Majalah Kedokteran Gigi Indonesia
Vol 10 No 3 – December 2024
ISSN 2460-0164 (print), ISSN 2442-2576 (online)
Available online at https://jurnal.ugm.ac.id/mkgi
DOI: http://doi.org/10.22146/majkedgiind.87352

RESEARCH ARTICLE

Silkworm cocoon (*Bombyx mori*) accelerates wound healing in skin excision: a study on macrophage and VEGF

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Submitted: 26th July 2023; Revised: 31st May 2024; Accepted: 26th November 2024

ABSTRACT

Silkworm cocoon (*Bombyx mori*) is a natural polymer composite and largely used as bio-functional material for wound healing. It consists of fibroin and sericin protein that has antibacterial effect. This study aimed to investigate the effect of silkworm cocoons (*Bombyx mori*) wound dressing on the number of macrophages and VEGF expression in skin excision. The subject of this study was 12 Wistar rats, which were grouped based on the duration of wound dressing application (3rd day and 6th day) and the use of dressing materials (n = 6). The rats were anesthetized with ketamin and xylazine prior to wound excision. A punch biopsy wound excision with 4 mm diameter of subcutaneous depth was made on both sides of the rat's back, with the right side dressed with silkworm cocoon as the treatment group and the left side dressed with moist gauze application as the control group. Hematoxylin-eosin (HE) staining was performed to observe the number of macrophage cells. Immunohistochemical staining using an anti-VEGF antibody was performed to observe the expression of VEGF. Data were analyzed using a two-way ANOVA and an Independent t-test with confidence interval of 95%. Statistical analysis demonstrated a significantly higher number of macrophages in the silkworm cocoon wound dressing group on 6 days post-application ($p = 0.026$) and significantly higher VEGF expression in the silkworm cocoon wound dressing group on 3 days post-application ($p = 0.002$) and on 6 days post-application ($p = 0.044$). Silkworm cocoon (*Bombyx mori*) wound dressing can increase the number of macrophages and VEGF expression in wound excision model in Wistar rat.

Keywords: *Bombyx mori*; macrophage; skin wound excision; VEGF; wound dressing

INTRODUCTION

Surface tissue damage occurs due to discontinuity and damaged or lost tissue substance. In the facial area, wounds commonly involve the skin and its underlying layers, such as excoriation, laceration, incision, and excision wounds. Excision wounds are open wounds, which if not appropriately managed, can affect the healing process. Wounds should be treated with adequate wound care, including the periodic use and replacement of wound dressings. Selection of a good wound dressing can improve treatment results and wound response to drugs; therefore, it is essential to select a dressing material that is absorbent, is not easily soluble, and can distribute drugs to the wound area optimally.[1,2,3,4]

Silk moth caterpillars (*Bombyx mori*) produce cocoons with structures, functions, and protective mechanisms that are nearly similar to human skin.[5,6] This similarity indicates that the overall structure of silk moth cocoons could be beneficial for wound repair.[7] Previous studies indicated that fibroin and sericin in silk moth cocoons can support the growth of epithelial cells, fibroblasts, keratinocytes, and endothelial cells.[5,7,8] The same studies also demonstrated that fibroin and sericin facilitate the migration and adhesion of L929 fibroblast cells and keratinocytes.[5,7]

In vitro studies showed that fibroin can assist cell migration and proliferation by acting as a cellular and molecular modulator.[5,6,7] Additionally, sericin is known to have antibacterial effects, particularly against *S. aureus* and *E. coli*, thus preventing infections that could impede the wound healing process.[7] Silk moth cocoons have the

Majalah Kedokteran Gigi Indonesia. December 2024; 10(3): 197-208
ISSN 2460-0164 (print)
ISSN 2442-2576 (online)

potential to be utilized as wound dressings that are believed to accelerate the wound healing process.

The success of an optimal wound healing process can be assessed through various methods, including clinical, histopathological, immunohistochemical, and biomechanical evaluations. One of the indicators that can be assessed histopathologically and immunohistochemically is the number of macrophage cells and VEGF expression, which play a crucial role in the early phases of wound healing.

