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Chitosan-Honey Biomembrane Contributes to the Healing Process of Burn Wound in Male White Rats

Biomembran Kitosan-Madu Berperan dalam Proses Penyembuhan Luka Bakar pada Mencit Putih Jantan

Muslim Suardi*[1], Wahyu Margi Sidoretno[1], Refika Sukriyani[2], Rieke Azhar[2]
[1]*Faculty of Pharmacy and Health Sciences-Universitas Abdurrab Pekanbaru Riau Indonesia*
[2]*Sekolah Tinggi Ilmu Farmasi (STIFARM) Padang Sumatera Barat Indonesia*
**Correspondence author: muslim.suardi@univrab.ac.id*

ABSTRACT

Chitosan and honey posses antibacterial activity. The research objectives were to prepare chitosan and chitosan honey biomembrane, and evaluate the influence of chitosan and chitosan-honey-PVA biomembrane on burn wound healing in male white rats. The skin irritation was tested on five healthy volunteers. Twenty-four rats were divided into four groups. Burns were induced on the dorsal skin using a 2-cm-diameter metal plate at 80-100 ºC for 5 seconds. The burn area was measured on days 3, 5, and 7 after injury. New blood vessels and bullae were observed histopathologically in scar tissue. Irritation was tested on five healthy volunteers. Bullae were observed in 5, 5, 5, 1, and 3 rats in the negative control, chitosan biomembrane, chitosan-honey biomembrane, and positive control (Sterile Amnion Lyophilized) groups, respectively. The wound area on day 5 after treatment was 3.06 ± 0.088, 2.29 ± 0.176, 2.64 ± 0.147, and 2.43 ± 0.201 cm^2 for the negative control, positive control, chitosan-PVA biomembrane, and chitosan-honey-PVA biomembrane groups, respectively. No significant difference was found between the positive control and chitosan-honey-PVA biomembrane ($p > 0.05$). Chitosan-honey-PVA biomembrane contributes to the healing process of burn wounds.

Keywords: Chitosan, honey, biomembrane, wound, healing.

ABSTRAK

Kitosan dan madu memiliki aktivitas antibakteri. Tujuan penelitian adalah membuat biomembran kitosan-PVA dan kitosan-madu-PVA, serta mengevaluasi pengaruhnya terhadap penyembuhan luka bakar pada tikus putih jantan. Iritasi kulit diuji pada lima sukarelawan sehat. 24 ekor tikus dibagi menjadi empat kelompok. Induksi luka bakar dilakukan pada kulit punggung menggunakan pelat logam diameter 2 cm, pada suhu 80-100 ºC selama 5 detik. Luas luka bakar diukur pada hari ke 3, 5, dan 7 setelah terjadinya luka. Pembuluh darah baru dan bula diamati secara histopatologis pada jaringan parut. Uji iritasi dilakukan pada lima sukarelawan sehat. Bula ditemukan pada kelompok kontrol negatif, biomembran kitosan-PVA, biomembran kitosan-madu-PVA, dan kontrol positif menggunakan Lyophilized Amnion Steril masing-masing pada 5, 5, 5, 1, dan 3 ekor tikus. Luas luka pada hari ke 5 setelah perlakuan pada kelompok kontrol negatif, kontrol positif, biomembran kitosan-PVA, dan biomembran kitosan-madu-PVA berturut-turut adalah 3,06 ± 0,088, 2,29 ± 0,176, 2,64 ± 0,147, dan 2,43 ± 0,201 cm^2. Tidak terdapat perbedaan bermakna antara kontrol positif dan biomembran kitosan-madu ($p > 0,05$). Biomembran kitosan-madu dapat berperan dalam proses penyembuhan luka bakar.
Kata kunci: Kitosan, madu, biomembran, luka, penyembuhan.

INTRODUCTION

Previous reserachers have developed membranes as film layers using synthetic polymers or natural polymers (biopolymers). In recent years, the use of biopolymers for packaging, film layers and biomembranes has increased due to the several advantages including lower costs and physicochemical properties that are easily decomposed by the environment (Kolybaba et al., 2003).

Chitosan is a natural polymer derived from crustacean waste, such as shrimp and crab shells. Chitin, the precursor to chitosan is the second most abundant polymer on Earth, after cellulose, and is found in the outer shell of crustaceas. Research on chitosan has progressed rapidly, driven by its abundance in nature, non-toxicity, and biodegradability. Degradable membranes made of chitosan often combined with other materials, have increased their using as adsorptive membranes and in biomedical applications (Vedula and Yadav, 2021).

