Research Article

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Effectiveness of Edamame (*Glycine max* L. Merril) Membrane in Accelerating The Wound Healing Process of Deep-Partial Thickness Burn

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ABSTRACT

The gold standard for deep-partial thickness burns is early excision and skin graft; however, many hospitals in Indonesia still use conventional treatment due to the high cost of surgery and the requirement of qualified medical professionals. This research aimed to study the effectiveness of edamame (*Glycine max* L. Merill) membrane as therapeutic innovation in deep-partial thickness burns. Forty-eight male Wistar rats with deep-partial thickness burns were randomly assigned to four groups, including control and treatment (silver sulfadiazine, the membrane with 40% and 60% edamame extract). Multiple serial measurements of wound healing parameters such as macroscopic evaluation, histopathologic, and hydroxyproline were examined on days 4, 10, and 16. Treatment groups of membrane edamame significantly improved wound healing compared to the control group. Macroscopically, histopathological findings and hydroxyproline assay confirmed the efficacy of the edamame membrane at 60%, which provided the best healing results. This study showed that edamame membrane is effective as deep-partial thickness burns wound dressing.

Keywords: burns, dressing, edamame, hydroxyproline, wound healing.

1. Introduction

Burns is a universal public health problem, estimated at around 180,000 deaths yearly [1]. The American Burn Association divided burns into four groups based on depth: epidermal, superficial-partial thickness, deep-partial thickness, and full-thickness burn [2]. According to a study by Wardhana in 2017, the highest prevalence of burns was full-thickness, followed by deep-partial thickness burns [3]. Deep-partial thickness might worsen into full-thickness burns when they do not get adequate treatments. Full-thickness burns are more severe than the other types because they might lead to a critical state or death due to respiratory distress and sepsis [4].

The primary purpose of burns treatment is to prevent infection that might inhibit the healing process of the wound itself [5]. Early excision and skin grafting are the standard gold treatment for deep-partial burns to decrease the risk of infection during the healing process [6]. However, many hospitals in Indonesia still use conventional treatment with silver sulfadiazine (SSD) cream due to the high cost of surgery and the requirement for qualified medical professionals [7]. SSD is a sulfa-derivative topical antibacterial capable of controlling infection and promoting healing in deep-partial thickness burns. Currently, there are studies regarding the long-term use of SSD cream. SSD cream was reported to form a dry, rough, and leather-like eschar on the wound surface that causes poor skin penetration and slows wound healing [5].

Wound healing is divided into three overlapping phases: inflammatory reaction, cell proliferation and synthesis of the extracellular matrix, and collagen maturation. In the proliferation phase, fibroblasts will move to the wound area and produce collagens so that the granulation tissue gradually covers the wound [6]. Collagens contain much hydroxyproline, one of the amino acids used as the biochemistry marker of wound healing [7].

Edamame (*G. max*) is a soybean harvested when immature and still tender green, about 60 days after planting [8]. Missouri Botanical Garden Herbarium lists this plant under the number 04796514 [9]. It contains potassium, ascorbic acid, iron, vitamins A, B1, C, E, and isoflavones [8]. Genistein is the primary isoflavone in soybean, and evidence suggests that genistein has anti-inflammatory, antioxidant, and antibacterial effects that promote wound healing [10]. Edamame-based gauze dressings had created and maintained a moist environment for 3-4 days. Therefore it supported the healing of the wound area and prevented new injuries after the removal of rough scar [11]. This study aimed to determine the role of topical treatment with edamame membrane on deep-partial thickness burn wound healing in rats.