Macrophages are cells that play an important role in the transition from the inflammatory to the proliferative phase.[9] Depletion studies have shown that the absence of macrophages in both healing phases leads to reduced bleeding and tissue formation, potentially causing the failure of the subsequent healing stages.[10]

Research on the effect of film dressings made from silkworm cocoons (*Bombyx mori*) on the inflammatory phase and the proliferative phase after skin excision (*in vivo* research on Wistar rats) has generated interest among researchers due to the potential of silkworm cocoons (*Bombyx mori*) as a dressing material. To the best of our knowledge, research on the use of silk cocoon is still limited, and no previous studies have reported its effect on macrophage cells and VEGF expression.[5,6,7] In this study, the wound healing indicators that were observed histopathologically and immunohistochemically were the number of macrophage cells and VEGF expression, which play an essential role in the inflammatory and proliferative phases.

MATERIALS AND METHODS

This study had been approved by the Ethics Committee of the Faculty of Veterinary Medicine of Universitas Gadjah Mada (ethical clearance number 00107/EC/FKH/Eks./2021). This research is a quasi-experimental laboratory study with a sample size calculated with resource equation method. Twelve Wistar rats (*Rattus novergicus*) were grouped based on the duration of wound dressing application (3 and 6 days) and the type of dressing material (n = 6). The subjects were selected based on inclusion criteria, which were males aged 3 to 4 months, weighing ± 250 grams, appearing healthy and physically active, and no anatomical defects. Exclusion criteria included post-operative pain and infection, death before the

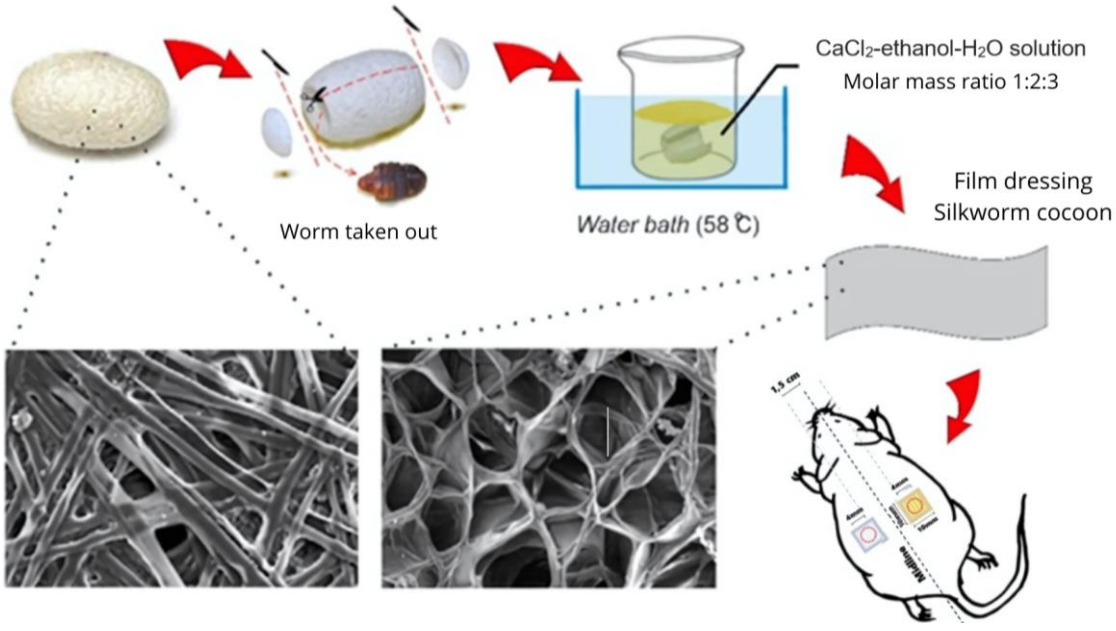

Figure 1. Process of making film dressing from silkworm cocoons (*Bombyx mori*)

