Original Article

The effect of red fruit oil (*Pandanus conoideus* Lamk.) emulgel on angiogenesis and collagen density in incisive wound healing in mice (*Mus musculus*)

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Abstract

Pandanus conoideus Lamk. or commonly known as red fruit oil (RFO) can be used to accelerate wound healing because it contains tocopherols, carotenoids, oleic acid, linoleic acid, and linolenic acid. The RFO in this study was formulated in the form of an emulgel because it has the most convenient and effective drug delivery system. The aims of this study were to determine the activity of RFO emulgel on increasing the amount of angiogenesis and collagen density in incised wound healing and to determine the optimal dose of RFO emulgel to increase the amount of angiogenesis and collagen density in incised wound healing. This was a true experimental study with a posttest only control group design that included five treatment groups: a positive control group (10% povidone-iodine), a negative control (gel base), and three groups that varied the concentration of RFO emulgel used at 5%, 10%, and 15%. Parameters observed were the amount of angiogenesis using Image Raster software and the percentage of areas of collagen density using ImageJ software. The data were analyzed using a one-way ANOVA test and continued with the least significant difference test. The results of this study showed that RFO emulgel was able to increase the amount of angiogenesis and collagen density in the wound healing process with $P = 0.000$. An increase in the amount of angiogenesis and collagen density occurred in mice treated with RFO compared to the positive and negative control groups. It can be concluded that RFO emulgel has activity toward increasing the amount of angiogenesis and collagen density in the wound healing of mice incisions. The optimal dose concentration of RFO emulgel for increasing the amount of angiogenesis and collagen density in incision wound healing was shown in RFO emulgel with a concentration of 15%.

Key words: Angiogenesis, collagen density, emulgel, incision wound, red fruit oil

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INTRODUCTION

Wounds are the loss or damage to some body tissues caused by sharp or blunt trauma, changes in temperature, exposure to chemicals, explosions, electric shocks, or animal bites.[1] Based on the cause, there are incision wounds, torn wounds, stab wounds, burns, and abrasions.[2] An incisional wound

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is a wound that occurs as a result of being cut by a sharp instrument.[3] With a percentage of 20.1%, incision wounds are the third leading cause of national injury prevalence in Indonesia.[4] The data show that the incidence of incision wounds in Indonesia is still quite high.

In wound care, a topical 10% povidone-iodine treatment is commonly used. Povidone-iodine was one of the first anti-infection treatments. Furthermore, povidone-iodine is a powerful antimicrobial agent for wound healing.[5] The use of 10% povidone-iodine as a wound treatment can also have negative side effects such as wound irritation and inhibition of the formation of fibroblasts.[6,7]

One of the efforts to find effective wound healing agents is by utilizing plants that have the potential to be used as treatments. Plants that are thought to have potential as an alternative to wound healing are red fruit plants (*Pandanus conoideus* Lamk), where the oil is used.[8] Compounds in red fruit oil (RFO) that have the potential to treat wounds include tocopherols, carotenoids, oleic acid, linolenic acid, and linoleic acid.[9]

RFO content can stimulate increased angiogenesis and collagen density. Oleic acid functions to induce cell proliferation and cell migration in the wound healing process.[10] Carotenoids and tocopherol compounds, besides stimulating angiogenesis in the wound healing process, also have a function as an antioxidant that can control the concentration of reactive oxygen species (ROS). In the wound healing process, it is important to control ROS concentrations because too high ROS concentrations will interfere with the proliferative process of wound healing, resulting in a slow process of re-epithelialization and less than perfect wound closure and healing.[11]

The content of tocopherols and carotenoids can function as antioxidants.[12] Antioxidants counteract free radicals, allowing transforming growth factor-beta (TGF-β) activity to be uninhibited and increasing fibroblast proliferation.[11] The increase in fibroblasts will also increase the synthesis of collagen and other extracellular matrices so that wounds heal faster.[13] The content of oleic acid, linoleic acid, and linolenic acid functions to accelerate the wound healing process by providing a therapeutic effect when treatment is carried out starting from day 0 of the inflammatory phase.[14]

Utilization of RFO for the treatment of wounds can be developed in the form of an emulgel. Emulgel is a topical preparation made by mixing emulsion and gel together. Incorporation of the emulsion into the gel makes it a controlled drug release system and can also solve problems such as phase separation as well as improve the stability of the preparation. In addition, emulgel is a preparation that has the most comfortable and effective drug delivery system.[15] Topical use of alternative drugs from RFO has not been widely studied, especially in the process of wound healing. In this research, a RFO emulgel preparation will be formulated using a combination of hydroxypropyl methyl cellulose (HPMC) and carbopol gel base. The combination of HPMC and carbopol produces a gel preparation that has better and more stable physical properties than a single-base gel preparation.[16]

Based on the background that has been described, this study aims to determine the activity of RFO emulgel formulation on increasing the amount of angiogenesis and collagen density in incised wound healing and to determine the optimal dose of RFO emulgel for increasing the amount of angiogenesis and collagen density in incised wound healing.