2. Material and Methods

2.1. Plant Collection and Edamame Membrane Preparation

The researchers collected edamame (*G. max*) seeds from PT. Mitra Tani Dua Tujuh in Jember, January 2020. We macerated the powder of edamame with 96% ethanol and evaporated it on a rotary evaporator. We added 40% and 60% extract with 2 g of hydroxypropyl methylcellulose (HPMC) until it rose viscous within 24 hr. Moreover, we added 0,18 g of methylparaben, 15 mL of propylene glycol, and 0,15 g of propylparaben, then mixed it with 100 mL of water that contains 5% of DMSO. We poured the mixtures into a glass plate in which there was sterile gauze with a size of 2.5 cm in length, 2.5 cm in width, and ±1 mm thick. We made the sterile gauze covered by the mixtures and stored it in the refrigerator till use (4 ˚C) [11,12].

2.2. Animal and Experimental Control

The researchers acclimated male Wistar rats weighing 200±50 g in the animal unit (12 hr light/dark cycle, temperature approximately 32 ˚C) for one weeks before experiments. We housed the rats in individual cages with free access to water and food pellets. We randomly divided forty-eight rats into four groups of 12. After creating burn wounds, we dressed each group in a different treatment: SSD cream (positive control group), membrane without extract (negative control group), the membrane with 40% edamame extract group, the membrane with 60% edamame extract group. We sacrificed four animals from each group on the serial days 4th, 10th, and 16th. We determine the healing process by macroscopic, biochemistry, and histopathologic evaluation. The Faculty of Medicine, University of Jember's ethics team, has approved this research with approval number 1352/H25.1.11/KE/2019 on 27 December 2019.

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2.3. Skin Burn Injury Induction

The researchers anesthetized the rats with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (15 mg/kg). We shaved the animals` backs and induced burn by applying hot aluminum plaque (2x2 cm). We heated aluminum plaque to 70 ˚C in the dry oven and touched it on the skin of the rats for 10 sec to create a deep-partial thickness burn wound. We allowed the temperature of the aluminum plate to drop within 5 sec before being put into the oven for the following induction. The same person performed all procedures to minimize the bias of differences in the force of application. Venter et al. validated this method in producing deep-partial thickness burns [13].

2.4. Treatment

The researchers divided the animals into four groups. We topically dressed group one (SSD) in SSD cream twice daily. We treated the control group 2 (Control) with a membrane without any extract. We applied membranes containing 40% (40% MEE) and 60% edamame extract (60% MEE) to group 3 and group 4. We renew the membrane every three days.

2.5. Wound Area and Closure Assessment

The researchers photographed burn wounds after creating a wound (first day) and on days 4, 10, and 16, with the same instrument (Samsung Galaxy S10 Mobile Phone) and settings, a fixed camera distance from the wound, and the exact position of rats. We analyzed the photos by MATLAB R2009 software. We got data from MATLAB software: pixels of wound image per pixel of one cm^2. We compared differences in the wound size on the first day, 4th, 10th, and also 16th, between groups.

2.6. Hydroxyproline Analysis

The researchers sacrificed some rats on the 4th, 10th, and 16th days. We took 100 mg of skin tissue from the burn scar area. We placed the sample on a petri dish, dried it at 60 ˚C for 12 h, and added 3-5 mL of HCl 6 N. We hydrolyzed the tissue at 130 ˚C for four h. We moved 2 mL of solution from the hydrolysis process to the Eppendorf tube and centrifuged it at 10000 rpm for 5 minutes. We took the supernatant, transferred it into a test tube, and evaporated it for 30-45 minutes at 60- 80 ˚C. We added 500 µL of a vaporized solution with 30 µL of Chloramine T and 470 µL of pH six citrate buffer and mixed it. We incubated the mixture for 20 minutes at room temperature. Moreover, we added 250 µL HClO4 0.4 M and 250 µL Ehrlich solution, mixed, and set it for 90 min at 60 ˚C. We centrifuged the fluid at 3000-4000 rpm for 5 min, then transferred the supernatant into a cuvet. We measured the absorbance value by spectrophotometer at 557 nm, then calculated towards the standard hydroxyproline curve to obtain the hydroxyproline levels [14,15].