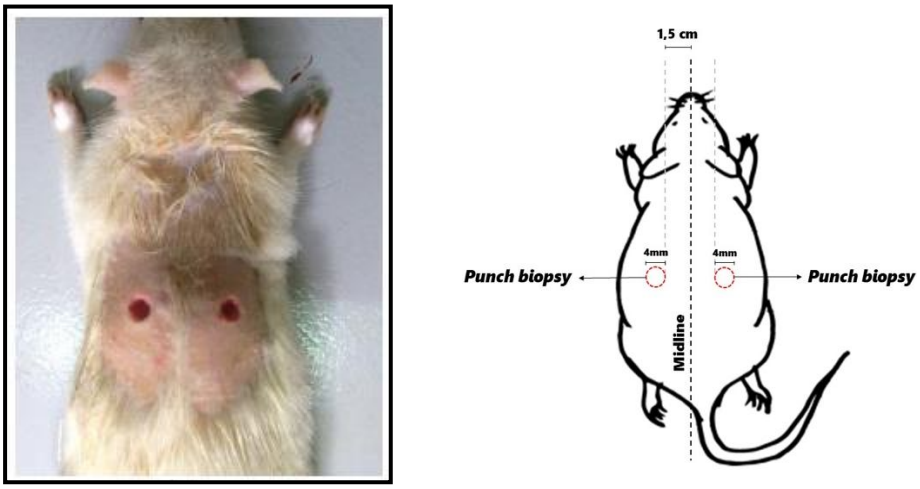

Figure 2. Excision wounds on the back skin of the rats with punch biopsy tools

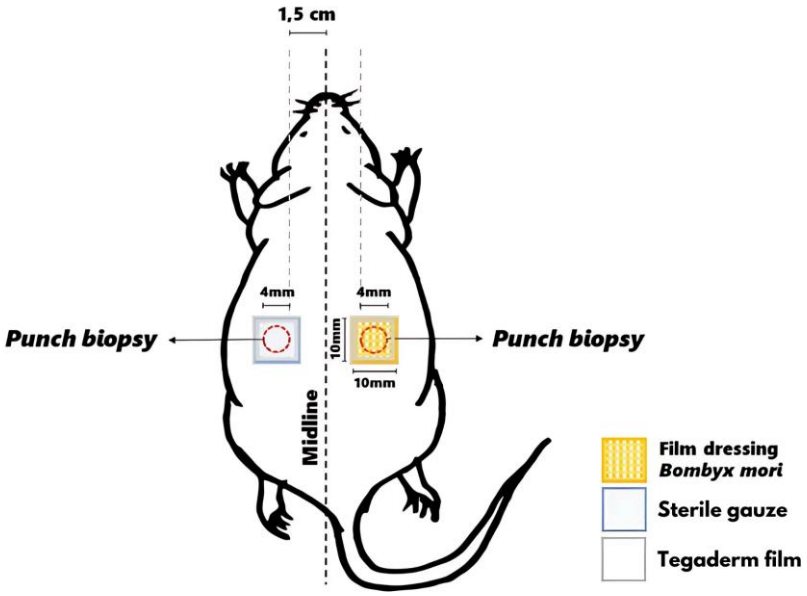

Figure 3. Application of film dressing from silkworm cocoons (*Bombyx mori*)

decapitation process, and unstable body weight (weight loss of <20% within a week).

The rats were adapted to a laboratory environment with temperature and humidity adjusted to laboratory standards for 1 week, given standard food and drink, and placed in individual cages. Then, simple randomization was carried out to determine the application duration group. The Wistar rats were anesthetized with intramuscular ketamin hydrochloride 10% (Ketamil®) and xylazine with a dose of 50 mg/kg and 10 mg/kg, respectively. Each rat was subjected to two excision wounds on the back, with the right side was intended for treatment, and the left side was functioned as the control.

Wound dressings were made from silkworm cocoons following the guidelines established by Yu et al.[7] The cocoons used were oval-shaped and cut at both ends to form a tube. The tube was further cut on one side to create a rectangular sheet measuring 4 x 1.5 cm. These silkworm cocoon sheets were soaked in a $CaCl_2$-ethanol-H_2O solution with a molar mass ratio of 1:2:8 in a beaker.

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ISSN 2460-0164 (print)
ISSN 2442-2576 (online)

The beaker containing the cocoon sheets was placed in a water bath at 58 °C for 90 minutes. Afterward, the cocoon sheets were rinsed four times with sterile aquadest at room temperature and dried with desiccant silica gel for 24 hours. The result of this process was a transparent film piece measuring 4 × 1.5 cm with a thickness of 0.7 mm, which was ready for use as a wound dressing. The wound dressings were sterilized using a sterilization pack and ethylene oxide gas (EOG) at 36 °C for 7 hours before application to the wounds (Figure 1).

