RESEARCH ARTICLE

Hydrolyzed VCO Cream Reduces Neutrophil Number and Increases Angiogenesis in Mid Dermal Burn Wound Healing

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Received date: Jan 9, 2023; Revised date: Apr 5, 2023; Accepted date: Apr 14, 2023

Abstract

BACKGROUND: Researches on plant active substances are conducted to find effective topical drugs for mid dermal burns wound. The 70% hydrolyzed VCO (hVCO) cream has been known to accelerate macroscopic healing of mid dermal burns compared to 30% and 50% hVCO creams. This study was conducted to further investigate the effectiveness of 70% hVCO cream and find the most effective doses of hVCO to heal mid dermal burns based on the macroscopic diameter, neutrophil number and angiogenesis.

METHODS: Thirty mid dermal burns-induced male *Rattus norvegicus* were randomly divided into 6 groups that were either treated with 70% hVCO cream, 100% hVCO cream, or basic cream for 6 or 12 days. Macroscopic appearance of mid dermal burn was documented, and the diameter was calculated. Hematoxylin Eosin (HE) examination was conducted to measure the neutrophil and angiogenesis in mid dermal burn wound.

RESULTS: The decrease of wound macroscopic size was found to be significant after 6 and 12 days of treatment (p=0.004 and p<0.001, respectively), with the smallest diameter was found in 70% hVCO group. The lowest number of neutrophils was found in the 70% hVCO group at day-6 (p=0.039) and day-12 (p=0.013). There was a significant increase of angiogenesis at day-12 (p=0.025) with the highest value obtained in the 70% hVCO group.

CONCLUSION: The 70% hVCO decreases the wound macroscopic size, decreases neutrophil number, and increases angiogenesis compared to the 100% hVCO. Hence, 70% hVCO isshown to be the most effective dose in accelerating mid dermal burns wound healing andcan be explored further as topical medication.

KEYWORDS: hVCO, neutrophil, angiogenesis, wound healing

Indones Biomed J. 2023; 15(3): 240-6

Introduction

Burn is a trauma with high mortality rate, especially in poor and developing country. There were 300.000 death case caused by burn every year, and the highest mortality rate found in Southeast Asia (11,6 deaths per 100.000 populations each year).(1) Management of burn injury is given by the depth, the surface area of injury, and the burn classification. Based on the depth, mid dermal burn wound is a wound which affected some part of dermis, including the peripheral nerve. One of the treatments that can be chosen for this case is wound care with topical drugs that play a role in burn healing process.(2,3)

Normally, burn healing occurs in three phases, namely inflammatory phase, proliferation phase, and remodelling phase. Neutrophils play an important role in the inflammatory phase by phagocyte the debris and foreign substances in tissue surface. Neutrophil number increase in the beginning and decreased in the end of inflammatory phase before the proliferation phase started.(4) In proliferation phase, a new skin structure is formed. The angiogenesis process play an important role as a nutrition transport way and tissue oxygen supply to granulation tissue formation.(5) Angiogenesis is induced by inflammatory factors such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet derivate growth factor (PDGF), transforming growth factor (TGF)-α and TGF-β.(6)

Many studies were conducted to obtained effective and efficient active substances to heal the burn wound. Virgin Coconut Oil (VCO) is one of the plant materials that has been widely used and studied for this purpose. The lipid acid of VCO has been known to have anti-inflammatory effect.(7,8) Through the hydrolysis process, the complex chain of VCO fatty acid split into the single chain which more effective than before. The anti-inflammatory effects hydrolyzed VCO (hVCO) also has been proved to have an antibacterial effect which is very important in wound healing process.(9,10) The 70% hVCO has been known to have the best effect in mid dermal burn healing compared to 30% hVCO and 50% hVCO in term of accelerating the wound closuremacroscopically.(11,12) This study was conducted to further investigate the effectiveness of 70% hVCO cream and to find the most effective dose of hVCO to heal mid dermal burn wound based on the macroscopic diameter, neutrophil number and angiogenesis.

