelializeation between chitosan groups compared to control groups at three days interval although the Chs took the highest mean rank as shown in (Table 1).

Table (1): Three days duration

3 Days	Mean Rank	
Groups	Inflammation*	Reepithelializeation
Cs	1.70	2.80
Ca	1.30	1.80
Chs	3.30	3.20
Cha	3.70	2.20
p-value	0.005*	0.305

*Significant difference *p*-value ≤ 0.05

On Freidman statistical test for seven days the *P*-value of inflammatory reaction was revealed a significant difference in inflammation capacity of chitosan groups comparing to control groups at seven days period, while *P*- value of reepithelialization was shown no significant difference between chitosan groups comparing to control groups at seven days interval although the Chs, Cha took the highest mean rank as shown in (Table 2).

Table (2): Seven days duration

7 Days	Mean Rank	
Groups	**Inflammation***	**Reepithelialization**
Cs	1.90	2.30
Ca	1.70	1.60
Chs	2.60	3.00
Cha	3.80	3.10
***p*-value**	0.022*	0.124

*Significant difference *p*-value ≤ 0.05

On Friedman test for fourteen days, the result of *p*-value for inflammatory reaction was displayed a significant difference in the inflammation capacity of chitosan groups comparing to control groups at fourteen days period, while *P*-value of reepithelializeation was shown no significant difference between the chitosan groups comparing to control groups at fourteen days interval although the Chs, Cha took the highest mean rank and as shown in (Table 3). Table (4) was explained which group among four groups causes the significance in results.

Table (3): Fourteen days duration.

14 Days	**Mean Rank**	
Groups	**Inflammation***	**Reepithelialization**
Cs	2.00	2.20
Ca	1.10	1.70
Chs	2.90	2.90
Cha	4.00	3.20
***p*value**	**0.002***	**0.221**

* Significant difference *p*-value ≤ 0.05

Table (4): Wilcoxon signed ranks test of histopathological findings for inflammation comparing between variables within the same period.

	Variables					
Duration	**Ca-Cs**	**Chs-Cs**	**Cha-Cs**	**Chs-Ca**	**Cha-Ca**	**Cha-Chs**
3 Days	0.357	0.039*	0.039*	0.043*	0.042*	0.705
7 Days	0.458	0.285	0.042*	0.102	0.039*	0.102
14 Days	0.059*	0.046*	0.034*	0.041*	0.034*	0.038*

* Significant difference *p*-value ≤ 0.05.

Among different durations of healing, each variable was compared as shown in (Table5).

Table (5): Kruskal-Wallis test and *p*-values for inflammation and reepithelialization between days

Groups	Days	Inflammations *P*-value	Reepithelialization *P*-value
Cs	3.00		
	7.00	0.007*	0.022*
	14.00		
Ca	3.00		
	7.00	0.013*	0.012*
	14.00		
Chs	3.00		
	7.00	0.029*	0.006*
	14.00		
Cha	3.00		
	7.00	0.003*	0.003*
	14.0		

* Significant difference *p*-value ≤ 0.05.

All groups showed significance in both inflammation and reepithelializeation. (Tables 6, 7) were shown the most active period in which healing process was faster.

Table (6): Inflammation. *p*-value for variables at 3, 7, 14 days of healing.

Variables	Duration	P-value	Duration	*p*-value	Duration	*p*-value
Cs	3	0.043*	7	0.045*	3	0.007*
	7		14		14	
Ca	3	0.014*	7	0.740	3	0.011*
	7		14		14	
Chs	3	0.163	7	0.116	3	0.014*
	7		14		14	
Cha	3	0.013*	7	0.016*	3	0.008*
	7		14		14	

* Significant difference *p*-value ≤ 0.05

Table (7): Reepithelialization. *p*-values for variables at 3, 7, 14 days of healing.

Variables	Duration	P-value	Duration	*p*-value	Duration	*p*-value
Cs	3 7	0.331	7 14	0.073	3 14	0.008*
Ca	3 7	0.041*	7 14	0.136	3 14	0.008*
Chs	3 7	0.034*	7 14	0.033*	3 14	0.008*
Cha	3 7	0.008*	7 14	0.041*	3 14	0.007*

* Significant difference *p*-value ≤ 0.05

DISCUSSION

Conveniently, chitosan influences every stage of wound healing.[26] This study showed an enhancement of inflammatory reaction by chitosan. This enhancement may be due to that the inflammatory cells at the first 3 days are more in number and more specific with high capacity for secreting more chemotactic factors in contrast to normal healing process, so the process appeared faster and healing takes a short duration to complete in comparison with control. This finding is in agreement with many studies.[27- 29] Furthermore, chitosan can enhance the release of PDGF-AB and TGF-β1from platelet; those cytokines play an important role in healing mechanism.[30] It has been reported that fibroblasts stimulated by chitosan molecules secrete interleukin-8 (IL-8) and other cytokines which in turn could induce angiogenesis, fibrosis and epithelialize-ation.[31] IL-8 is known to be angiogenic and chemo-attractive to both endothelial and epidermal cells.[32] Chitosan has a free active group (N-acetyl-D-glucosamine) which has a pioneer role in healing process. [33] Although the presence of suture is considered as a source of infection [34], the antimicrobial property of chitosan plays an important role in accelerating wound healing and prevents infection [35].Several studies suggest that the amino groups of the chitosan when coming in contact with physiological fluids are protonated and bind to anionic groups of the microorganisms, resulting in the agglutination of the microbial cells and inhibition of growth. [35-37] Topical application of chitosan in managing burn wounds can induce the formation of granulation tissue in the early stages of wound healing and by activation of fibroblasts which produces collagenase enzyme that is responsible for remodeling in the wound healing process and enhances re-epitheliazeation.[28,38] Reepithelializeation, in this study, showed no significant variation between groups even when higher in groups treated with chitosan.This result may be due to chitosan that was used in the study did not have sufficient molecular weight and degree of deacetylation to enhance re-epithelializeation process [38-41] as wound-healing effects of chitosan could be affected by the factors of molecular weight,[28,42] de-acetylation degree.[35,43] Also, the concentration of chitosan that was used may be not enough to induce re-epithelializeation. The effect of chitosan was affected by concentration of chitosan itself, the higher chitosan concentration is with a higher initial effect, but the lower chitosan concentration having a longer influence time.[44]Also reepithelializeation reduction may be due to the short period of chitosan application, due to the plaster that was placed after surgical procedure not staying for long period postoperatively, it stayed about (15) minute as a maximum due to the normal activity of animals after awaking from anesthesia which led to removal of the plaster from the back of animal easily when they ran, jumped and digging their tunnels, this led to washing of chitosan from wounds when applied in the powder form.

CONCLUSION

Chitosan induces the acceleration of wound healing by enhancing inflammatory reaction and preventing wound infections.

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