An excision punch biopsy wound with a subcutaneous depth of 4 mm in diameter was created on both sides of the rat's back, with the right side as the treatment group and the left side as the control group (Figure 2). The excision wound on the treatment side was covered with a film dressing made from silkworm cocoon (*Bombyx mori*), while the excision wound on the control side was covered with gauze soaked in NaCl solution (Figure 3). The condition of the rats remained healthy from before the operation until the time of sacrifice, with no weight loss exceeding 20%. Clinically, no signs of infection were observed in the post-operative area. The rats were anesthesized using ketamine and xylazine intraperitoneally prior to decapitation. Tissue samples from the wound areas were taken at 3 days post-application (H+3) and 6 days post-application (H+6). Tissue samples were processed, stained with hematoxylin and eosin (HE) for the observation of macrophage cell count, and immunohistochemically stained for the observation of VEGF expression. The data collection was then carried out.

The data were analyzed using IBM SPSS Statistics 22 (IBM, United States). Initially, they were subjected to Shapiro-Wilk normality test and Levene's homogeneity test. Macrophage cell count was analyzed using a two-way ANOVA and an independent t-test. The two-way ANOVA was used to analyze the mean difference within the study groups, while the independent t-test was used to analyze the mean difference between groups. Confidence interval of 95% ($p < 0.05$) was used. Meanwhile, VEGF expression were analyzed using the Paired sample t-test.

RESULTS

Observation of macrophage cells in the wound's peripheral connective tissue was conducted using a light microscope with a magnification of 400x. Each sample was photographed in six fields of view after hematoxylin-eosin staining on the 3rd and 6th days. The total count of macrophage cells in each field of view and the average number of macrophage cells was obtained. Macrophage cells were identified by the presence of single-nucleus cells (mononucleus) shaped like a horseshoe pattern or resembling kidney cells with a bluish-purple color (Figure 4). The observation results of the number of macrophage cells in the wound's peripheral connective tissue in each treatment group at different durations of wound dressing application were calculated for the mean and standard deviation, as presented in Table 1.

The mean number of macrophage cells in the peripheral connective tissue of the wound in the treatment group was higher than the control group, both at the 3rd and 6th days post-excision. The mean macrophage cell number in the peripheral connective tissue of the wound on day 3 post-excision in the treatment group (9.5000) was higher than the control group (8.6383). Similarly, on day 6 post-excision, the mean number of macrophage cells in the treatment group (16.8617) was also higher than the control group (10.7500). The highest number of macrophage cells was observed in the treatment group on the 6th day post-excision (Table 1). To assess the significance of the differences in the number of macrophage cells in the peripheral connective tissue of the wound between observation times, treatment groups, and the interaction of observation times and treatment groups, a two-way ANOVA analysis was conducted. The results are presented in Table 2.

As shown in Table 2, the number of macrophage cells in the peripheral connective tissue of the wound in the treatment group was significantly higher than that in the control group (3rd and 6th days) with a p-value of 0.009. Similarly, there was a significant difference in the number of macrophage cells between the control group

and the treatment group with a p-value of 0.046. However, the p-value for the interaction between the observation time and treatment group is 0.125 ($p > 0.05$), indicating that there is no interaction between the treatment group and observation time, or the treatment group is not influenced by the observation time. The independent t-test was conducted to compare the mean number of macrophage cells between the treatment groups at each observation time. The results of the research indicate that there is a significant difference ($p < 0.05$) in the mean number of macrophage cells between the treatment groups only at the 6th ($p = 0.026$).

