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Effect of Robusta coffee extract gel on fibroblast and collagen during proliferative phase of IIB degree-burn on Long Evans rats

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ABSTRACT

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IIB Degree-burn takes longer to heal because only a small epithelium component remains. It requires a topical agent that can accelerate the wound healing process. One of the wound healing signs is the increased collagen formation as an extracellular matrix produced by fibroblast in the proliferative phase. This study aimed to prove the effect of Robusta coffee extract gel as a topical agent on increasing the number of fibroblasts and collagen density in IIB degree-burn treatment on Long Evans rats. This study used 24 Long Evans rats which were divided into three groups, namely positive control (silver sulfadiazine), negative control (gel base), and treatment group (2.5% of Robusta coffee extract gel). The results showed a higher number of fibroblast ($p < 0.05$) and a denser collagen density ($p < 0.05$) of the Robusta coffee extract gel compared to the control group on day 8 and day 14. These results indicated that the number of fibroblasts and collagen increases that can scavenge free radicals and stimulate the release of cytokines that play a role in increasing fibroblast proliferation. Robusta coffee affects expanding the number of fibroblasts and collagen density to be an alternative topical agent of second-degree burns treatment.

ABSTRAK

Proses penyembuhan luka bakar derajat IIB membutuhkan waktu yang cukup lama. Hal ini dikarenakan komponen epitel yang tersisa hanya sedikit sehingga memerlukan sediaan topikal yang dapat mempercepat proses penyembuhan luka tersebut. Salah satu tanda proses penyembuhan luka adalah terjadinya peningkatan pembentukan kolagen sebagai matriks ekstraseluler yang dihasilkan oleh fibroblast pada fase proliferasi. Tujuan penelitian ini adalah untuk membuktikan efek gel ekstrak kopi Robusta sebagai agen topikal terhadap peningkatan jumlah fibroblas dan kepadatan kolagen pada perawatan luka bakar derajat IIB tikus Long Evans. Penelitian ini menggunakan 24 ekor tikus yang dibagi menjadi 3 kelompok yaitu kontrol positif (silver sulfadiazine), kontrol negatif (basis gel), dan kelompok perlakuan (gel ekstrak kopi Robusta 2,5%). Hasil penelitian menunjukkan jumlah fibroblas yang lebih tinggi ($p < 0.05$) dan kepadatan kolagen yang lebih rapat ($p < 0.05$) antara gel ekstrak kopi Robusta dibandingkan dengan kelompok kontrol baik pada hari ke-8 maupun hari ke-14. Hasil ini menunjukkan bahwa ada peningkatan fibroblast dan kolagen yang dapat melindungi dari radikal bebas dan merangsang pelepasan sitokin yang berperan dalam meningkatkan proliferasi fibroblast. Gel kopi Robusta dapat meningkatkan jumlah fibroblast dan kepadatan kolagen sehingga gel ini dapat menjadi alternatif agen topikal dalam perawatan luka bakar derajat IIB.

Keywords:

topical agent;
burn;
deep partial thickness;
gel;
Robusta coffee

INTRODUCTION

A burn is one of the biggest global health problems. It is estimated around 11 million cases annually worldwide.¹ In Jember, East Java, Indonesia, the burn prevalence reaches 70 cases from January 2014-October 2016, with the most cases severe burn.² IIB-Degree burns have a fairly high prevalence in Indonesia, there were 104 of 414 cases during 2013-2015.³ In the II-degree burns, the damage usually extends to the reticular dermis. After the injury, the burn will cause redness and paleness or splotchy skin followed by the appearance of bullae in the injured area. At this stage, the patients are complaining of pain and decreased capillary refill when pressure is applied to the injury. Patients with IIB-degree burns are able to heal spontaneously without surgery.^{1,4}

