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ORIGINAL ARTICLE

Effect of Chitosan/Propolis Biodegradable Film on Full Thickness Wound Healing in Rats

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Abstract

Objective- The objective of the present study was to assess effect of propolis in combination with chitosan biofilm on excisional wounds.

Design- Experimental Study.

Animals- Male healthy Wistar rats.

Procedures- Sixty-four rats were randomized into four groups of 16 rats each. Group I: Animals with wounds treated with 0.9% saline solution. Group II: Animals with wounds were dressed with chitosan biofilm. Group III: Animals with wounds were treated topically with propolis and Group IV: Animals with wounds were treated topically with propolis and dressed with chitosan biofilm. Wound size was measured on 6, 9, 12, 15, 18 and 21days after surgery. Histological studies were performed on three time points of 7, 14 and 21 days post-wounding.

Results- Planimetric studies and quantitative histological studies and mean rank of the qualitative studies demonstrated that there was significant difference (p<0.05) between group IV and other groups.

Conclusion and clinical relevance- It was concluded that the propolis with chitosan biofilm had a reproducible wound healing potential in excisional wounds in rats.

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1. Introduction

The skin is the biggest organ of the human being and animals, and has many functions. Therefore, the healing of a skin wound dis- plays an extraordinary mechanism of cascading cellular functions which is unique in nature. As healing and regeneration processes take place in all parts of the human body, this review focuses on the healing processes of the skin and highlights the classical wound healing phases.[1]

Propolis is a generic name of a mixture of resinous substances collected by honeybees from parts of plants, buds, and exudates in the north temperate zone, extending from the Tropic of Cancer to the Arctic Circle. The main sources of propolis are poplar, willow, birch, elm, alder, beech, conifer, and horse-chestnut trees.[2] An inclusive spectrum of positive biological activity of propolis with respect to the human body larpastey results from the anti-oxidative effects of polyphenols.[3] Propolis is believed to have antiseptic, antibacterial, antimycotic, astringent, spasmolytic, anti-inflammatory, anesthetic, antioxidant, antifungal, antiulcer, anticancer, and immunomodulatory effects.[4,5] Some results confirm the propolis therapeutic efficacy, throughout quantitative and qualitative analyses of collagen types I and III expression and degradation in wounds matrix, indicating that propolis could have favorable biochemical environment supporting re-epithelization.[6,7] Biological activities of propolis on wound repair and tissue regeneration might be correlated to its antimicrobial, anti-inflammatory, and immunomodulatory properties.[8,9] The mechanisms of the anti-oxidative activity of polyphenols are different, such as the ability of inhibiting the appearance of ROS, chelating ions of metals involved in the ROS creation, and scavenging ROS, thus interfering with the cascade of reactions leading to the peroxidation of lipids and synergistic cooperation with other Antioxidants.[10]

Chitosan is a non-toxic cationic biopolymer usually obtained by alkaline deacetylation from chitin, which is the principal component of crustacean exoskeletons. [11] Chitosan presents with biocompatibility, chelating capacity and also antimicrobial effects against a broad range of gram positive and gram-negative bacteria as well as fungi.[12,13] Previous in vitro studies have demonstrated the significant biofilm efficacy of chitosan nanoparticles (CNPs).[14,15]

To the best knowledge of the authors the literature is poor regarding potentiation effects of propolis in combination with chitosan nanoparticle biofilm on wound healing in full thickness infected wounds with antibiotic resistant gram positive bacteria. Therefore, the present study aimed to study effects of combination of propoplis loaded chitosan nanoparticle biofilm on wound healing in full thickness infected wounds with methicillin resistant Staphylococcus aureus.

2. Materials and Methods

The study was approved by the institutional animal research ethics committee. Sixty-four adult healthy male Wistar rats weighting 200–220 g were used and housed in individual cages under constant temperature (22º C) and humidity with natural light/dark cycle, and had *ad libitum* access to chow and water throughout the study.

Preparation of MP (Carrier)

To prepare 100 g of Macrogol ointment, 40 g of polyethylene glycol 3350 (Ineos Manufacturing, Deutschland GmbH, Germany) was mixed with 60 g of polyethylene glycol 400 (DOW Chemical Company, USA). The two ingredients were heated in water bath at 65° C until complete melting and then allowed to cool down to room temperature while stirring until the mixture was congealed.