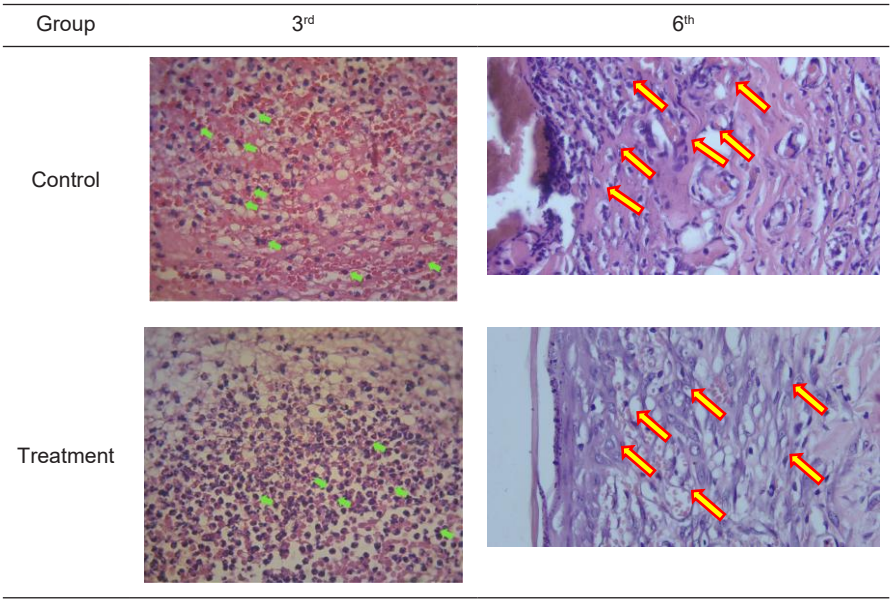

Figure 4. Microscopic image of the histological appearance of macrophage cells (green and red-yellow arrows) stained with HE at a magnification of 40x. It is evident that there are more macrophage cells at 6th day compared to 3rdday

Table 1. Mean and standard deviation (SD) of the number of macrophage cells in the peripheral connective tissue of the wound in each treatment group based on the duration of wound dressing application post-excision.

The Duration of Wound Dressing Application	Group	Mean	Std. Deviation (SD)	N
3rd	Control group	8.6383	4.72487	6
	Treatment group	9.5000	3.01482	6
6th	Control group	10.7500	2.60579	6
	Treatment group	16.8617	5.13330	6

Table 2. The statistical analysis using Two-way ANOVA showed the significance of the differences in the number of macrophage cells in the peripheral connective tissue of the wound for the following factors

	Sig.
Observation Time	.009**
Treatment Group	.046*
Observation Time* Treatment Group	.125

Note: *p < 0.05, **p < 0.01, ***p < 0.001

Table 3. The independent t-test showing the differences in the mean number of macrophage cells between the control group and the treatment group based on observation time