Methods

VCO Hydrolyzation

VCO (HerboristTM VCO, PT. Victoria Care, Jakarta Indonesia) was hydrolyzed by taking 50 grams of oil which then added with NaOH ethanol by 70% and 100% of the total saponification value. To turn the soap (sodium salt of fatty acids) into free fatty acids, the mixture was then acidified with diluted HCl. After shaking the acidified liquid, 50 mL of n-hexane was added to extract it, that later created two distinct layers: the lower layer and the upper layer, both containing the hexane fraction. Then, fraction 1 of the hexane fraction was formed. To obtain fraction 2, extraction was carried out once again at the bottom layer. Fifty grams of Na_2SO_4 anhydrous were added to two fractions, which were then mixed and dried after 15 minutes. The hydrolyzed oil was then dried in a water bath to evaporate the hexane from the dry hexane fraction.

hVCO Cream Production

Creation of the cream was carried out with oil emulsion in water with the smelting process. The ingredients were first weighed according to a predetermined formula, then each oil phase material (VCO, stearic acid, ethyl alcohol, isopropyl myristate, span, lanolin, paraben propel) was inserted into the glass cup and melted in a porcelain cup over the bath water at 70°C. The water phase material was dissolved in a glass cup placed on a water bath at the same temperature of 70°C. After being melted and dissolved, the oil phase material was mixed into the water phase in a hot state and was carried out by mixing with a mixer or using mortar and pestle after the cold cream mass. The manufacturing process was carried out two times according to the formula made, 70% and 100% hVCO.

Animal Treatment

The experiments were conducted following the institutional guidelines, and the protocol was approved by the Health Research Ethics Committee of the Faculty of Medicine Diponegoro University (No.: 11/EC/H/FK-UNDIP/II/2019). Thirty 8- to 10-weeks-old male Wistar rats (*Ratus norvegicus*) with weight range between 120–150 grams were included in this study as samples. Rats were acclimated for 7 days in the individual cage at the same room, and received standard feed and water *ad libitum*. After the acclimatization, mid dermal burn wound was induced to rats' skin. Rats were then divided to six groups randomly, and treated with 70% VCO cream for six days (group A1), 100% hVCO cream for six days (group B1), basic cream for six days (group C1), 70% hVCO cream for twelve days (group A2), 100% hVCO cream for twelve days (group B2), and basic cream for twelve days (group C2).

Induction of Mid Dermal Burn Wound

Rats' back was cleaned and shaved before the burn wound induction. Rats then anesthetized with 10% ketamine intramuscular. Mid dermal burn wound was obtained by attaching a 10 mm diameter iron rod with a weight 50 grams for fifteen seconds which was previously immersed in 1000C boiling water for three minutes. The macroscopic appearance of the mid dermal burn wound was then documented 15 seconds after the induction and followed by the hVCO cream application to the wound.

hVCO Cream Treatment

Soon after burn wound was induced to rats, cream was applied thinly on the wound surface according to the treatment groups, 70% hVCO for group A, 100% hVCO for group B, and basic cream for group C. Wound care and application of topical cream was done once daily for six days for group 1 and twelve days for group 2.

Histological Examination

Just before sacrificed, rats were anesthetized with lethal dose of 10% ketamine intramuscular, then diameter of wound was documented and measured macroscopically with manual calliper. After the rats showed some death signs, skin was resected for about 8 mm long from the wound edges until as deep as the edge of muscle. Tissue was fixed by buffer formalin 10% for five hours, then the paraffin block was made. Small part of tissue from paraffin block was used to evaluate the number of neutrophil and angiogenesis with Haematoxylin Eosin (HE) staining. Microscopic examination with 400x magnification was conducted by an anatomy pathologist to count the number of neutrophils and angiogenesis in four different field, with the mean result used as the data for each sample.