Formation of the propolis paste

To prepare 50 g of propolis paste, 15 g of propolis (Bee Propolis, Holistic Herbal Solutions, LLC, USA) was mixed well with 35 g of MP in a sterile mortar to obtain a creamy paste.

Preparation of chitosan biofilm

The chitosan nanoparticles were prepared based on a procedure described by others. [16] A 2.5 mg/mL chitosan solution was prepared by dissolving LMW or VLMW chitosan in a 0.05% (v/v) acetic acid solution and leaving it under stirring for 24 h. The pH was adjusted to 5.5with a 0.5 M sodium hydroxide solution and diluted in deionized water to the final desired concentrations. The tripolyphosphate (TPP) was dissolved in deionized water to a final concentration of 0.25 mg/mL. TPP and chitosan solutions were filtered through a 0.45 µm membrane (Millipore). Then, the TPP solution was added to the chitosan solution drop wise (0.3 mL/min) at different TPP: chitosan ratios under vigorous magnetic stirring at room temperature. The resulting suspension was dissolved in 100 mL of 1% acetic acid and stirred for 24 h at room temperature. The obtained solution was then filtered through G4 sand filter in order to remove the impurities

and undissolved particles. The prepared plain polysulfone (PSf/TiO2) membrane (100 cm^2) was pasted on the glass plate separately using tape with thickness of 1 mm. The stuck membrane was washed with distilled water and wiped with smooth tissue paper. A thin film of saturated polyvinyl alcohol solution was brush coated on the substrate. Chitosan (30 mL) was slowly poured in the center of the substrate and spread evenly throughout the substrate. Further, the thin film was dried at 60° C for 4 h in a hot air oven. After drying, the membrane was allowed to reach room temperature, and was then washed with 1% NaOH to remove excess acetic acid. Finally, the membrane was washed with distilled water until the washed water reached neutral pH. The same was repeated for bare PSf membranes.[17] The obtained membranes were used to dress the wounds.

Wound creation and animal Grouping

Rats were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg of BW) and xylazine (5 mg/kg of BW), the hair on their back was shaved and the skin cleansed with 70% alcohol solution. Following shaving and aseptic preparation, a circular excision wound was made by cutting away approximately 300 mm^2 full thickness of predetermined area on the anterior-dorsal side of each rat. Postoperative pain was controlled using meperidine (Hameln, Germany); 10 mg/kg were injected subcutaneously once daily for three days.

Sixty-four rats were randomly selected and allocated into four groups of 16 rats each. Group I: Animals with wounds treated with 0.9% saline solution. Group II: Animals with wounds were dressed with chitosan biofilm. Group III: Animals with wounds were treated topically with propolis paste application once daily at a concentration of 200 mg/kg on the created wound and Group IV: Animals with wounds were treated topically with topical propolis paste application once daily at a concentration of 200 mg/kg on the created wound and were dressed with chitosan nanoparticle biofilm and dressed with chitosan biofilm. All the test formulations were applied for 7 days starting from the day of wounding. In each group 12 animals were served for histological and 4 for planimetric studies.

Planimetric studies

Wound-healing property was evaluated by wound contraction percentage and wound closure time. Photographs were taken immediately after wounding and on days 6, 9, 12, 15, 18 and 21 post-operation by a digital camera while a ruler was placed near the wounds. The wound areas were analyzed by Measuring Tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc, San Jose, CA, USA) and wound contraction percentage was calculated using the following formula: Percentage of wound contraction = $(A_0 – A_t) / A_0 \times 100$

Where A_0 is the original wound area and A_t is the wound area at the time of imaging.[18] The animals were left in separate cages for four days at room conditions for acclimatization. Animal houses were in standard environmental conditions of temperature (22±3° C), humidity (60±5%), and a 12 h light/dark cycle. The animals were maintained on standard pellet diet and tap water. All rats were closely observed for any infection and if they showed signs of infection were separated, excluded from the study and replaced.