The proliferative phase plays an important role in restoring tissue integrity by replacing lost tissue and repairing damaged tissue during the wound healing process.⁵ Proliferation of fibroblast cells occurs in this phase to synthesize collagen.⁶ Collagen is a major protein component in the extracellular matrix to control the inflammatory response to injury.⁷ The density of collagen in the wound area determines the strength of the tissue to prevent the risk of wound dehiscence. However, non-optimal wound care will cause a slower wound healing process.⁸

Moist wound treatment promotes the healing process of burn by providing a topical agent, such as silver sulfadiazine.⁹ Another way to create a moist environment is to use standard modern dressings.¹⁰ However, the number of modern dressings is very limited, especially in the peripheral area. These problems may cause a physical and psychological burden on the patient because the treatment takes longer.¹¹ Therefore, natural ingredients that can

stimulate the wound healing process and abundant materials can be an alternative therapy with economic value. One of these ingredients is Robusta coffee.

Previous studies showed a positive effect of Robusta coffee extract in accelerating the healing process of incisional wounds in mice, indicating the number of fibroblasts increases.¹² Robusta coffee contains ingredients such as chlorogenic acid, caffeine, flavonoids, tannins, and saponins, which have antioxidant effects so that those ingredients can ward off excess free radicals at the wound base.¹³ Flavonoids in coffee have antimicrobial effects that work through the destruction of cell membranes and denaturation of cell proteins. Besides, flavonoids have anti-inflammatory effects.¹⁴ Nowadays, there have been no previous studies that found the use of Robusta coffee extract gel in the healing process of IIB degree-burn. This study was conducted to prove the effect of Robusta coffee extract gel as a topical agent on increasing the number of fibroblasts and collagen density in IIB degree-burn treatment on Long Evans rats.

MATERIALS AND METHODS

Animals

This study used 24 male Long Evans rats aged 2-3 months weighing 150-200 g. Rats were randomly divided into three groups: the 2.5% Robusta coffee extract gel group, the positive control group that was given silver sulfadiazine, and the negative control that was given the gel base. Rats were used in the experiment after one week of acclimatization. The research was conducted after obtaining approval from the Ethics Committee of the Faculty of Medicine, the Universitas Jember, Jember (ref:1508/H25.11/KE/2021).

Extract preparation

Robusta coffee used in this study was obtained from the Coffee and Cacao Research Institute in Jember. Two hundred g of Robusta coffee beans were grinded into powder. The powder was extracted using the ultrasonic method with 1.5 L of ethanol at 40°C for 30 min. The results were filtered using filter paper on a vacuum Buchner funnel and concentrated using a rotary evaporator.

Gel preparation

Methylparaben, nipasol, and propylene glycol were mixed into a glass beaker. Then, the carbopol and distilled water were mixed into the mortar in a ratio of 1:20. 4% triethanolamine was added slowly while stirred. The propylene mixture was put into a mortar containing carbopol and distilled water, then stirred to form a homogeneous gel mass. Robusta coffee extract and remaining distilled water were added to the mortar until homogeneous.

Burn wounds model

Rats were anaesthetized with a mixture of ketamine and midazolam intraperitoneally. Each rat was shaved on its back and cleaned with 70% alcohol. Based on Akhoondinasab *et al.*¹⁵ modifications were carried out under the supervision of a clinically experienced burn and wound consultant plastic surgeon and experimental research related to burns may avoid bias without

the need for a blind examiner. The burn was made using an iron plate with 2 x 2.5 cm width, immersed in boiling water at 98°C for 10 min. The hot iron plate was attached to the back of the rats for 20 seconds.

The wound was cleaned with normal saline and Savlon. Then, the gel base, silver sulfadiazine, and coffee extract gel were applied every 2 days according to each group. The wound was covered with a transparent film, sterile gauze, and plaster. Skin tissue was taken by biopsy on day 8 and day 14 to evaluate wound healing microscopically. The tissue was stored in 10% NBF solution for histopathological slides using HE staining.