The Treatment Group based on Observation Time.	Sig.
3rd	.714
6th	.026*

Note: *p < 0.05

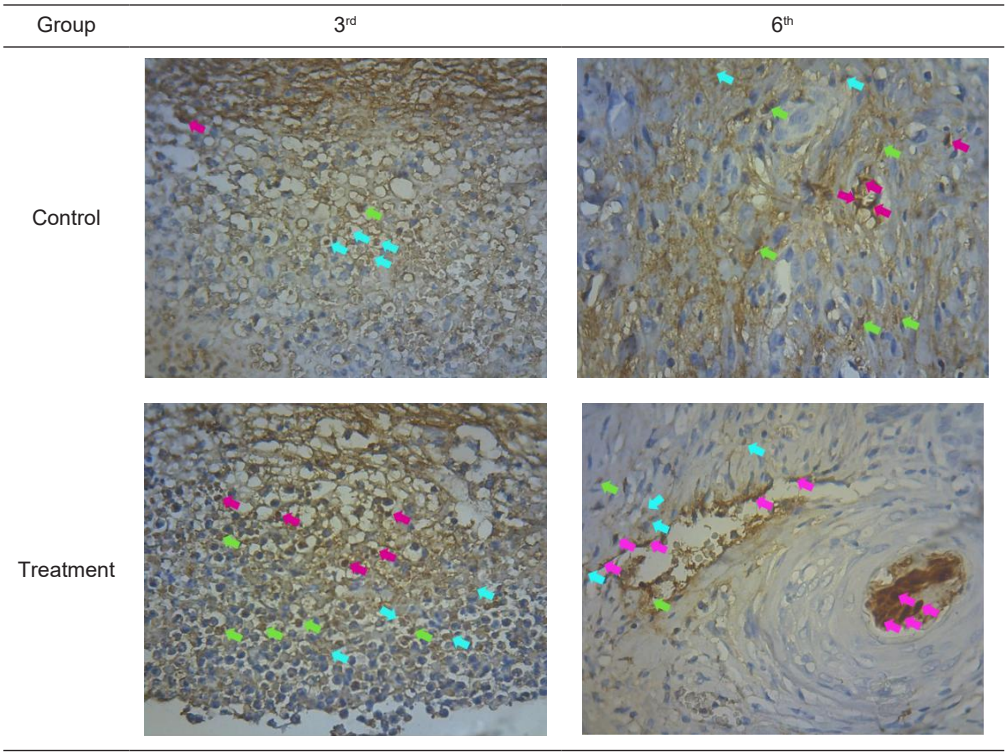

Figure 5. Microscopic images of the histological expression of VEGF (pink arrow indicating strong VEGF immunoreactivity, green arrow indicating moderate immunoreactivity, and blue arrow indicating weak immunoreactivity) during the wound healing process of the excision wound on the back skin of Wistar rats at magnifications of 40x and 400x

Table 4. The mean and Standard Deviation (SD) of VEGF expression in the inflammatory cells of the wound area for each treatment group at different durations of wound dressing application after wound excision

The Duration of Wound Dressing Application	Group	Mean	Std. Deviation (SD)	n
3rd	Control group	2.0550	.71082	6
	Treatment group	3.3883	.39035	6
6th	Control group	4.1117	.62159	6
	Treatment group	4.7783	.34342	6

The observation of VEGF expression was conducted in 3 large fields of view, which revealed immunoreactivity in the cytoplasm of inflammatory cells around the wound area (Figure 5). Each area was further used for the calculation of the mean VEGF expression in

Table 5. The statistical analysis using Two-way ANOVA was conducted to assess the significance of the differences in VEGF expression in the inflammatory cells of the wound area for the following factors

	Sig.
Observation time	.000***
Treatment Group	.000***
Observation time* Treatment Group	.145

Note: ***p < 0.001

the inflammatory cells. VEGF expression was considered positive when a brown color was present in the cytoplasm of inflammatory cells in the wound area.

To assess the significance of the differences in VEGF expression in the inflammatory cells of the wound area between observation times, treatment groups, and the interaction of observation times and treatment groups, a two-way ANOVA analysis was conducted. The results are shown in Table 5.

An independent t-test was conducted to compare the mean VEGF expression in the inflammatory cells of the wound area between the treatment groups at each observation time. The results of the research showed a significant difference ($p < 0.05$) in the mean VEGF expression in the inflammatory cells of the wound area between the treatment groups both on the 3rd and 6th days.

DISCUSSION

The excision wound model can be used in research for skin wound healing process because it allows for the assessment of epithelization, granulation tissue formation, scar formation, contraction, and angiogenesis in one model.[11,12] Secondary wound healing in the dermal layer involves a complex physiopathological process of skin healing due to significant loss of tissue substance. This causes the wound-healing process in the dermal layer to be followed by the formation of scar tissue. Analysis of secondary healing`s biological processes in response to various forms of dermal substitution is important to produce an efficient therapeutic product to stimulate wound healing.[13]

Optimization of wound healing can be done by following several basic principles, including (1) maintaining wound moisture, (2) ensuring adequate blood supply, and (3) minimizing infection. These principles can be facilitated by the use of an ideal wound dressing material that can protect and cover the wound, maintain moisture, is permeable to air so that the tissue receives an adequate supply of oxygen and can inhibit the growth of pathogens without inhibiting tissue growth.[14]

Silkworm (*Bombyx mori*) produces cocoons that have a structure, function, and protection mechanism similar to human skin so they can be useful for wound repair.[5,6,7] Many studies have shown that the content of fibroin and sericin as the two main proteins that make up the structure of silkworm cocoons are highly biocompatible materials and have good regenerative abilities for human body tissues.[5,7] Silkworm cocoons have the potential to be used as wound dressing materials that help accelerate the wound healing process. This study used silkworm cocoons which went through a degumming process for 90 minutes, and they were used as a basis for wound dressings in skin excision wounds in the Wistar rats.