Honey is a natural product well known for its benefits , particularly in Indonesia. It is rich in phenolic compounds, enzymes, and sugars, exhibiting antioxidant, anticarcinogenic, anti-inflammatory, and antimicrobial properties. Honey is valuable for health maintenance, cosmetics, and pharmaceuticals. Its efficacy in inhibiting bacterial growth, eliminating wound odors, and promoting wound healing has been proven. Honey can accelerate wound care with minimal scarring, effectively peel thickened skin, and increase fibroblasts production, which plays a role in wound healing (Puspitasari, 2007; Jull et al., 2008; Lay-flurrie, 2008; Scepankova, et.al, 2021). To date, no research has been reported on the formulation of chitosan-honey-PVA combination in biomemranes for wounnd healing.

METHOD

Equipment and Materials

The equipments used included a rotary microtom (Thermo), microscope (Olympus BX 51.DP2-BSW (DP 20), pH meter, refractometer, balance, animal balance, oven, magnetic stirrer, metal plate, and standard laboratory glassware.

The materials used were male white rats (*Rattus novergicus domestica)*, chitosan, honey, sterile amnion liofilisation (SAL), glycerol (Brataco), polivynil alcohol (Brataco), 2% lactic acid solution (Brataco), phenolphthalein, 0.9% NaCl , 10% formalin, acetone, xylol, liquid paraffin, 70% ethanol, 96% ethanol, haematoxylin, eosin, 0.4 N HCI, silver nitrate, ethyl ether, and distilled water.

Transformation of Chitosan from Shrimp Shells

The transformation process was performed following the Rinaudo method (2006). Deproteination was carried out by treating shell shrimp in 1.0 M sodium hydroxide solution while demineralization was done by treating the shell 1.0 M hydrocholoric acid solution. The deacetylation process involved treating the shells with 45-50% sodium hydroxide (NaOH) to remove acetyl group from chitin polymer obtain chitosan.

Protein-free Test

The protein-free test was determined by the Biuret method with CuSO4 solution. If no purple color was formed, the chitosan from deproteinized shrimp shell residue was considered protein-free (Sudarmadji, 1994).

Evaluation of Materials Used

The materilas used were evaluated accordinng to official references **(Anonymous, 1975; Anonymous 1995).**

Preparation of biomembrane

Chitosan-Honey-PVA biomembrane was prepared according to the following formulas.

Tabel I. Formula of biomembrane

Ingredients	FI	F2	F3
Chitosan solution 4% in lactic acid 2% (g)	3	3	-
Honey (g)	1	-	-
Glycerol (g)	0,4	0,4	-
Polivinyl Alcohol (g)	0.5	0,5	-
Sterile amnion (g)	-	0.5	ad 10
Distilled Water (g)	ad 10	ad 10	-

Chitosan was dissolved in lactic acid solution in a weighed beaker glass, mixed until homogenous using a magnetic stirrer for 6 hours, and then combinned with PVA solution. The mixture was heated on a water-bath, stirred until transparent for 6 hours and then mixed with glycerol, honey, and distilled water to a total weight of 10 grams. The acidity of solution was measured using a calibrated pH-meter. The solution was poured into a mould (Petri dish) and dried at an ambient temperature for 3 days. The biomembrane obtained was stored in a desiccator (Eldin et al., 2008). SAL was prepared using sterile amnion, mixed with PVA, placed in a 15 cm Petri dish, and lyophilizing (Madigan, et.al, 2022). .

Evaluation of Biomembrane

The organoleptic properties of the biomembrane were evaluated visually (Anoymous, 1995). The pH of biomembrane was measured using a calibrated pH-meter (Anonymous, 1995). One gram of biomembrane was crushed and mixed with distilled water to a volume of 10 mL. A cleaned and dried electrode was inserted into the mixture and the pH was recorded.

Irritation Test

The irritation test was performed as follows. A 2 x 2 cm^2 biomembrane was attached to the inner forearm of 5 volunteers' skin and covered with a patch or dressing to prevent irritation from external factors. The skin was observed for any signs of irritation, such as redness, swelling, itching, or burning, after 24 hours. The irritation level was evaluated and compared to the positive and negative control groups (Wasitaatmaja, 1997).

Evaluation of Healing Process of Burn Wound

The evaluation of healing process of burn wound was conducted as follows: Twenty-four acclimated rats were divided into 4 groups (n=6) after a 7-day acclimatization period. During acclimatization, the rats were fed a standard diet and monitored for health and weight. The rats were anesthetized using ethyl ether. The fur on the dorsal of the mouse was shaved, and 70% alcohol was applied as an antiseptic. A burn wound was created using a 2 cm diameter metal plate heated to 80°-100°C, which was then applied to the skin for 5 seconds (Vogel, 2002).