Observation of histopathological slides

The histopathological observation was carried out using a light microscope with 400x magnification connected to an optical camera. Fibroblasts were identified as spindle-shaped cells or oval, flat, and purple in HE staining. These cells were observed in 6 fields of view, selected zig-zag from top to bottom in each slide, and then the average was calculated.¹⁶ Meanwhile, collagen was identified as long pink fibers, which were interpreted in the scoring system. The scoring was determined based on Rachmanita *et al.*,¹⁷ study in 4 fields of view, selected zig-zag from top to bottom in each slide, and then the average was calculated. The scoring system can be seen in TABLE 1.¹⁷

TABLE 1. Collagen density scoring

Score	Interpretation
0	No collagen fibers in the wound area
1	Collagen fibers in the low wound area (<25%)
2	Collagen fibers in moderate wound areas (26-50%)
3	Collagen fibers in tight wound areas (51-75%)
4	Collagen fibers in very tight wound areas (76-100%)

Data analysis

Research data analysis used the SPSS program. Data of the study were shown as mean ± standard deviation (SD). The fibroblast’s statistical data analysis was carried out using the One Way Anova and continued using the Post Hoc LSD test. Further, the observation results of collagen density were tested using the Kruskal-Wallis’ and followed by the Mann-Whitney’s. The $p < 0.05$ indicated a significant result.

RESULTS

Number of fibroblasts

Fibroblasts have spindle-shaped cells or oval, flat, and purple colored in HE staining. FIGURE 2 shows the fibroblasts in the burn day 14 of the negative control

group (FIGURE 2a), Robusta coffee extract gel (FIGURE 2b), and positive control group (FIGURE 2c). The number of fibroblasts was significantly higher after being given Robusta coffee extract gel on the 8th and 14th day (59.27 ± 7.18 and 64.3 ± 9.93) than in the negative control group (51.07 ± 5.93 and 53.08 ± 5.33), but it did not differ much compared to the positive control group (59.98 ± 5.27 and 62.71 ± 8.77), as shown in FIGURE 1. Burn treatment with Robusta coffee extract gel showed a significant difference in the number of fibroblasts compared to the negative control group on day 8th and day 14th ($p < 0.05$), but there was no significant difference with the positive control ($p > 0.05$). It was indicated that the Robusta coffee extract gel significantly increased the number of fibroblasts (FIGURE 1).