Statistical Analysis

Data was analysed with SPSS ver.25 software for Windows 10 (IBM Corporation, Armonk, NY, USA). One-way Anova test followed by Post-Hoc test was used to analysed data with normal distribution. Otherwise, the Kruskal Wallis test and Mann Whitney test was performed. The result was considered significant if the p-value<0.05.

Results

Macroscopic Size of the Wound

In all groups, the diameter of the wound formed soon after induction has same size as the iron rod (Figure 1). Over time, the surface area of the wound appeared to be larger on day-6, and then became smaller on day-12 (Figure 2). The statistical analysis showed a significant difference in the wound diameters between the three groups that were treated by topical cream for 6 days (A1, B1, C1 groups, with p=0.004) and between the three groups that were treated for 12 days (A2, B2, C2 groups, with p<0.001). For both the 6 days and 12 days of treatment, the smallest wound diameter were found in 70% hVCO groups (with A1 group: 14.00±1.00 mm and A2 group: 8.20±0.84 mm) (Figure 3).

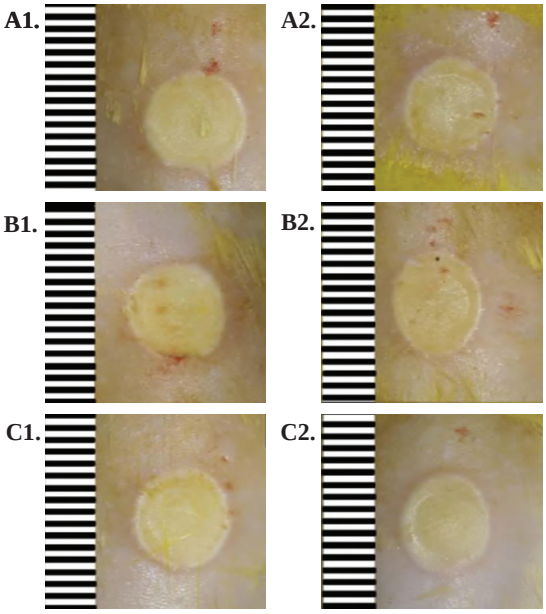

Figure 1. Macroscopic appearance of mid dermal burn wound 15 seconds after the iron rod induction prior to the treatment. Each bar (black-white): 1 mm.

Neutrophil Numbers

Distribution of neutrophils in histological preparation was counted and shown in Figure 4. Neutrophil counts were found to be lower in the groups that were treated by the topical creams for 12 days (A2, B2, C2 groups) compared to the groups that were treated for 6 days (A1, B1, C1 groups). There was significant difference between groups that were treated by the topical creams for 6 (p=0.039) and

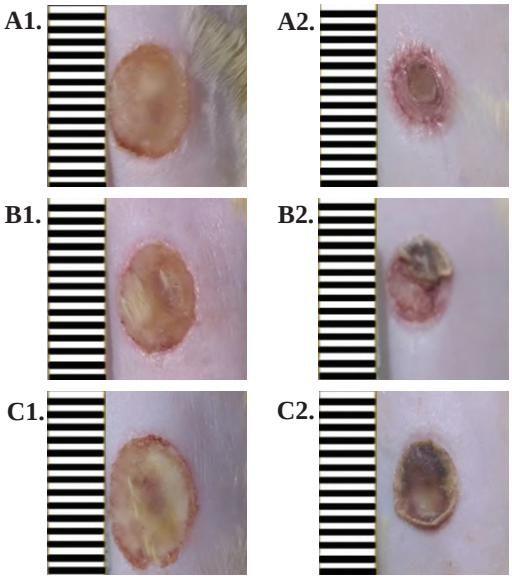

Figure 2. Macroscopic appearance of mid dermal burn wound size after 6 and 12 days of treatment. Each bar (black-white): 1 mm.