Determination of hydroxyproline levels

On the day 21 after surgery, a piece of skin from the healed wound area was collected and analyzed for hydroxyproline content. As a major part of collagen, hydroxyproline has an essential role in collagen stability. The collagen is the major component of extracellular tissue, which gives support and strength. The hydroxyproline contents were estimated using a method described by others.[21] Briefly, tissues were dried in a hot air oven at 60–70° C to constant weight and were hydrolyzed in 6N HCl at 130° C for 4 h in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4M perchloric acid and color was developed with the help of Ehrlich reagent at 60º C and measured at 557 nm using UV-visible spectrophotometer (CamSpec M330, Cambridge CB2 4BG, UK).

Histological and quantitative morphometric studies

The tissue samples were taken on 7, 14, 21 days after surgery from periphery of the wound along with normal skin and fixed in 10% buffered formalin, dehydrated and embedded in paraffin wax, sectioned at 5 µm and stained with hematoxylin and eosin (H&E) and Masson's trichrome stains. Photomicrographs were obtained under light microscope to assess the predominant stage of wound healing. Three parallel sections were obtained from each specimen. Cellular infiltration including the number of mononuclear cells, poly morphonuclear cells and fibroblastic aggregation were quantitatively evaluated. Acute hemorrhage, congestion, vascularization, epithelialization, collagen production and density were also

evaluated qualitatively. Morphological findings were scored using image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). The histological parameters were classified according to the intensity of occurrence in five levels (- absence; + discrete; ++ moderate; +++ intense; ++++ very intense).[16]

Statistical Analysis

Differences among groups in excisional model, hydroxyproline level test were evaluated by Kruskal–Wallis variance analysis. When the *p*-value from the Kruskal–Wallis test statistics was statistically significant, multiple comparison tests were used to know differences. Student's t-test was used for evaluation of mechanical test results. Comparison among days was assessed by Mann–Whitney U-test. The Bonferroni correction was applied for all possible multiple comparisons. SPSS 11.5 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A *p*-value was set at 0.05.

3. Results

Planimetric studies

Wound contraction percentage in different groups during the course of study is shown in Table 1. The healing rate of wounds in group IV was significantly different compared to the control group ($p< 0.05$).

Assessment of hydroxyproline content in wounds

Proline is hydroxylated to form hydroxyproline after protein synthesis. Hydroxyproline contents in the groups I to IV were found to be 57.47±3.12, 63.82±2.39, 77.51±2.73 and 87.38±3.93 mg g^{-1}, respectively. Hydroxyproline contents were increased significantly in the group IV which implies more collagen deposition compared to other experimental groups ($p<0.05$).

Histomorphometrical findings

There were significant differences in comparisons of group IV and other groups, particularly in terms of cellular infiltration, acute hemorrhage, congestion, edema, collagen production and density, reepithelialisation and neovascularization. During the study period, scores for reepithelialisation and neovascularisation were significantly higher in group V rats than other groups ($p<0.05$). Polymorphonuclear (PMN) and mononuclear (MNC) cell count, fibroblast cell proliferation and also Mean Rank of the qualitative study of acute hemorrhage, edema and collagen production score in group IV were significantly higher than those of other experimental groups ($p<0.05$) (Table 2) (Figures 1-4).

4. Discussion

Skin wound healing is a dynamic and highly regulated process of cellular, humoral and molecular mechanisms which begins directly after wounding and might last for years. Every tissue disruption of normal anatomic structure with consecutive loss of function can be described as a wound.[19] The healing process is activated when platelets come into contact with exposed collagen leading to platelet aggregation and the release of clotting factors resulting in the deposition of a fibrin clot at the site of injury. The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing. Inflammatory cells also arrive along with the platelets at the injury site providing key signals known as growth factors. The fibroblast is the connective tissue cell responsible for collagen deposition required to repair the tissue injury. The collagen is the main constituent of extra cellular tissue, which is responsible for support and strength.[20]

Several reports have demonstrated that there is a beneficial effect of chitosan as a biologically active dressing in wound management. It has been reported that the application of chitosan to the open wounds in dogs induced

Table 1. Effect of propolis and/or chitosan biofilm on circular excision wound contraction area (mm^2). Values are given as mean ± SEM.

Groups	Wound area in days (mm^2)					
	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
I	255.73±0.91	103.27±1.36	87.78±0.69	44.18±1.28	22.88±1.28	7.20±0.19
II	248.15±0.55	97.63±0.61	82.52±1.60	41.51±0.30	17.18±1.10	4.30±0.39
III	220.09±0.44	70.55±0.12	40.14±0.05	10.40±1.20	5.4±0.26	00.70±1.50
IV	210.63±1.51*	56.23=0±0.49*	21.36±0.10*	2.04±0.25*	1.10±0.30*	00.37±0.05*

The treated groups are compared by Student t test with other groups. *: The mean difference is significant at the 0.05 level vs other experimental groups.