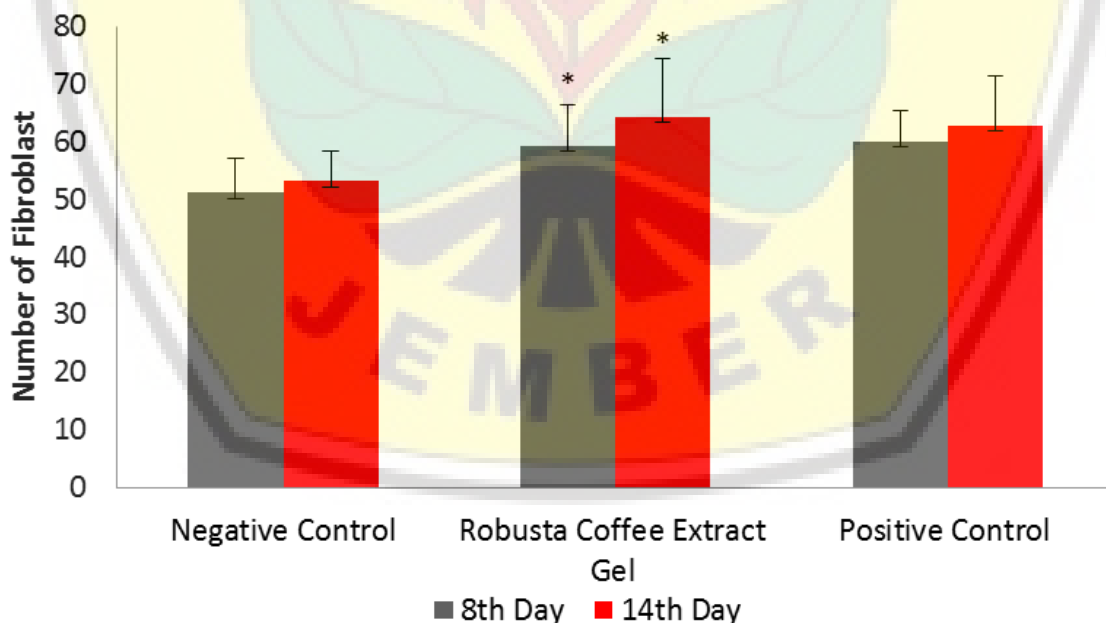


FIGURE 1. Number of fibroblast (mean ± SD) in each group on day 8th and day 14th. Post Hoc LSD Test; * $p < 0.05$

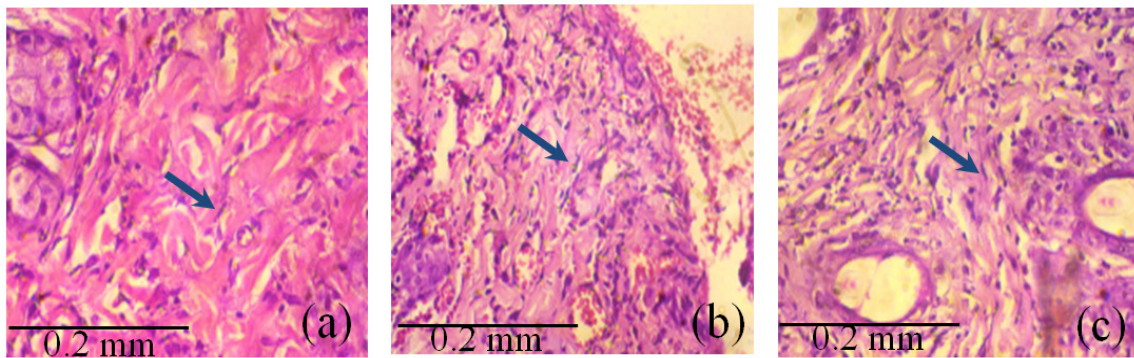


FIGURE 2. Photomicrographs of fibroblast cells in burn day 14th with magnification 400x; (a) negative control; (b) Robusta coffee extract gel; (c) positive control group.

Collagen density

A scoring system was used to measure the collagen density. FIGURE 3 shows that on the 8th and 14th days, the Robusta coffee extract gel has denser average collagen (2.75 ± 0.33 ; 3.25 ± 0.54) compared to the positive control (2.73 ± 0.44 ; 3.13 ± 0.45) and the negative control (2.25 ± 0.35 ; 2.65 ± 0.45). Histopathological description of the collagen density on day 14 of the negative control (FIGURE 4a), Robusta coffee extract gel (FIGURE

4b), and positive control (FIGURE 4c) can be seen in FIGURE 4. Burn treatment with Robusta Coffee extract gel showed a significant difference in collagen density compared to the negative control group on the 8th and 14th days ($P < 0.05$). However, there was no significant difference between the Robusta coffee extract gel group and the positive control group ($p > 0.05$). It was indicated that the Robusta coffee extract gel significantly increases the density of collagen (FIGURE 3).