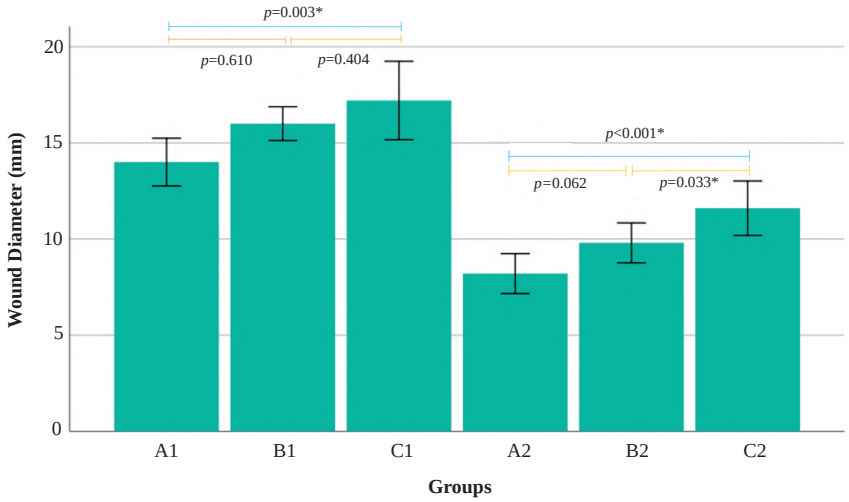

Figure 3. The diameter of mid dermal burn wound size diameter after 6 and 12 days of treatment. Data were shown in mean±SD. Significant if $p<0.05$, tested with Post- Hoc.

12 days ($p=0.013$), with the lowest number of neutrophils was found in the 70% hVCO-treated groups (with group A1: 86.00±20.51 and group A2: 32.20±1.92) (Figure 5).

Angiogenesis

The presence of angiogenesis in histological tissue was shown in Figure 6. Angiogenesis was found to be higher during the proliferation phase after 12 days of treatments compared to 6 days of treatment. The angiogenesis result on day 6 was not normally distributed between group A1, B1, and C1, and the Kruskal-Wallis result was not significant ($p=0.874$). There was significant difference of angiogenesis between group A2, B2, and C2 ($p=0.025$), with group A2 was showing the highest number of angiogenesis (13.60±6.02).

Discussion

Burn wound has 3 zone of wound namely coagulation zone, static zone, and hyperemic zone. White zone on the rat's back (Figure 1) which appears on areas exposed to heat shown the coagulation zone. On the outside of coagulation zone, static wound was found. This zone was a reversible area with low level of inflammation sign and perfusion. Cells in this zone often become damaged and die within the first 48 hours so the wound became wider.(13,14)

The wound size is narrowed along the healing process. As seen in this study, the wound size in groups treated for 12 days were smaller than in groups treated for 6 days in