Table 2. Intensity of histological parameters assessed in experimental animals.

		Histological parameters				
Groups	**Days**	**Acute Hemorrhage**	**Congestion**	**Vascularization**	**Epithelialization**	**Collagen**
I	7	+++	+++	-	-	-
	14	++	++	+	+	+
	21	++	++	+	+	+
II	7	++	++	++	+	+
	14	+	-	++	++	++
	21	-	-	++	++	++
III	7	++	++	++	-	+
	14	+	-	++	+	++
	21	-	-	++	++	++
IV	7	+*	+*	++*	++*	++*
	14	-	-	+++*	++*	++*
	21	-	-	+++*	+++*	+++*

Classification of histological parameters according to the intensity of occurrence: - absence; + discrete ;++ moderate; +++ intense; ++++ very intense. Histopathological damages were assessed as explained under material and methods on days, 7, 14 and 21 of lesion. *p<0.05 vs other experimental groups.

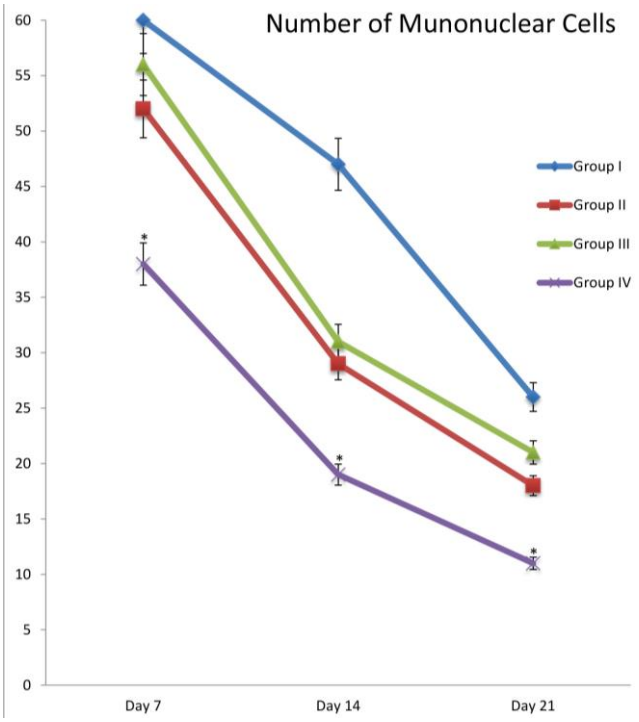

Figure 1. Line graph indicating number of mononuclear cells in excisional model of the rat's skin in experimental groups. Results were expressed as mean ± SEM. * p<0.05 vs other experimental groups.

exudate, which has a high growth factor activity, and induced infiltration by inflammatory cells and granulation tissue formation accompanied by angiogenesis.[21,22] Chitosan-membrane-based wound products have been investigated both in laboratory animals and humans, however, are still at the early stages of development. Since 1980, chitosan and its derivatives have been used in skin and wound management products in Japan. Beschitin W, an artificial skin prepared from chitin threads, has been developed for human use and is on the market.[23,24] Chitosan microspheres have been demonstrated to bear robust antimicrobial activity against S. aurous.[25]

We selected chitosan as a dressing material due to its biocompatibility, biodegradability, hemostatic activity, anti-inflectional activity and property to accelerate wound

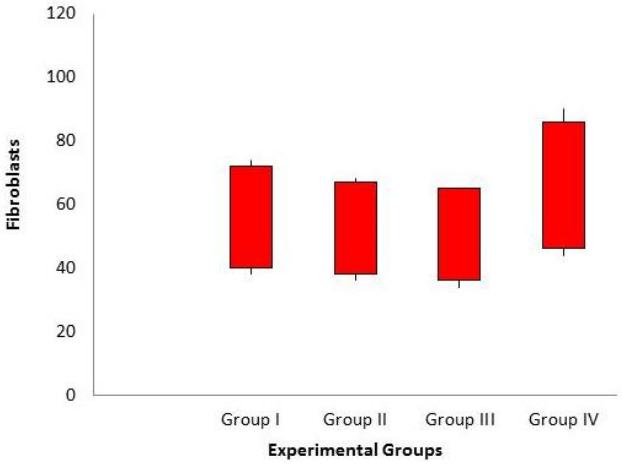

Figure 2. Box plots of number of fibroblasts in excisional model of the rat's skin in experimental groups. Results were expressed as mean ± SEM.