The rats were divided into four groups: Group I (control), Group II (treated with chitosan-PVA biomembrane), Group III (treated with chitosan-honey-PVA biomembrane), and Group IV (treated with SAL).

Wound care was performed daily at the same time in each group. Burns were cleaned with 0.9% NaCl physiological solution using sterile gauze. Dead skin and scabs were removed with tweezers, and the wound was rinsed with physiological solution. After cleaning, the wound was covered with the assigned biomembrane. The diameter of the burn wound was measured on days 3, 5 and 7. Wound healing was evaluated based on the decrease in wound area and histopathological observations using a microscope. On day 7, the mice were euthanized by cervical dislocation, and a 6.25 cm^2 of tissue sample was taken from the subcutaneous depth. The tissues were processed into paraffin blocks and histopathological preparations were created using hematoxylin-eosin staining. Microscopic observations were performed to evaluate neocapillaries, new blood vessels formation, and the presence of bullae.

Data Analysis

The impact of biomembrane and SAL on the burn wound area was analyzed using Two-way ANOVA ($\alpha = 0,05$), Non-parametric Freadman Test, and Student T-test.

RESULTS AND DISCUSSION

Materials Used

All of materials used met requirements for organoleptics, lost on drying, and ash content according to official references (Anonymous, 1975; Anonymous, 1995). No protein impurities found in transformed chitosan. The chitosan, transformed from shrimp shell, fulfilled the requirements of reference.

Evaluation of Biomembrane

The pH of membrane solution F1 and F2 were 5.49 ± 0,017 and 5.67 ± 0.01, respectively. While the pH of membrane of F1 and F2 were 4.88 ± 0.01 and 5,30 ± 0.06, respectively. The pH of all formulas was within the range of 4.2 to 6.5, making them suitable for topical use (Wasitaatmaja, 1997).

Evaluation of Biomembrane Stability

The biomembrane examination was conducted visually. The results showed that each formula yielded a transparent and odorless solid in the case of the chitosan-PVA biomembrane, while the chitosan-honey-PVA biomembrane had a slightly yellowish, transparent solid form with a distinct honey smell. The stability examination was performed at room temperature (28.5 °C) and cold temperature (5 °C) to determine changes in shape, color and stability of the biomembrane due to low temperatures and during storage at room temperature (Martin, 1993). Both biomembrane formulas remained stable, showing no changes in shape or color at cold temperature. At room temperature, the chitosan-PVA biomembrane remained stable whereas the honey-chitosan-PVA biomembrane underwent a color change, turning brownish on the fourth day, with increasing intensity on the fifth day. This indicates that honey plays a role in the color change likely due to the inversion of sucrose by enzymes in honey into reducing sugars such as glucose and fructose.

Irritation Skin Test

No irritation symptoms, such as redness and itchiness were observed on the skin of all volunteers (n=5). It can be assumed that the chitosan-PVA and chitosan-honey-PVA biomembrane are save to be used as topical treatment of burn wound.

The Impact of Biomembrane on Burn Wound Healing

The burn wound area of control, chitosan-PVA biomembrane, chitosan-honey-PVA biomembrane, and SAL groups were 3.06, 2.64, 2.43, and 2.29 cm2, respectively. There were no significant differences in burn area among the SAL, chitosan-PVA biomembrane and chitosan-honey-PVA biomembrane groups ($p > 0.05$). However, significant differences were observed between the negative control and the SAL (positive control), chitosan-PVA biomembrane and chitosan-honey-PVA biomembrane on the burn wound area ($p < 0.05$). These result suggest that the chitosan-PVA biomembrane and chitosan-honey-PVA biomembrane can reduce the burn wound area.

Tabel II. The measuring of burn wound area after treatment

Group	Wound Area (cm^2)		
	Day-3	Day-5	Day-7
Negative Control	3.14 ± 0.00	3.14 ± 0.000	3.06 ± 0.088
Chitosan-PVA Biomembrane	3.14 ± 0.00	3.03 ± 0.083	2.64 ± 0.147
Chitosan-Honey-PVA Biomembrane	3.09 ± 0.127	2.91 ± 0.205	2.43 ± 0.202
Positive Control (SAL)	3.06 ± 0.088	2.78 ± 0.152	2.29 ± 0.176

Microscopic Evaluation: Burn Wound Histopathology
Histopathology observations of burn wounds revealed the formation of more than two new blood vessels in all groups. This indicates that burn wound healing occurred in all of animal subjects.