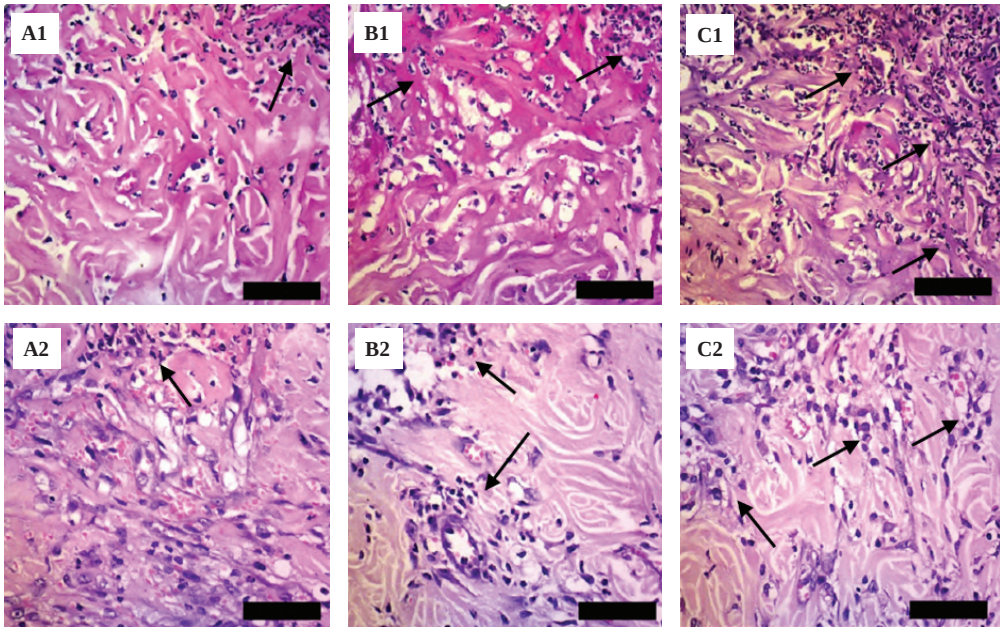

Figure 4. Histological tissue examination of neutrophil number after 6 and 12 days of treatment. Black arrow: neutrophil. Black bar: 50 µm.

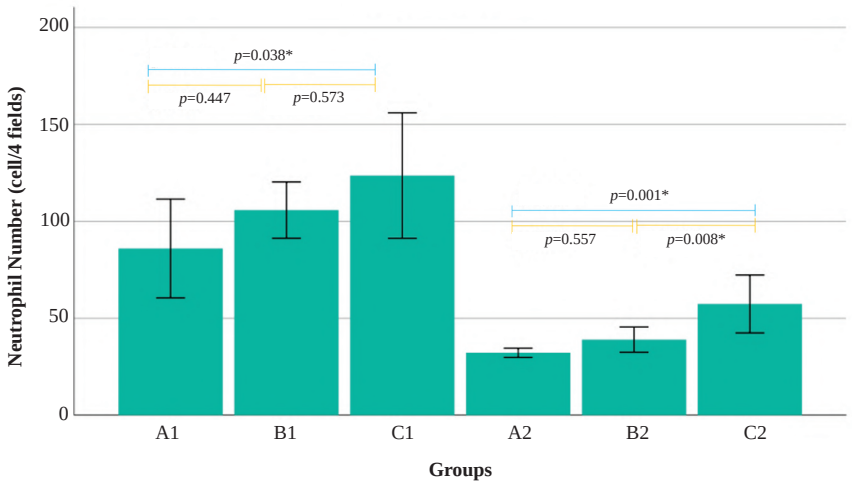

Figure 5. Neutrophil number after 6 and 12 days of treatment. Data were shown in mean±SD. Significant if $p<0.05$, tested with Post-Hoc.

all treatment groups. Groups who received 70% hVCO had smaller wound size than the others both after 6 and 12 days of treatment. This result is consistent with a study who found that application of 70% hVCO on mid dermal burn wound speeds up the burn wound healing time faster than 35% hVCO, 50% hVCO, and bioplacenton in terms of wound size.(11) A study of fermented VCO (fVCO) also shown that consumption of fVCO speeds up the wound healing faster than control group.(15) Another study compares the application of VCO, conventional silver sulfadiazine cream, and no treatment on diabetic wound healing, where it proves that VCO could promote and accelerate wound healing faster than silver sulfadiazine application and the no treatment group.(16)

In the cellular level of wound healing process, neutrophil plays an important role in inflammatory phase. Soon after trauma occurs, damaged tissue produces chemoattractant substance and draw neutrophil to migrate to the wound edges. Neutrophil number will increase rapidly in the beginning of this phase and decrease in the end of inflammatory phase after apoptotic and phagocytosis process. The large number of neutrophils can lead inflammatory phase prolonged and make the healing process become slower and increase the complication risk.(17,18) This study showed that the number of neutrophils were lower after 12 days of treatment compare to 6 days of treatment, in which the lowest was found in the group receiving 70% hVCO. This result indicates that in day-6, proliferation phase might begin and neutrophils start to decrease due to apoptosis and phagocytosis by macrophages. Neutrophil numbers indicate the developmental of burn wound healing, where fewer neutrophils found in the 70% hVCO group reflects a faster inflammatory phase with the treatment. Other than the antibacterial effect, the anti-inflammatory property of VCO