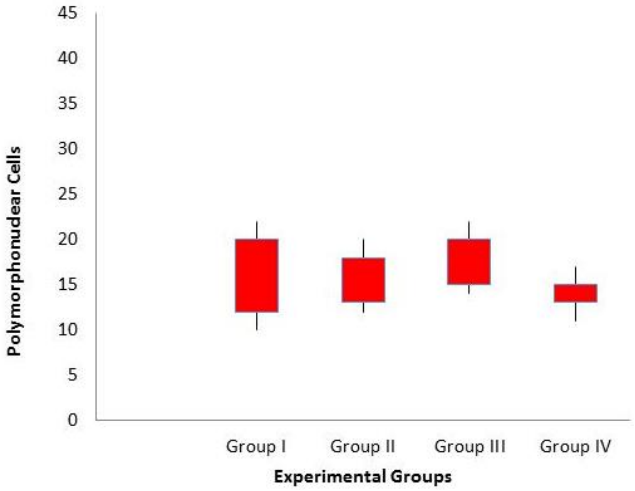

Figure 3. Box plots of number of polymorphnuclear cells in excisional model of the rat's skin in experimental groups. Results were expressed as mean ± SEM.

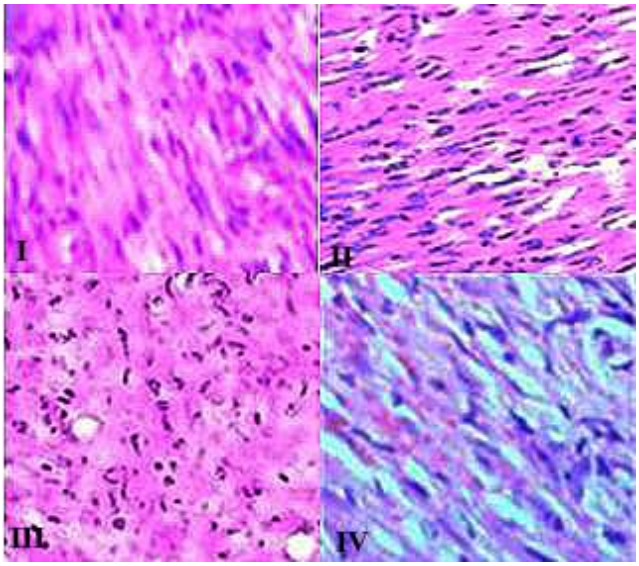

Figure 4. Representative histological micrographs on day the 14 following wound creation in excisional wound model. Wounds with surrounding skin were prepared for histological microscopic evaluations using Masson trichrome staining (×400).

healing.[26] The N-acetyl glucosamine (NAG) present in chitin and chitosan is a major component of dermal tissue which is essential for repair of scar tissue. Its positive surface charge enables it to effectively support cell growth and promotes surface induced thrombosis and blood coagulation. Free amino groups which are present on the chitosan membrane surface may form polyelectrolyte complexes with acidic groups of the cellular elements of blood.[26] It has several advantages over other type of disinfectants because it possesses a higher antimicrobial activity, a broader spectrum of activity, a higher killing rate and a lower toxicity toward mammalian cells. However, synthetic polymers are available at a lower price than biopolymer chitosan, substitution of chitosan by these synthetic polymers could reduce the price of chitosan-based films with safe effect on their functionality.[26]

Antimicrobial properties of propolis are essentially due to the flavonoid content and in particular to the presence of pinocembrin, galangin, and pinobanksin. Pinocembrin also exhibits antifungal properties. Other compounds with well-established effects are ester of coumaric and caffeic acids.[27] Some studies have highlighted the role of propolis as the solvent employed for the extraction of propolis that may influence the potency of its antimicrobial activity.[28] Propolis also shows anti-inflammatory effects against acute and chronic models of inflammation, but how propolis induces this effect is still unclear. Others verified that propolis inhibits, in a concentration dependent manner, COX activity from lung homogenates of saline- or LPS-treated rats.[29] The finding of others showed that application of propolis increases the wound healing rate and reepithelialisation of diabetic wounds in rodents. It has also proposed other roles for propolis in decreasing neutrophil infiltration and normalizing macrophage influx into wounded area.[30]