During the inflammatory process, broken blood vessels cause bleeding, prompting the body to respond with vasoconstriction, shrinking the broken vessel ends. Signs of an inflammatory reaction become apparent, characterized by redness (rubor), increased temperature (calor), pain (dolor), and swelling (tumor). Cellular activity involved the movement of leukocytes through blood vessel walls (diapedesis) towards the wound. Leukocytes secrete hydrolytic enzymes that digest bacteria and wound debris. Lymphocytes and monocytes then appear, participating in destroying and consumption of wound debris and bacteria through phagocytosis. In this phase, the wound healing process begins, involving various body elements, including blood vessels, fibroblasts and epithelial cells. Initially, blood in the wound clots, followed by an inflammatory response that clears away dead cells and bacteria. Epithelialization occurs rapidly, protecting wounds from external contamination and reducing the risk of infection.

In the negative control group, chitosan-PVA biomembrane group, chitosan-honey-PVA biomembrane group, and positive control group 5, 5, 1, and 3 bullae occurred, respectively. Interestingly, while there were 3 bullae in the positive control group, only one bulla was observed in the chitosan-honey-PVA biomembrane group (Figure 3).

This suggests that the chitosan-honey biomembrane has an advantage in terms of bulla formation. This may be attributed to the characteristics of honey, such as its high osmolarity, which is particularly beneficial when applied to wounds. Honey's high osmosis ability enables it to draw water from wounds, promoting rapid drying and reducing bacterial growth. The addition of honey to the

biomembrane can attract water from bullae, preventing the growth of microorganisms that trigger infection.
Scepankova and co-workers (2021) reported that honey have some advantages in wound healing, including accelerated dermal repair and epithelialization, promotion of angiogenesis, immune response enhancement, and reduction of healing-related infections with pathogenic microorganisms.

The burn wound area in the positive control group was not significantly different from that in the chitosan-honey-PVA biomembrane group ($p>0.05$). Notably, the chitosan-honey-PVA biomembrane had comparable effect to SAL in terms of burn wound area. To date, no references have been found on the use Chitosan-Honey-PVA biomembrane for treating burn wounds. However, Radoor et.al. (2021) reported that the Chitosan-Honey-PVA biomembrane exhibits good antibacterial activity againts *Staphylococcus aureus* (Gram-positive bacteria) and *Escherichia coli* (Gram-negative bacteria).