MATERIALS AND METHODS

Materials

The animal model used in this study was the BALB/c strain of mice (*Mus musculus*) obtained from mice farms at the Polytechnic of Health, Malang, East Java, Indonesia. The ingredients used are RFO, carbopol, HPMC, triethanolamine (TEA), propylene glycol, BHT, distilled water, Tween 80, Span 80, methyl paraben, propyl paraben, 10% povidone-iodine, ketamine, and xylazine, while the tools used are glassware, a spatula, an analytical balance, label paper, a tripod, bunsen, plastic wrap, aluminum foil, a mouse cage, a dry patch, sterile gauze, scalpels, razor blades, surgical scissors, tweezers, microscopes, and digital cameras.

Methods

Red fruit oil emulgel manufacturing

The first stage is the preparation of the base gel, starting with a sprinkle of carbomer and HPMC over hot, distilled water at 70°C in each mortar. After that, the preparation was crushed and then allowed to stand for 15 min until it was completely dispersed. Then, TEA was added to the carbomer base. Next, HPMC was added to the carbomer and TEA mixture and then crushed until homogeneous.

The second stage is the preparation of the emulsion by heating the oil and water phases separately at 70°C. The water phase consists of aquadest, Tween 80, and propylene glycol. Meanwhile, the oil phase consists of RFO, Span 80, BHT, methyl paraben, and propyl paraben. The aqueous and oil phases were mixed and stirred at 9600 rpm for 30 min. The last step is the base gel and emulsion that have been formed, mixed little by little, and homogeneously crushed to form a stable emulgel preparation.

Treatment

The mice must be intramuscularly sedated with a 1:1 mixture of ketamine and xylazine before the wound can be created.[17] Some of the hair on the mice's back was shaved, and an incision was made 1 cm long and

0.2 cm deep using a razor in that area. The mice that had been incised underwent treatment that was divided into five groups: the negative control group (K−) was given a gel base, the positive control group (K+) was given 10% povidone-iodine, the treatment group was given an RFO emulgel for a concentration of 5% (P1), the treatment group was given an RFO emulgel for a concentration of 10% (P2), and the treatment group was given an RFO emulgel for a concentration of 15% (P3). Wound care was given twice a day for 14 days. On the 14th day, the mice were euthanized using the cervical dislocation technique. Next, the mice were placed on a fixation board for surgery, and the skin was taken. Then, histopathological skin preparations were made with hematoxylin and eosin (HE) staining. All procedures used in animal research have been declared ethically feasible in accordance with seven WHO standards, with a certificate number of passing ethical review: Reg. No.: 240/KEPK-POLKESMA/2021.

Microscopic observation

Observations were made using HE dye on an Olympus microscope equipped with a × 400 magnification camera or Optilab in five fields of view.[18] The observation of the amount of angiogenesis was carried out by manually counting using the Image Raster application. The observation of collagen density was processed using ImageJ 1.52V software. The data generated are in the form of a percentage of the area of collagen density formed.[19]

Data analysis

This research was conducted by means of statistical analysis using SPSS software version 26 for windows (Armonk, New York, USA) for Windows. The data obtained were tested using one-way ANOVA followed by the *post hoc* Fisher's least significant difference (LSD) test to determine if there was a significant difference ($P < 0.05$).