2.7. Histopathologic Evaluation

The researchers took the treated skin after the sacrificed animals and stored it in a 10% formalin-containing solution. We stained the sections with hematoxylin-eosin (HE) and observed under a light microscope at a 400x lens magnification in five fields of view, and the result was average. We carried out the observations by the blinding method. The histopathological examination includes an examination of fibroblast and epithelialization.

2.8. Statistical Analysis

The researchers reported all data as the mean ± standard deviation (mean ± SD). We used a statistical software package, SPSS, to perform statistical analysis. We tested the data for normality and homogeneity of variance, then analyzed it with analysis of variance (ANOVA), followed by Post hoc multiple comparisons. Statistical significance was accepted at $p<0.05$.

3. Results and Discussion

Figure 1 shows a deep-partial thickness burn on the first day. This burn was under the characteristic of deep-partial thickness burns, based on the American Burn Association, in which the skin typically is white in color, splotchy red, dry, and blisters may occur. We evaluated the wound healing process on multiple serial days of 4, 10, and 16 after burning induction. We measured the wound area, hydroxyproline, number of fibroblasts, and epithelial thickness as wound healing parameters. Figure 2 shows every group's macroscopic evaluation of the wound healing process.

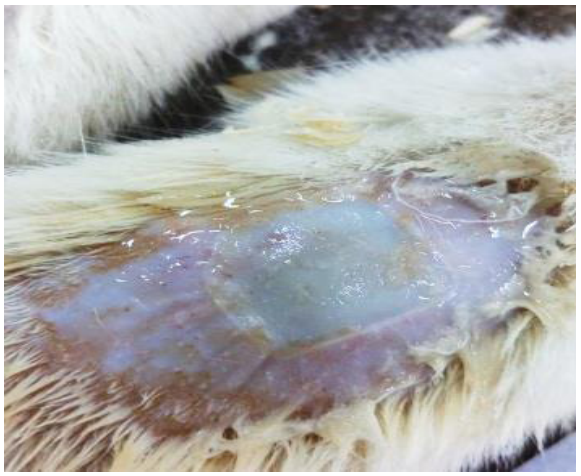

Figure 1. A Deep-partial thickness burn wound after induction. The wound appears white, splotchy red, dry, and has blisters.

Table 1 shows the wound area of every group. On day 4, the 60% MEE group showed a significant reduction ($p>0.05$) in the wound size compared with other groups. In contrast, the average wound area between SSD, control, and 40% MEE groups was almost similar, and there was no significant difference between the groups ($p<0.05$). On the 10th-day induction, the wound area was significantly narrower in all groups compared with the 4th day. The difference was significant between all groups except SSD and 40% MEE groups ($p>0.05$). Furthermore, on the 16th day of the experiment, the group threatened with SSD, 40% MEE, and 60% MEE showed a significant reduction ($p<0.05$) in the wound size compared with the control group. The 60% MEE group gave the highest percentage of wound closure (Table 2).

Edamame (*G. max*) extract contains some active components. One of them is isoflavones, which are phytoestrogens [16]. The main isoflavones are genistein, daidzein, and glycitein. Edamame has genistein at the highest level, among other isoflavones [17]. These phytoestrogen/isoflavones have similar efficacy as estrogens. Estrogens play a role in increasing the expression of the TGF-β growth factor to stimulate fibroblasts' proliferation and accelerate the rate of epithelization [18,19]. Genistein also stimulates the proliferation of fibroblasts directly by modula-