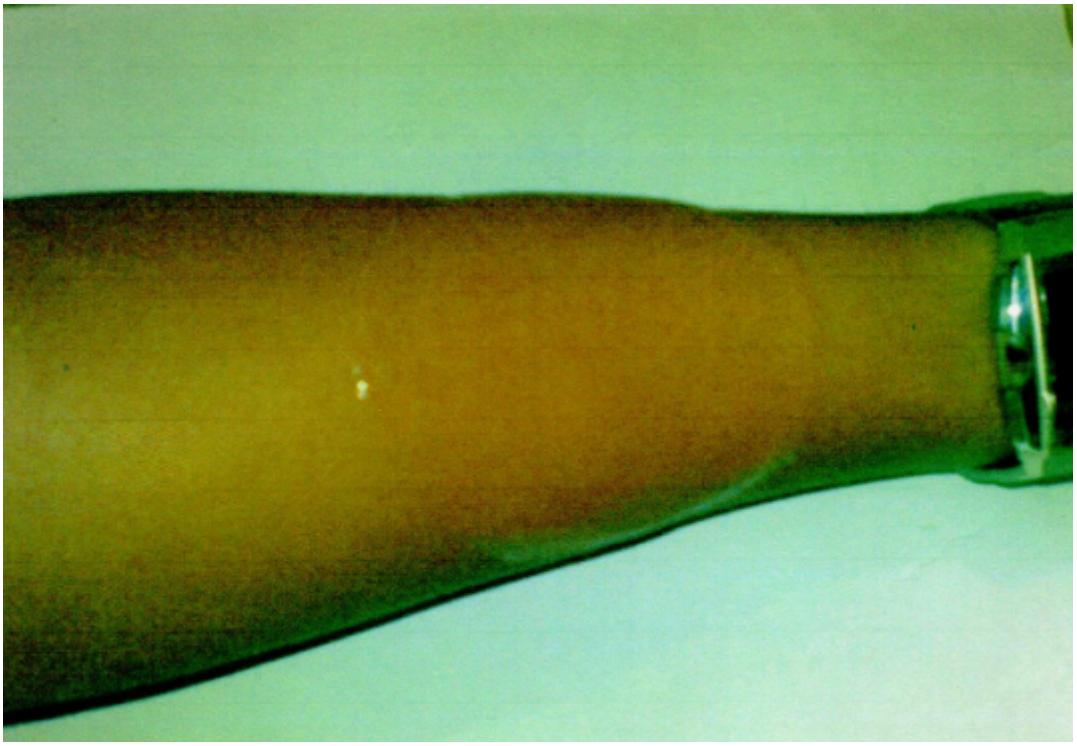

Figure 1. Chitosan-Honey-PVA Biomembrane

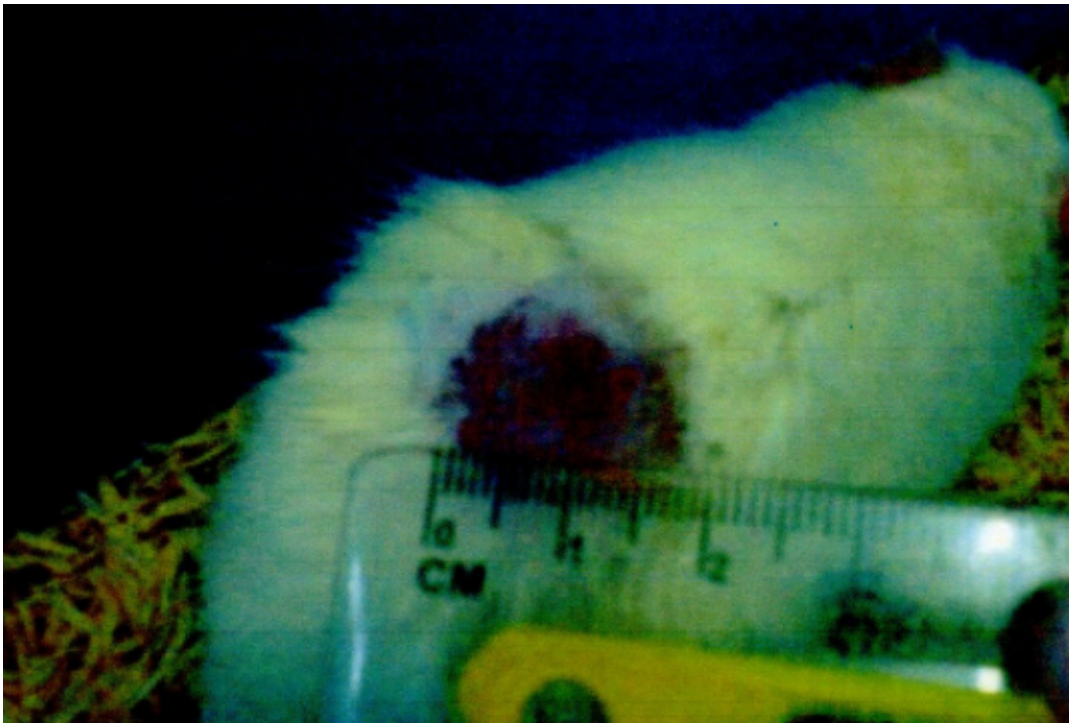

Figure 2a. Burn wound of positive control using SAL.