Wound healing in mice is generally faster than in humans but comparable.[15,16] Observations in this study were conducted on the 3rd and 6th days. This timing was chosen to observe macrophage cells and VEGF expression during the inflammation phase, which peaks on the 3rd day, and the proliferation phase, which peaks on the 6th day.[15,17,18,19,20]

Macrophage cells are known to play a crucial role in the early stages of wound healing. The inflammation phase is characterized by the recruitment of immune cells, including resident macrophages (M0), neutrophils, and monocytes, from the blood to the injury site. Subsequently, they work with various types of cells inside and around the injured skin to regulate the repair process.[6,21] Macrophage cell activity becomes visible early on the second day during the wound healing process, both as resident M0 macrophages and M1 macrophages, which function as pro-inflammatory cells.[22]

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ISSN 2460-0164 (print)
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The benefits of biomaterial characteristics of wound dressing from silkworm cocoons (*Bombyx mori*) and the function of fibroin and sericin proteins component keep the wound environment moist.[5,6,7,8] Maintaining the wound environment in humid conditions prevents the occurrence of dehydration, accelerates re-epithelization, and further facilitates wound healing processes.[23,24] The moist condition on the edge of the excision wound facilitates the migration of neutrophilic and macrophage cells to the wound area to digest foreign objects and bacteria.[25] In addition, the migratory of neutrophils and monocytes or macrophages into the injury area triggers the release of cytokines and growth factors such as VEGF, FGF, TGF-β1, and TGF -α which accelerates the onset of the proliferation phase characterized by the abundance of fibroblasts in the area of the wounds.[26,27] This was seen on the 3rd and 6th days of observation in the treatment group that showed a higher number of macrophage cells compared to the control group.

The average number of macrophage cells in the *Bombyx mori* silkworm cocoon wound dressing treatment group on the 3rd day did not show a significant increase ($p = 0.714$) compared to the control group, but on the 6th day, there was a significant increase ($p = 0.026$). The number of macrophages increased during the inflammation phase, reaching their maximum concentration during the proliferation phase, and gradually decreased during the remodeling phase. The increase in the number of macrophage cells in the treatment group is due to the antibacterial and anti-inflammatory effects of the silkworm cocoon. The high number of macrophage cells on the 6th day is caused by the healing process which reaches its peak during the proliferation phase on the 6th day.[15] The high number of macrophage cells on the 6th day is also due to the healing process which remains in transition from mid to late-phase inflammation.[22,28]

The number of wound macrophage cells significantly increases in acute wounds and remains high for several days.[29] This study used an excision wound model with a subcutaneous wound depth of 4 mm, which is considered to be the contributing factor to the high number of macrophage cells on the 6th day. The high number of macrophage cells on the 6th day is a result of the early proliferation phase, where approximately 85% of macrophages in the wound area have a pro-inflammatory M1 phenotype that switches to M2 anti-inflammatory macrophages from day 5 to 7.[24] The 6th day is the peak of the proliferation phase in mouse wound healing, where macrophages play a role in anti-inflammation, tissue regeneration, and angiogenesis. The dominant macrophages during this proliferation phase are M2 macrophages.[14,22]

Macrophage cells play a crucial role during inflammation and proliferation in normal wound healing. They promote the recruitment and proliferation of fibroblasts and express several key growth factors that stimulate angiogenesis. One of these key growth factors that stimulate angiogenesis is the vascular endothelial growth factor (VEGF). VEGF is a major regulator of new blood vessel growth and an important inducer of blood vessel permeability.30 [31] VEGF also plays a role in stimulating the proliferation and migration of endothelial cells.30, 31, 32, 33[30,31,32,33]

Similar observations about VEGF expression as an indicator of the wound healing process were made, with VEGF protein serving as one of the proangiogenic mediators. This study observed VEGF expression in both wound dressing groups on the 3rd and 6th days after skin excision. Preservation of moist conditions on the excision site with the application of *Bombyx mori* silkworm cocoon wound dressing accelerates the migration of neutrophils and macrophage cells to the wound area for phagocytosis of the foreign bodies and bacteria. This process stimulates the release of cytokines and growth factors, including VEGF protein which accelerates the onset of the proliferation phase characterized by an increased number of fibroblasts in the wound area and an increased activity of VEGF expression.[26,27] The average expression of VEGF protein in the treatment group was observed to be higher on both the 3rd and 6th days compared to that in the control group.