RESULTS AND DISCUSSION

Red fruit oil emulgel's effect on the number of angiogenesis

The results of the one-way ANOVA test analysis showed $P = 0.000$ ($P < 0.05$), so it was considered that there was a significant difference between the K(+) group and the K(−) group and the treatment groups (P1, P2, and P3). The analysis was continued with the Fisher's LSD *post hoc* test, which showed that there was a significant difference ($P < 0.05$) between the K(−) group and the treatment groups (P1, P2, and P3), but there was no significant difference ($P > 0.05$) with a K(+) group. These findings indicate that 5%, 10%, and 15% RFO emulgel doses have an effect on angiogenesis, as evidenced by an increase in the amount of angiogenesis in mice. A significant difference with $P < 0.05$ was also found between the P1 and P3 groups, but not between the P2 and P1 groups ($P > 0.05$). These results can be interpreted as indicating that RFO emulgel with a concentration of 15% has a greater effect on angiogenesis in incision wound healing than concentrations of 5% and 10%.

The dosage must be precise and take the desired therapeutic impact into consideration. If the therapeutic dose given is too small, the drug will not be effective, and if the therapeutic dose given is excessive, it will cause toxic effects.[20] The dosage form chosen can also affect the therapeutic process in wound healing; for example, emulgel preparations are chosen because they are more stable and can simultaneously reduce surface tension and interfacial tension so that the active substance carried will be more easily absorbed by the skin and provide a therapeutic effect.[21]

Figure 1 shows the lowest average number of angiogenesis seen in mice in the K(−) group. This is because the K(−) group was only given gel-based therapy, where the gel base itself did not contain any active substance or efficacious drug content to assist in the wound healing process. The K(+) group also showed lower results when compared to the treatment group. This is because the process of forming fibroblasts and growth factors in the form of fibroblast growth factor can be inhibited due to the administration of 10% povidone-iodine, so that ultimately angiogenesis is also inhibited.[22] In addition, the use of povidone-iodine results in longer healing by leaving scars because 10% povidone-iodine has an irritating effect.[23]

Figure 1 shows that in each treatment group (P1, P2, and P3), there was an increase in the amount of angiogenesis. The average number of angiogenesis in the treatment group showed a greater increase than in the K(+) and K(−) groups. This is probably due to the presence of active substances such as carotenoids, tocopherols, oleic acid, linolenic acid, and linoleic acid in RFO emulgels, which have a role in the emergence of angiogenesis, so that the presence of these ingredients can increase the amount of angiogenesis and cause incision wound healing to occur more quickly. There is a study that stated that the more angiogenesis found, the faster tissue repair would occur, so that the wound healing process would be faster.[24] The findings of this study were also demonstrated in the examination of mice tissue using HE staining to detect visible angiogenesis [Figure 2].

The healing process of incision wounds can also be affected by the presence of linoleic acid contained in RFO. Linoleic acid functions by inducing the migration of inflammatory cells and increasing the formation of new blood vessels (angiogenesis) in wound tissue. Linoleic acid increases the production of vascular endothelial growth factor (VEGF), which occurs after the activation of endothelial cells and fibroblasts. The increased production of proangiogenic factors by linoleic acid can lead to increased vascularization and angiogenesis.[25] Another fatty acid that has a role in the process of angiogenesis in wound healing

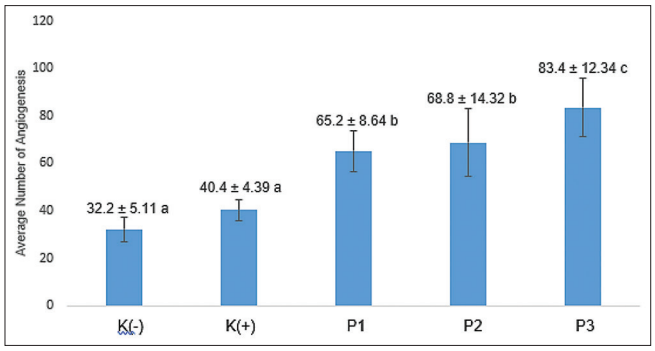

Figure 1: Histogram of average angiogenesis

is oleic acid. Oleic acid functions to increase angiogenesis by inducing the activation of AKT 1 and 2. Another function of oleic acid is that it can induce cell proliferation and cell migration in the process of wound healing.[10]

RFO contains β-carotene and α-tocopherol in addition to unsaturated fatty acids. Both of these compounds also play a role in the process of angiogenesis.[26] Hormozi *et al*. stated that β-carotene compounds can induce the expression of angiogenic genes and stimulate cell differentiation, in this case the angiogenic genes that affect the induction of angiogenesis, namely VEGF.[27] α-tocopherol compounds play a role in the process of angiogenesis by stimulating the emergence of VEGF which plays a role in promoting the process of angiogenesis by taking up free radicals. In addition, α-tocopherol also plays a role in stimulating VEGF production through the PI3K/AKT pathway resulting in increased angiogenesis, so it can be seen that the presence of α-tocopherol can accelerate the wound healing process.[28] According to Hu *et al*., α-tocopherol compounds induced increased proliferation, migration, and the ability of VEGF to appear in endothelial cells.[29] in this case, α-tocopherol became one of the active substances used in wound dressings and demonstrated increased angiogenesis in wounds.