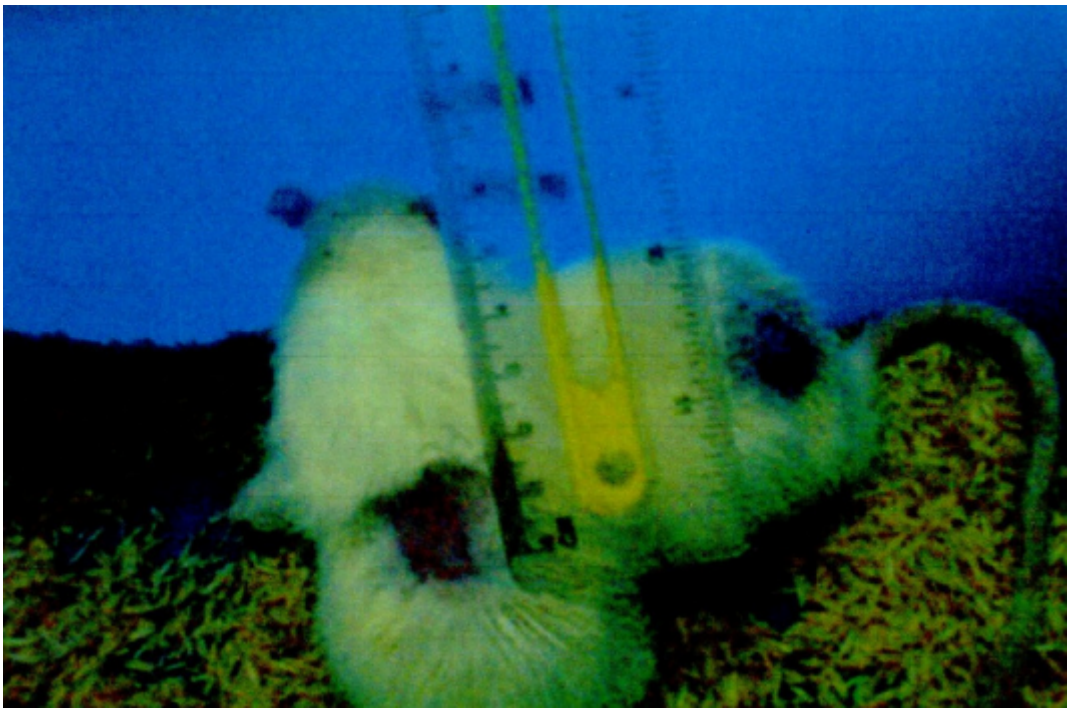

Figure 2b. Burn wound after treatment using positive control, SAL.

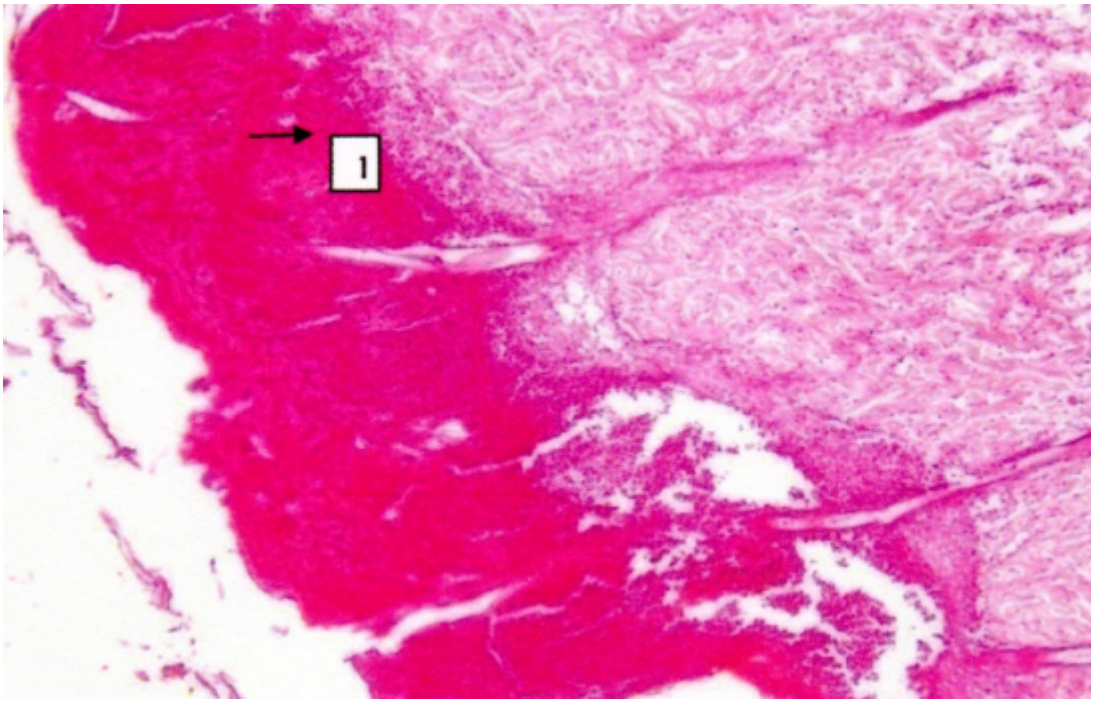
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Figure 3a. Histopathology observation of burn wound after treating using Chitosan-Honey-PVA biomembrane. No bulla was found, but only solidified of leucocyte.