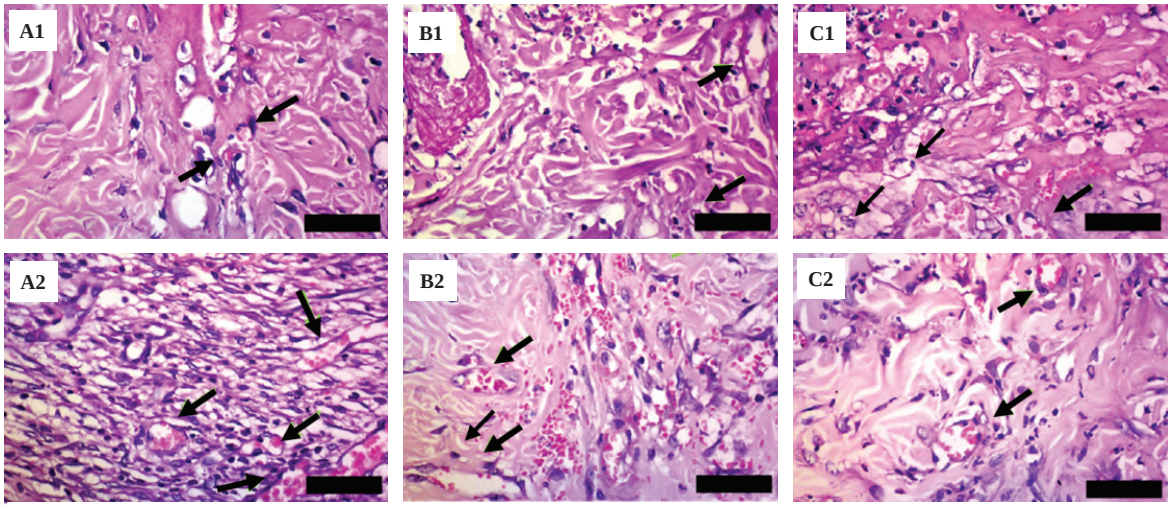

Figure 6. Histological tissue examination of angiogenesis appearance after 6 and 12 days of treatment. Black arrow: angiogenesis. Black bar: 50 µm.

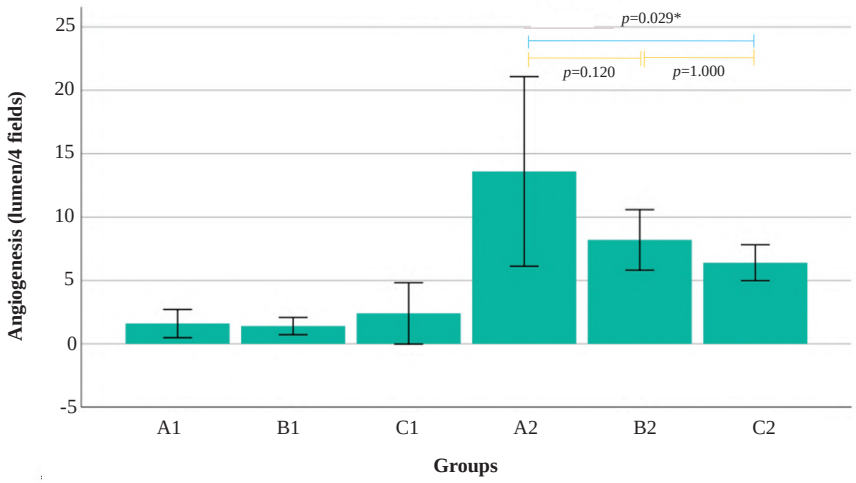

Figure 7. Angiogenesis after 6 and 12 days of treatment. Data were shown in mean±SD. Significant if $p<0.05$, tested with Post-Hoc.