In excisional wound model there was a significant decrease in wound area in propolis and/or chitosan treated animals. This indicated improved collagen maturation by increased cross linking. The balance between synthesis and breakdown and so deposition of collagen is important in wound healing and development of wound strength.[31] Hydroxyproline is a major component of the collagen that permits the sharp twisting of the collagen helix. It helps on providing stability to the triple-helical structure of collagen by forming hydrogen bonds. Hydroxyproline is found in few proteins other than collagen. For this reason, hydroxyproline content has been used as an indicator to determine collagen content.[32,33] Increase in hydroxyproline content in group V indicated increased collagen content, since hydroxyproline is the direct estimate of collagen synthesis. Mechanical testing is sensitive to changes that occur during the progression of wound healing, and can be used as a tool to measure the quality of healing.

Biomaterials derived from natural products can provide materials with greater complexity and composition. In order to mimic the extracellular matrix (ECM) conditions of the wound and to provide a scaffold for the fibroblasts for collagen deposition, ECM-based therapies have gained popularity.[34] A phase I clinical trial using fibroin to enhance wound healing is currently underway. Finally, there have been numerous marine polysaccharide hydropastes like marine collagen from *Stomolophus nomurai meleagris*, *Oncorhynchus keta*, *Lates calcarifer*, *Stichopus japonicas*, and *Salmo salar*, alginate from

Macrocystis pyrifera, chitosan from crabs and shrimps, which are bioactive and increase wound healing rates in mice.[35]

In the present study, histopathological examination and scoring revealed that there was a significant difference by means of wound healing scores in group IV compared to other experimental groups. Propolis with chitosan biofilm decreased the maturation time of granulation tissue and wound contraction which means that it enhanced reepithelialisation with significant effect on inflammatory infiltration and number of fibroblasts in time-dependent activity. In conclusion, propolis with chitosan nanoparticle biofilm resulted in significant improvement of full thickness wound healing in excisional wound healing in rats.

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Conflict of Interests

None.

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چکیده

تأثیر لایه زیست‌تخریب‌پذیر کیتوزان/پروپولیس بر روی التیام زخم‌های تمام ضخامت پوست در موش صحرایی

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هدف- هدف از انجام این مطالعه کاربرد لایه زیست‌تخریب‌پذیر کیتوزان/پروپولیس بر روی التیام زخم‌های تمام ضخامت پوست در موش صحرایی بود.

طرح- مطالعه تجربی.

حیوانات- موش صحرایی نر سالم نژاد ویستار.

روش کار- در این مطالعه شصت و چهار حیوان به‌طور تصادفی به چهار گروه ۱۶ تایی تقسیم شدند. گروه اول: ایجاد زخم بدون به‌کارگیری درمان. گروه دوم: ایجاد زخم همراه با کاربرد لایه کیتوزان بر روی زخم به‌تنهایی. گروه سوم: ایجاد زخم همراه با کاربرد پروپولیس. گروه چهارم: ایجاد زخم همراه با کاربرد لایه کیتوزان همراه با پروپولیس. سطح زخم‌ها در روزهای ۶، ۹، ۱۲، ۱۵، ۱۸ و ۲۱ اندازه‌گیری شدند. مطالعات بافت‌شناسی در سه بازه زمانی روزهای ۷، ۱۴ و ۲۱ انجام پذیرفت.

نتایج- مطالعات پلانیمتری و بافت‌شناسی نشان داد که تفاوت معناداری بین گروه چهارم و سایر گروها وجود داشت ($p<0.05$).

نتیجه‌گیری و کاربرد بالینی- کاربرد لایه زیست‌تخریب‌پذیر کیتوزان/پروپولیس دارای توانای بالقوه بهبود التیام زخم است که با استناد بر این مطالعه می‌توان از آن به‌صورت عملی برای التیام زخم استفاده کرد.

کلمات کلیدی- پروپولیس، لایه کیتوزانی، زخم برداشتی، رت.