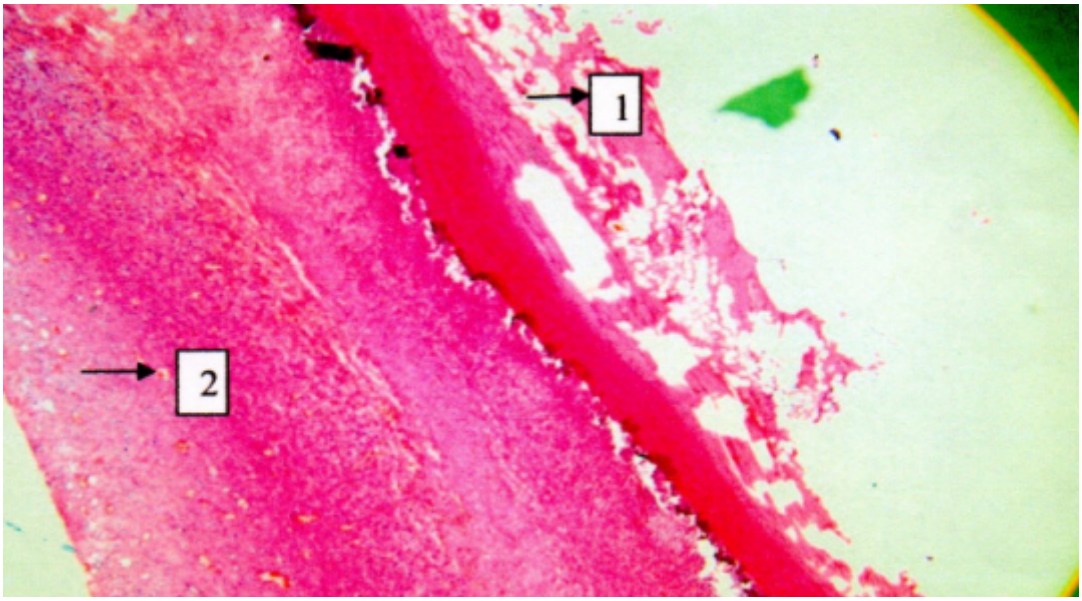

Figure 3b. Histopathology observation of burn wound after treating using positive control, SAL. Bulla and solidified of leucocyte were found.

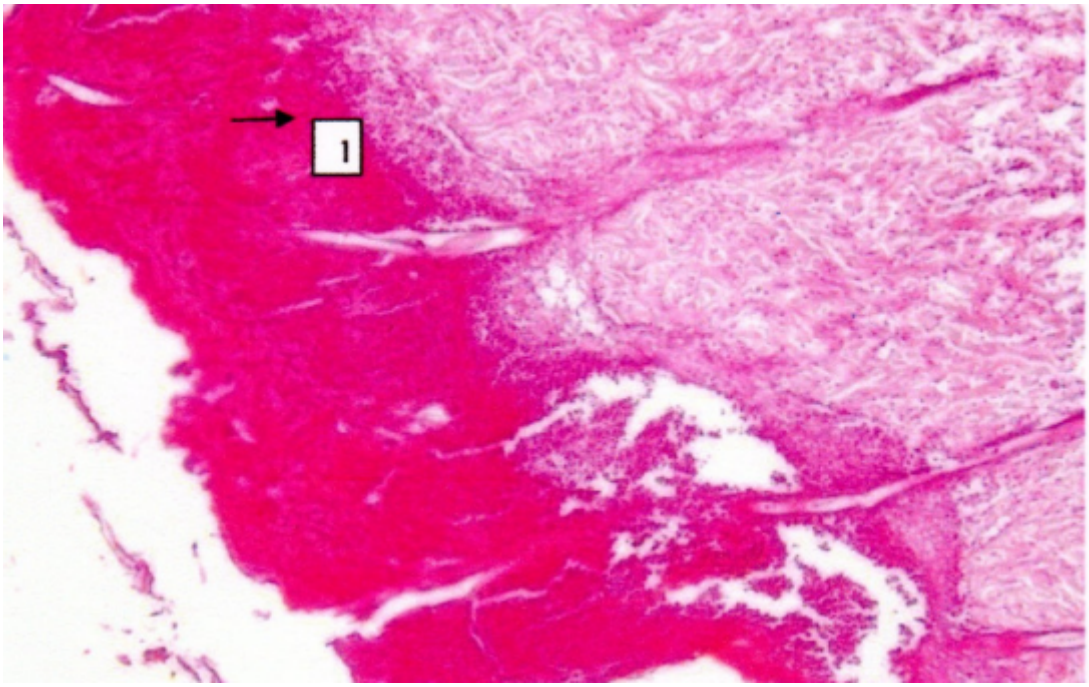

Figure 3c. Histopathology observation of burn wound after treating using chitosan-honey biomembrane. No bulla found, but only solidified of leucocyte.