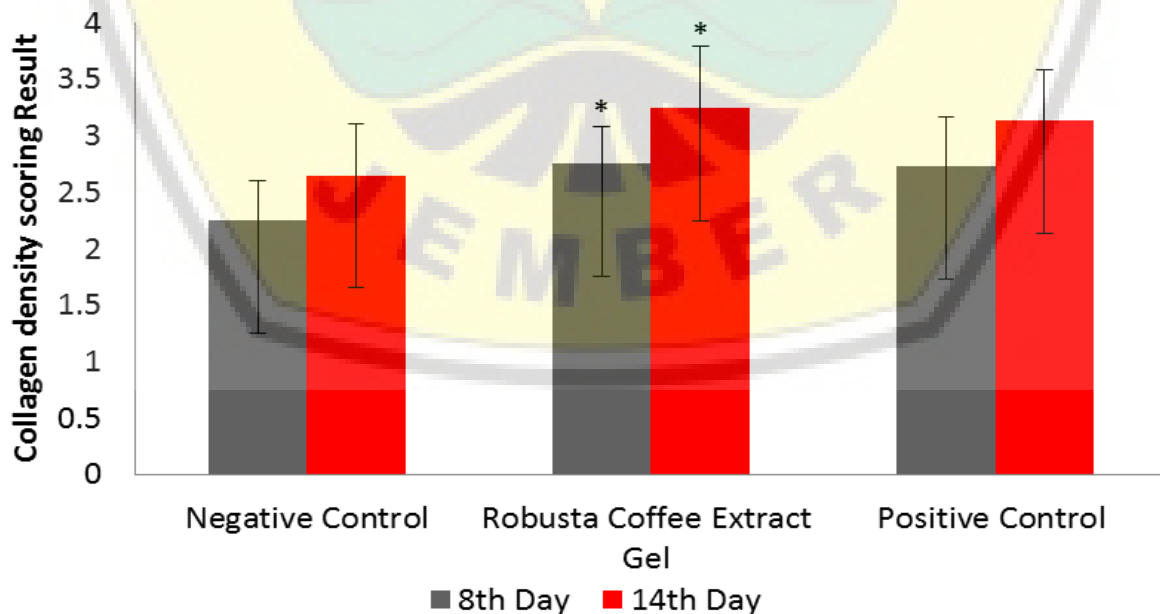


FIGURE 3. Collagen density scoring results (mean ± SD) for each group on 8th and 14th post-burn. Mann-Whitney; * $p < 0.05$

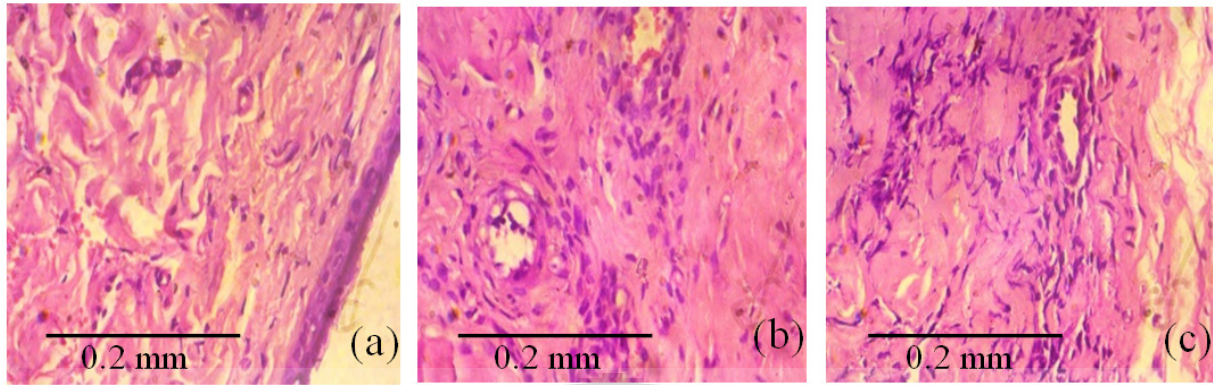


FIGURE 4. Histopathological of collagen density in day 14 burn with magnification 400x; (a) negative control; (b) Robusta coffee extract gel; (c) positive control.