Carotenoid and tocopherol compounds, apart from stimulating angiogenesis in the wound healing process, also have a function as an antioxidant that can control ROS concentrations. Carotenoids are strong ROS-stabilizing compounds that can control oxidative stress well in wounds. In the process of wound healing, controlling ROS concentrations to keep them low is important because if the concentration of ROS from oxidative stress reactions increases continuously, it will interfere with the proliferative process in wound healing, resulting in slow re-epithelialization of the wound and incomplete wound healing and closure.[11]

Red fruit oil emulgel's effect on increasing collagen density

The results of the statistical test using one-way ANOVA obtained $P = 0.000$ which indicated that there was a

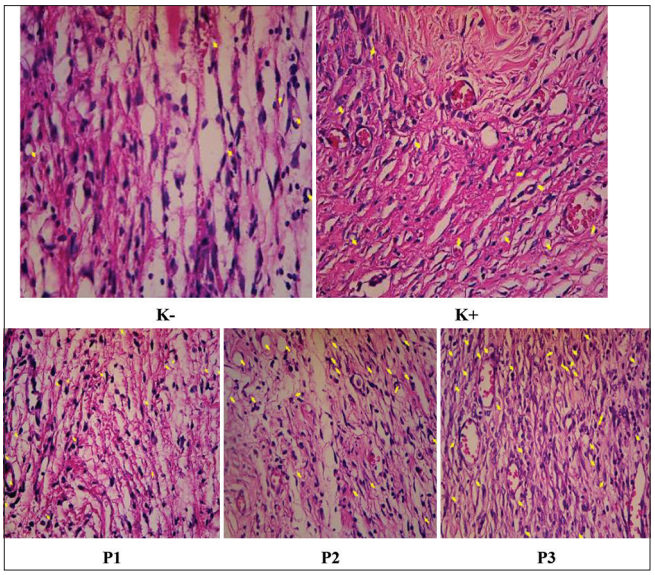

Figure 2: Results of observations of angiogenesis using HE staining at K(−), K(+), P1, P2, and P3, with × 400

significant difference ($P < 0.05$) in all the treatment groups. The differences between the groups were followed by the *post hoc* Fisher's LSD test which showed that there were significant differences ($P < 0.05$) between the K(−) and P1, P2, and P3 groups, but not significantly different ($P > 0.05$) with group K(+). Hence, it can be interpreted that giving RFO emulgel with concentrations of 5%, 10%, and 15% has activity toward increasing collagen density in incision wound healing, which is faster than giving 10% povidone-iodine. A significant difference ($P < 0.05$) was also shown between the P1 and P3 groups, but not significantly different ($P > 0.05$) from the P2 group. Hence, it can be interpreted that increasing the density of collagen by giving a RFO emulgel with a concentration of 15% has faster activity compared to giving a RFO emulgel with a concentration of 5% and 10%. This is in accordance with the theory, which states that using the right therapeutic dose when administering drugs will provide optimal therapeutic effects.[30]

Figure 3 shows that the K(−) group has the lowest percentage of collagen density. This was because the K(−) group was only given a gel base without any medicinal content that was efficacious to help the wound healing process. When compared to the K(−) group, the K(+) group, which received 10% povidone-iodine treatment, showed a greater increase in collagen density. Povidone-iodine is an antiseptic that can kill Gram-positive and Gram-negative bacteria (including those with antibiotic resistance), fungi or yeast, viruses, and protozoa.[31] Thus, the use of 10% povidone-iodine for wound treatment can increase collagen density because the inflammatory phase can pass earlier.[7]

In the treatment group that was given RFO emulgel at P1, P2, and P3, there was an increase in the percentage area