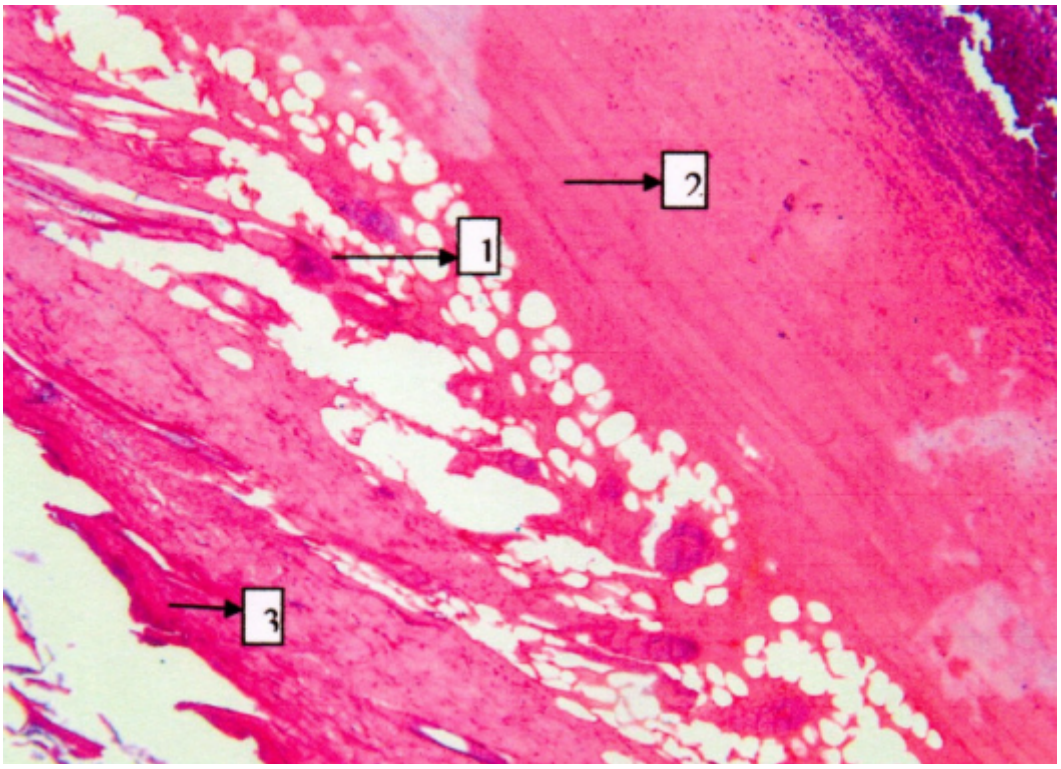

Figure 3d. Histopathology observation of burn wound of positive control. Bulla, solidified of leucocyte, and fibrolas proliferation were found.

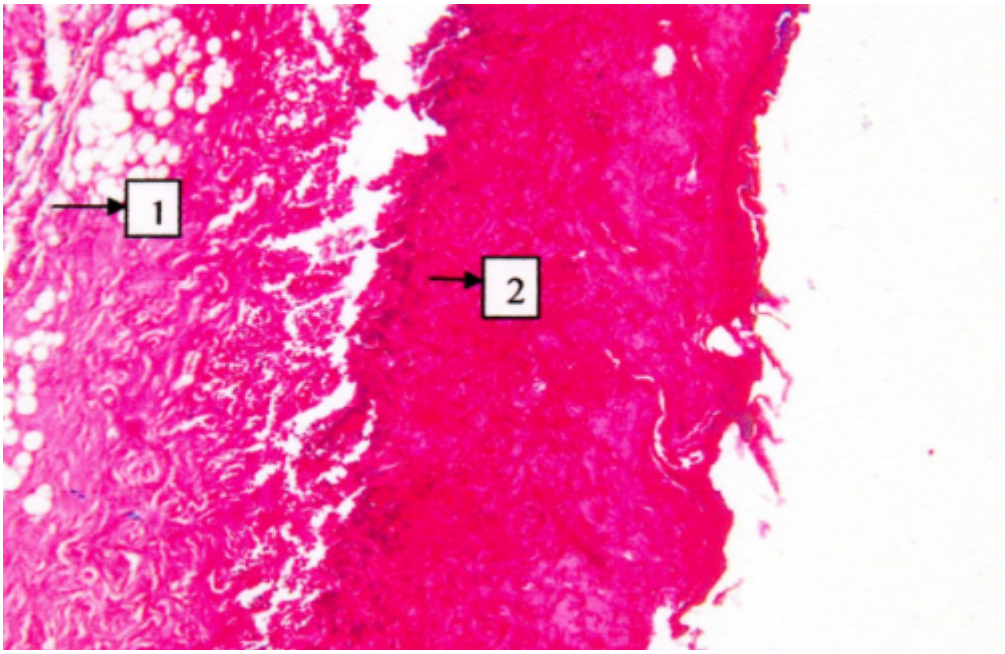

Figure 3e. Histopathological observation of burn wound after treating using chitosan-PVA biomembrane. Bullae and solidification of leucocyte were observed.

CONCLUSION

Chitosan-PVA biomembrane and Chitosan-Honey-PVA biomembrane contributed to decrease in burn wound area and a reduction in bulla formation. Chitosan-PVA and Chitosan-Honey-PVA biomembrane can enhance the burn wound healing process. These findings show promise for development of burn wound therapy.

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