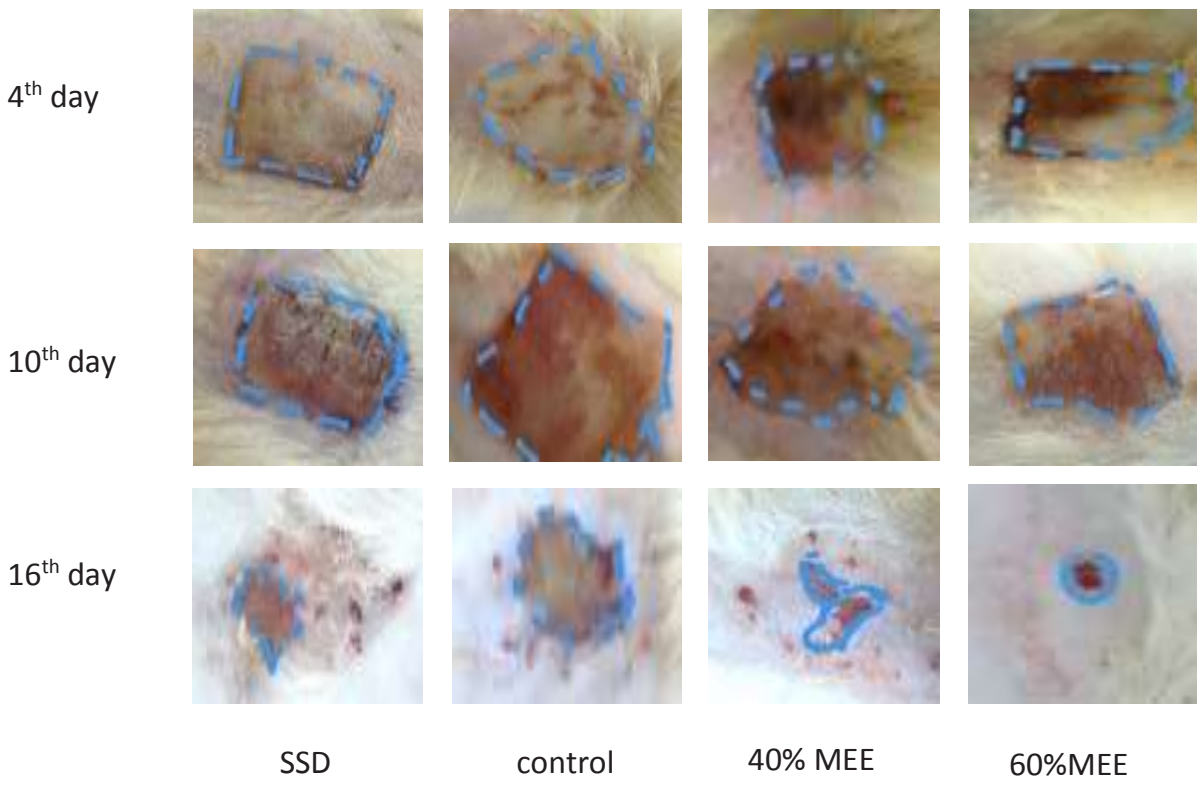

Figure 2. Macroscopic observations of the healing site of skin burn in different groups on days 4, 10, dan 16

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Table 1. The Average of Wound Area on Different Days

Day	SSD (mm^2)	Control (mm^2)	40% MEE (mm^2)	60% MEE (mm^2)
4	389.25±5.56	389±4.76	385.75±11.67	369.25±6.02
10	219.75±12.606	267±10.424	200.75±6.396	128.25±16.111
16	103.5±5.802	201.25±10.21	99±6.055	83.25±14.384

Each value represents the mean ± SD (n=4)

ting estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) [20]. Phytoestrogen also controls tissue inhibitor of metalloproteinase (TIMP) expression to inhibit excess collagen degradation [19]. Therefore, a keloid and a hypertrophic scar will not be formed [21].