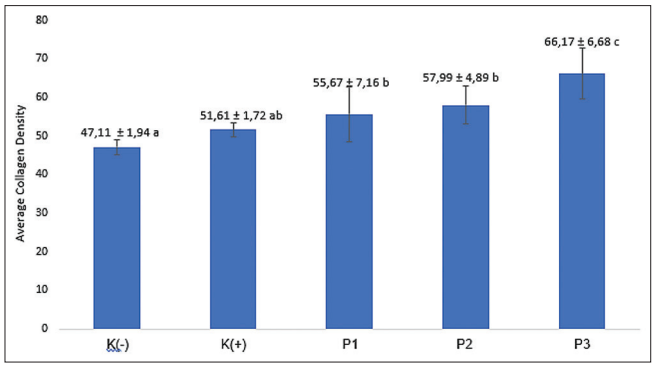

Figure 3: Histogram of the average percentage area of collagen density

of collagen density that was greater than the K(+) and K(−) groups. The greatest percentage of areas of increased collagen density was shown in mice in the P3 group, which were given a 15% concentration of RFO emulgel. This is consistent with previous research findings that RFO contains active ingredients such as tocopherols, carotenoids, oleic acid, linolenic acid, and linoleic acid.[9] The findings of this study were also demonstrated in the examination of mice tissue using HE staining to detect visible collagen density [Figure 4].

RFO contains active substances that are able to detoxify ROS, namely tocopherols and carotenoids, so they can act as antioxidants.[12] Tocopherol is easily oxidized, so free radicals will oxidize antioxidants and protect other compounds from damage due to the oxidation process.[32] Meanwhile, carotenoids work by reducing singlet oxygen ($^1O_2$) and deactivating other free radicals.[33] A study states that tocopherols and carotenoids can help the process of wound healing and collagen synthesis by preventing the damaging effects of free radicals and ensuring the stability and integrity of biological membranes.[34]

Oleic acid can accelerate the inflammatory phase by eliminating pathogens so as to prevent extensive tissue damage and can play a role in inducing TGF-β3 to produce collagen type 3.[35,36] Collagen plays an important role at wound healing process, because collagen is able to repair damaged or lost tissue.[24] Linoleic acid works as a precursor of arachidonic acid by having metabolic effects in producing prostaglandins, thromboxane, and leukotrienes. These substances act as inflammatory mediators that can accelerate the inflammatory process. Linolenic acid includes omega 3, which is an important precursor with the bioactive properties of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[14] EPA and DHA are anti-inflammatory agents that reduce inflammation. In addition, EPA has been shown to increase the number of fibroblasts and stimulate collagen formation. EPA plays a role in increasing the number of interleukin 6 cytokines so that it can increase collagen production by fibroblasts.[37]

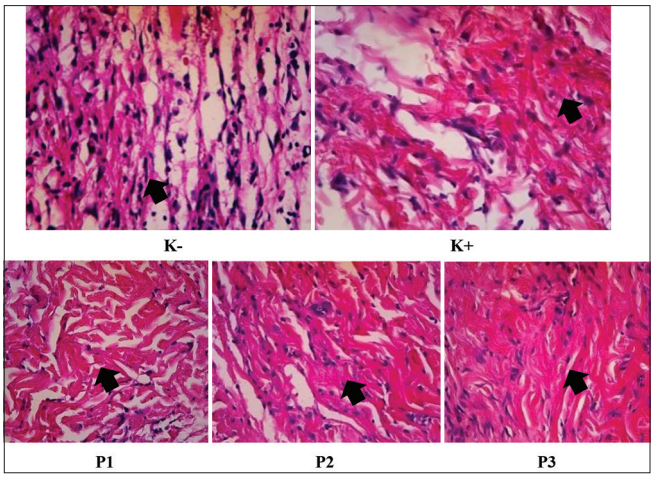

Figure 4: Results of observations of collagen density using HE staining at K−, K+, P1, P2, and P3 with × 400

CONCLUSION

This study shows that the use of RFO emulgel on mice incisions can improve conditions by increasing the amount of angiogenesis and collagen density. Administration of RFO emulgel with various concentrations showed an increase in the amount of angiogenesis and collagen density compared to the control group, which was only given a gel base or 10% povidone-iodine. The increase in the amount of angiogenesis and collagen density in the 5%, 10%, and 15% RFO concentration groups did not show a significant difference, but the highest increase was shown in the 15% RFO emulgel concentration, so it can be concluded that the optimal dose is the 15% concentration. The effect of RFO emulgel on wound healing is thought to be due to the presence of tocopherols, carotenoids, oleic acid, linolenic acid, and linoleic acid in red fruit.

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Conflicts of interest
There are no conflicts of interest.

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