DISCUSSION

In the burn wound healing process, there is a process of restoring tissue integrity by substituting lost tissue and repairing damaged tissue. During the proliferative phase, the process of epithelialization, angiogenesis, and collagen synthesis in the wound area will occur; this leads to the formation of an extracellular matrix to restore vascularity and form granulation tissue.⁵ Fibroblasts are cells with a significant role, these cells will proliferate to synthesize collagen.⁶ Collagen is a major protein component in the extracellular matrix that can control the inflammatory response to injury through its influence on mitogenesis, differentiation, and cellular migration.⁷ The density of collagen can strengthen the tissue. However, insufficient collagen deposition in the wound area can increase the risk of wound dehiscence.⁸

The wound healing process in the negative control group was slower than in the other groups. It is indicated by the lower number of fibroblasts and collagen density. The negative control only contains a gel base. It does not affect the wound healing process because there is no active substance but only as a vehicle. This result is strengthened by Fuadi *et al.*¹⁸ showing that wounds treated without active ingredients had lower fibroblast than others. On the

contrary, the number of fibroblasts and collagen density in the positive control is higher due to silver sulfadiazine having antibacterial properties by preventing folic acid synthesis. Silver sulfadiazine plays a role in fibroblast proliferation to produce collagen and fibronectin.¹⁹

The substance of antioxidant chlorogenic acid, caffeine, and flavonoids in Robusta coffee bean extract caused an increase in the number of fibroblasts and collagen density compared to the negative control group on the 8th and 14th days. Excessive free radicals can disturb the activity of TGF- β in fibroblasts proliferation to synthesize collagen during the proliferative phase.^{20,21} Humaryanto also showed that the administration of Robusta coffee extract could assist the wound healing process by increasing the number of inflammatory cells and fibroblast cell proliferation during the proliferative phase.¹² The results of this study were strengthened by Kenisa *et al.*²² that the administration of Robusta coffee extract was able to significantly increase the number of fibroblasts so that the full thickness healing process was faster than the control group. Shahriari *et al.*²³ reported that the administration of green coffee bean extract improved the healing process of full-thickness wounds in rats. Coffee bean extract and its chemical constituents can promote keratinocytes

and fibroblasts' proliferation and migration, which are highly important in the wound healing process.²³

Yaqin and Nurmilawati²⁴, the composition of Robusta coffee bean extract, namely caffeine, flavonoids, and chlorogenic acid, could inhibit the growth of *Staphylococcus aureus* bacteria colonies. The flavonoid contained in Robusta coffee has an antibacterial effect by damaging bacterial cell membranes and denaturing cell proteins to kill the bacteria.¹⁴ Prevention of infection during the wound healing process will accelerate the healing process towards the proliferation phase, so fibroblast cell proliferation occurs faster.²⁵ It caused the number of fibroblast cells in the Robusta coffee extract group to be higher than in the negative control group on the seventh day. In line with Yaqin and Nurmilawati²⁴, Yuwono²⁶ reported in his study that Robusta coffee has a strong zone of inhibition against methicillin-resistant *Staphylococcus aureus* (MRSA).

The gel dosage form in the extract can provide a cold sensation and reduce pain. Acute pain after an injury may cause hemodynamic changes and anxiety in the experimental animals, resulting in slower wound healing. Pain reduces macrophage activity which affects the performance of TGF- β in stimulating fibroblast proliferation.²⁷ Lack of pain control causes a prolonged catabolic phase, increasing glucagon, corticosteroids, and insulin hormone resistance which can inhibit wound healing.¹⁸

This study showed that Robusta coffee extract gel had a positive effect on burn healing by increasing the number of fibroblast cells and collagen density. A previous similar study revealed that coffee extract had an impact on healing burns in male rats.²⁸ The limitation of this study is that the observation of the burn healing process is limited at the end of the proliferative phase (14th day) so the wound that is formed has not

healed completely. In addition, Robusta coffee extract is only made in the form of gel preparation. Therefore, further research is needed on the process of burn healing to the remodeling phase or the use of Robusta coffee extract in other preparations such as ointments or creams.

CONCLUSION

The results of this study indicates that the Robusta coffee extract gel can accelerate the healing process of second-degree burns by increasing the number of fibroblasts and collagen density. Therefore, it can be an alternative topical agent in the IIB degree-burn treatment.

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