Genistein, a primary active MEE compound, is also an antioxidant, antimicrobial, and anti-inflammatory in accelerating burn healing [22]. Normal oxygen metabolism stimulates the production of reactive oxygen species (ROS) or free radicals, such as phagocytosis. Excessive free radicals are dangerous because they release various inflammatory mediators such as NF-κB and TNF to recruit neutrophils and other immune cells to the wound area, leading to tissue damage [23]. Genistein prevents cell death caused by ROS with the donor hydrogen ion mechanism and increases glutathione as a natural antioxidant [24,25]. As an anti-inflammatory agent, genistein suppresses the stimulation COX-2, so the secretion of pro-inflammatory molecules such as IL-1β, IL-6, IL-12, and TNF-α are inhibited [26]. Genistein was reported to have an antibacterial effect. Genistein represses topoisomerase's function and inhibits bacteria's DNA metabolism [27]. Edamame (*G. max*) also contains a lot of vitamins A, C, and E to help speed up the wound healing process. Vitamin A plays a role in relieving inflammation by increasing collagen synthesis, and vitamin C help to strengthen and stabilize the collagen structure. Meanwhile, vitamin E is an essential antioxidant for wound healing [28].

This finding motivated us to investigate the healing effects of edamame membrane on deep-partial thickness burn wound healing in the experimental animal model. Our study showed a significant difference ($p<0.05$) regarding the mean percentage of wound closure between days 4, 10, and 16 of the experiment in all groups. However, the membrane with 60% edamame extract seemed more effective. These results are also supported by hydroxyproline levels (Table 2) and histological findings (Table 3).

Table 2 shows the hydroxyproline levels of control and treated rats on different days of analysis. Hydroxyproline assessment on the 4th day after injury showed that 60% of MEE groups had the highest hydroxyproline level, while control groups had the lowest level. The difference was significant between all groups ($p<0.05$). An increased level of hydroxyproline is required for a faster wound healing rate. On the 10th day, membrane group hydroxyproline levels increased along with fibroblasts' proliferation process and collagen deposition. Sixteen days after induction, the hydroxyproline levels were going down as a sign that the proliferation phase would be complete, except for the control group, which maintained increasing hydroxyproline due to the deceleration of the proliferation phase. On the 16th day, the 60% MEE group had the lowest level of hydroxyproline among other groups. Hence, the 60% MEE group gave the best result for wound healing biochemically and macroscopically.

Histological assessment includes the counting of fibroblasts and the measurement of epithelial thickness. Table 3 presents that the control group had the smallest average number of fibroblasts compared to other groups on days 4, 10, and 16. MEE significantly increased fibroblast proliferation compared to the control group ($p>0.05$). However, statistics showed no difference between the MEE and SSD groups. Meanwhile, the 60% MEE groups gave the highest number of fibroblasts among other groups on days 4, 10, and 16.

Genistein accelerates burn wound healing by enhancing the proliferation of fibroblasts and keratocytes [24]. Data from days 4th, 10th, and 16th proved it when comparing the MEE and control groups. The Epithelial thickness and fibroblasts count each day always showed MEE significantly higher than the

Table 2. The average of hydroxyproline levels on different days based on μg per 100 mg skin tissue

Day	SSD	Control	40% MEE	60% MEE
4	5198±272.58	2625±305.54	6368±312.24	7708±577.39
10	9681±711.93	7230±1092.68	9858±54.46	12213±632.61
16	6575±1461.19	9125±938.45	3660±702.06	3288±1006.26

Each value represents the mean ± SD (n=4)

Table 3. The Average of Fibroblasts on Days 4. 10. and 16

Day	SSD	Control	40% MEE	60% MEE
4	20.73±5.53	11.23±0.81	22.58±5.27	24.38±3.30
10	26.2±4.64	16.75±1.42	25.8±3.93	29.83±2.87
16	41±4.97	28.88±6.32	42.63±7.46	50.2±3.79

Each value represents the mean ± SD (n=4)

control group. Furthermore, the increased fibroblast proliferation was accompanied by a large amount of collagen build-up due to the subcutaneous VEGF and TGF-β release by genistein [24]. Collagen contains the amino acid hydroxyproline, which plays a role in twisting the collagen triple-helix structure [23]. Therefore, hydroxyproline is used to measure the production of collagen. Hydroxyproline level decreased on day 16 in the MEE group, with 60% MEE showing the lowest level. This indicated that the MEE group had reached the remodeling phase and reduced collagen synthesis. So hydroxyproline production also decreased [29]. Although the hydroxyproline level showed decreased collagen, fibroblast as collagen-forming cells still increased on the last day. This phenomenon is expected due to the lack of negative feedback from collagen production [30].