could suppress proinflammatory cytokines production and indirectly lowered the neutrophil count.(7) Another study compares the effect of nanosilver collagen cream with silver sulfadiazine cream, where it was found that application of nanosilver collagen creams cannot lowered neutrophil count better than silver sulfadiazine cream, showing it's advantage in suprpressing the inflammation.(19)

The smallest number of neutrophils was found in group receiving 70% hVCO, both in 6 or 12 days of treatment. This finding was similar with a study, where hVCO has been proven to have antibacterial effect for skin pathogen bacteria like *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*.(20) Similar study has been done with the conclusion that hVCO has the most antibacterial ability for *Pseudomonas aeruginosa*.(10) The antibacterial effect of VCO mainly influenced by single chain of lauric acid level which increases with the hydrolyzed process. A study has found the similar result and conclude that inhibition zone of *E. coli*, *S. aureus*, *Salmonella typhimurium* and *B. cereus* becomes larger as lauric acid level increases in hVCO.(21) Besides antibacterial effect, VCO also been shown to have anti-inflammatory effect with suppressing pro-inflammatory cytokines which that affect neutrophil number.(5) The anti-inflammatory ability of VCO also shown in other study which conclude that topical VCO application suppress tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-6, IL-8, and IL-5 level.(22)

Angiogenesis as a part of skin components plays an important role in new tissue formed process which connected with wound healing process. Angiogenesis stimulated by vascular endothelial growth factor (VEGF), a pro-inflammatory cytokine produced by endothelial damage and also by neutrophils.(23) Angiogenesis peaked in the proliferation phase, so if the wound healing timeline on samples were consistent, angiogenesis might appear to started in day-6. Angiogenesis then continues to increase, as found after 12 days. The increasing number of angiogenesis formed indicates that the wound healing process has gone further than the fewer angiogenesis. This study results shown that there was a significantly increases of angiogenesis after 12 days of treatment, where the group that received 70% hVCO have more new blood vessels than two other groups, indicating that hVCO accelerates the angiogenesis process. This result is supported with a study which found that fVCO increases the VEGF receptor 2 (VEGFR2) level. VEGFR2 is a protein receptor which play important role in angiogenesis process.(15) While another study showed that the application of VCO resulted in more new developed blood vessels compared to those who receive conventional silver sulfadiazine cream and those who do not receive any treatment.(16)

A 70% hVCO has the highest effectiveness for wound healing because the levels of fatty acids as active elements found in VCO can only use from concentrations of 0.0001% to 70%, so that the fatty acids that can be hydrolyzed are only 70%. The lack of effectiveness of 100% hVCO is caused by damage to active substances due to chemical hydrolysis process.(24) In this study, the neutrophil count was only assessed on day-6 as the end of inflammatory phase and on day-12 where wound healing was still in the proliferative phase. Hence, it is necessary to conduct further study to examine the effect of hVCO creams in burn wound healing with longer assessment period.

Conclusion

The 70% and 100% hVCO are effective to wound healing process by suppressing the neutrophil number in day-6 and day-12, as well as by increasing the angiogenesis in day-12.

The 70% hVCO is more effective in healing the mid dermal burn wound, since it decreases the wound macroscopic size, decreases neutrophil number, and increases angiogenesis better than the 100% hVCO. Hence, the use of 70% hVCO cream can be explored further as topical medication formid dermal burn wound.

Authors Contribution

SKP planned the study, collected the data, performed the analysis, and wrote the manuscript. YB, SB, NS, UB were involved in giving critical revision for important intellectual content. All authors read and approved the final manuscript.

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