The activity of VEGF protein expression in the *Bombyx mori* silkworm cocoon wound dressing treatment group on the 3rd day (p=0.002) and the 6th day showed a significant increase (p=0.044). VEGF protein increases at the early points following skin injury, and the level of VEGF protein remains elevated in wound fluid for at least one week in surgical wounds.[34] This is consistent with the results of this study, where the activity of VEGF protein expression increased significantly in both the control and treatment groups on the 3rd day, with a continued increase observed until the 6th day (p=0.000). VEGF protein is believed to be one of the most important proangiogenic mediators during the wound healing process.[35] The increase in VEGF protein expression activity is also in line with the increase in macrophage cells during the inflammation and proliferation phases. VEGF protein expression reaches its peak during the inflammation phase on the 3rd day and peaks during the proliferation phase around the 5th and 6th days.[15]

VEGF is a central regulator of angiogenesis that induces endothelial cell proliferation, and chemotaxis, and increases vascular permeability mediators (nitric oxide and prostacyclin) during both physiological and pathological conditions.36,37,38[39,40,41] VEGF protein, originally identified as a vascular permeability factor,[9] can induce vascular permeability with a potency of several thousand times higher than that of histamine. This underlies the observed increase in VEGF expression activity on the 3rd and 6th days in this study. Other studies have found that VEGF is a strong positive regulator of angiogenesis and stimulates endothelial cell functions necessary for new blood vessel formation, such as proliferation, migration, differentiation, and survival.[36,37,38,39,40,41] The importance of VEGF as a mediator of neovascularization is highlighted by studies which show that even the loss of one copy of the VEGF gene can result in early embryonic death.[36,42]

The number of macrophage cells in the control group and the treatment group on the 3rd day did not show a significant difference, but the expression of VEGF on the same day showed a significant difference. The presence of VEGF in the wound area is not only influenced by macrophage cells. This growth factor is also produced by several other cells such as keratinocytes, endothelial cells, and fibroblasts. Keratinocyte proliferation, which forms the epithelial layer during wound healing, is known to actively express VEGF. Endothelial cells and fibroblasts that are actively proliferating during the wound-healing process will also express VEGF.[30,43] The presence of these cells in the wound area contributes to the high expression of VEGF on the 3rd day. The silk cocoon wound dressing material may also influence these cells, but our study did not observe this effect.

Tissue damage initiates a continuous process in the body to restore tissue structure and homeostasis. Infiltration of inflammatory cells and vascular remodeling is key to this process.[44] Macrophages can secrete various anti- and pro-inflammatory growth factors. The secretion of VEGF by macrophages plays a crucial role in inducing angiogenesis in tissue repair.[45] The production of VEGF by macrophages supports angiogenesis by activating VEGF receptors on endothelial cells and providing an autocrine effect to further increase the number of macrophages in the wound area. The ongoing angiogenesis process then triggers VEGF production by endothelial cells, further increasing VEGF expression in the injured area for vessel maturation.[46,47] The same phenomenon is observed in this study, where the increase in the number of macrophage cells in the excision area from day 3 to day 6 post-excision is parallel to the increased expression of VEGF in the excision area at the same observation time.[48]

Bombyx mori has the potential to be used to accelerate wound healing. However, further study is needed to assess its comprehensive effect on wound healing. Our study only assessed the effect of *Bombyx mori* on the 3rd and 6th days of wound healing. A longitudinal study with more time and a longer examination period should be done to elucidate the effect of this material in wound healing comprehensively. Further study should also assess which phase of wound healing is affected more by this material. This will indicate the best time for application of this material in the

clinical setting. The effect of *Bombyx mori* as an antibacterial agent should also be assessed to elucidate the full mechanism of this material in affecting wound healing.

CONCLUSION

The application of wound dressing from silkworm cocoons (*Bombyx mori*) can increase the number of macrophage cells and VEGF expression activity on the 3rd and 6th days post skin excision in Wistar rats.

CONFLICT OF INTEREST

The authors declare no competing interests.

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