Table 4 presents the result of epithelial thickness measurement from each group. The control group has the thinnest epithelial thickness compared to the other group. Meanwhile, the 60% MEE group has the thickest epithelial thickness. Microscopic observation on day 4 in the SSD group and 60% MEE group showed that the structure of epithelial tissue of the wound was more visible when compared to the 0% MEE and 60% MEE group. However, on days 10 and 16, the SSD group had thinner epithelial thickness than the 40% MEE and 60% MEE groups.

Statistics showed that on the 4th day, the average epithelial thickness of all groups had no difference ($p>0.05$). On the 10th and 16th days, there were significant differences between the MEE group with the SSD and control groups. 40% MEE and 60% MEE groups have thicker epithelial than the SSD and control groups. This indicates that edamame extract can accelerate the process of re-epithelization. On the 16th day, the SSD group showed no significant difference from the control group. This may occur because the SSD group formed a scar that was very hard and difficult to remove from the skin. The macroscopic observations show this rough scar.

In this study, 60% MEE and 40% MEE are more effective than the SSD group. It is due to the rough eschar formed in the SSD group from the 4th until the 10th-day afterburn induction. The rough eschar` was very difficult to remove from the skin. Meanwhile, 40% and 60% of MEE groups formed soft eschar, which can be removed before day 10. Hence, the 40% and 60% groups result in a higher percentage of wound closure, and a faster proliferation phase compares to the SSD group. The other study by Sutejo et al. investigated the healing effects of crude edamame extract and silver sulfadiazine on burn wounds in rats. Histopathological examination and hydroxyproline analysis were used to evaluate healing effects. They used crude edamame extract and SSD cream for 15

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Table 4. The Average Epithelial Thickness on Days 4. 10. and 16

Day	SSD (µm)	Control (µm)	40% MEE (µm)	60% MEE (µm)
4	11.13±3.29	8.9±2.47	10.25±2.58	14.73±2.07
10	23.52±1.62	20.78±1.58	26.92±0.43	31.17±2.17
16	36.65±1.54	34.33±2.17	41.70±3.40	45.20±2.29

days to improve the burned skin wounds in the rat models [15]. According to this improved research, the edamame membrane is more beneficial since it has a more extended usage than crude extract. This dressing only needs to be applied once for three days, whereas crude extract needs to be smeared twice daily. Edamame membrane contains humectants, so it moisturizes the wound and does not cause another injury when removed. This research also performs serial measurements multiple times to describe the phases of burn wound healing.

4. Conclusions

We concluded that the edamame membrane causes the acceleration of wound healing in deep-partial thickness burn-proven macroscopically, histopathological and biochemical assays. Our findings indicated that this edamame membrane could be applied to treat burns and can be considered an alternative therapy for wound healing. This membrane is superior to cream preparations because it has a longer usage time, acts as a moisturizer, and prevents the formation of rough scars.

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Conflict of Interest

The author/editor has no conflicts of interest, financial or otherwise, to declare.

Statement of Contribution of Researchers

Concept – I.R.S., D.D.W.; Design – I.R.S., D.D.W.; Supervision – I.R.S., D.D.W.; Resources – I.R.S., D.D.W.; Materials – B.S.E., A.N.A., S.F.Y.; Data Collection and/or Processing – B.S.E., A.N.A., S.F.Y.; Analysis and/or Interpretation – I.R.S., D.D.W.; Literature Search – B.S.E., A.N.A., S.F.Y.; Writing – I.R.S., D.D.W., B.S.E., A.N.A., S.F.Y.; Critical Reviews – I.R.S., D.D.W.

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