Topical Modified-Olive Oil (Dalethyne) for Bacterial-Infected Wound: A Study in the Rats

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Abstract

Open skin wound often leads to the infiltration of pathogens. Inadequate wound healing process might lead to systemic infections. Therefore, proper wound care is needed to prevent further possible complications. This study was aimed to investigate the effect of dalethyne administration on the wound healing process in open skin wounds infected by bacteria.

We conducted an in vivo experiment in rats. The skin of the rats was cut with a 1-cm incision along the torso on the left, and the right side then was inoculated with Staphylococcus aureus, Pseudomonas aeruginosa, Streptococcus epidermidis and Escherichia coli for 24-hour. Afterward, vehicle (glycerine) or dalethyne were administered to the wounds. Macroscopic and microscopic observations were done on Day 1 and Day 6 after treatment administration.

Dalethyne application resulted in a significant wound closure rate compared with the vehicle only (glycerine) on skin wounds infected with Pseudomonas aeruginosa. No significant difference in wound closure rate for skin infected with Staphylococcus aureus, Staphylococcus epidermidis, and E.coli after topical administration with dalethyne versus vehicle (glycerine). However, microscopic observation showed a higher tendency of the healing process, as demonstrated by neutrophil and mononuclear infiltration in the wound areas.

Dalethyne showed a beneficial healing rate in a rat model of bacteria-infected wounds. However, due to the short term observations in the present study, the more prolonged wound healing effect of dalethyne, still need to be investigated.


Keywords: Modified olive oil, dalethyne, skin wound, antibacterial.

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Introduction

The skin wound is an injury on the skin caused by trauma, tear, cut, or incision or wound. When the skin is impaired, pathogens can infiltrate the skin so that it will be susceptible to infections.¹ The most common pathogens infecting the skin includes Staphylococcus aureus, Streptococcus sp., and Pseudomonas aeruginosa.²³

Many strategies have been applied to manage open wounds so that the skin repair will result in the restoration of tissue integrity and prevent further systemic infection.¹ Improper wound healing process might lead to severe damage, such as the loss of skin, inadequate blood flow to the epidermis, and aggravation of inflammatory state that will eventually lead to chronic wounds.¹ Studies have shown that wound dressings containing topical antiseptics or antibiotics are an excellent option to limit the growth of bacteria and accelerate the process of wound healing. With growing resistance in pathogens against antimicrobial and antiseptic agents, the development of new topical antiseptic is urgently needed.⁴⁵

Studies have shown that olive oils and ozonated olive oils were shown to have a good effect on skin wound healing as well as
antimicrobial activity.\textsuperscript{6-9} Dalethyne is an ozonated olive oil, first introduced in India in 2015.\textsuperscript{10,11} Dalethyne has 18 side chains, categorized into four major groups, which are peroxides, aldehydes, iodines, and anisidines. Peroxides, iodines, and anisidines are active compounds that helped in the process of wound cleaning in epidermis and dermis. This mechanism will help in creating a conducive environment for tissue repair. Peroxides also can induce macrophage and neutrophil activities and aides in the process of phagocytosis and debridement. Meanwhile, the aldehyde group of dalethyne worked in the hypodermic layer.\textsuperscript{10}

The result from the \textit{in silico} study demonstrated that dalethyne could inhibit the growth of various positive and negative gram bacteria. The findings from \textit{in silico} study shown the promising potential of dalethyne as a broad-spectrum antibacterial agent.\textsuperscript{12} Dalethyne also has other beneficial activities such as suppressing inflammation, oxidative stress, and modulating growth and inflammatory factors, which will help towards wound healing.\textsuperscript{10} Previous \textit{in vivo} studies had also reported the acceleration of wound healing in MRSA-infected rats.\textsuperscript{13} Therefore, this study was aimed to investigate the acute effect of dalethyne administration on the wound healing process in open skin wounds infected by bacteria in the rats.

Materials and methods

Design

This study was an \textit{in vivo} experiment conducted using Sprague-Dawley male rats, 3-months old weighing about 150 – 200 grams. The Ethics Committee has approved the study before the start of the research (No. 0041/UN2.F1/ETIK/2019). Incision wounds were inoculated with four types of bacteria, which were \textit{Staphylococcus aureus} (SA), \textit{Pseudomonas aeruginosa} (PA), \textit{Staphylococcus epidermidis} (SE) and \textit{Escherichia coli} (EC). The standard group consists of rats that were incised but not inoculated with bacteria. Three pairs of incisions were done in each rat, which was the vehicle (glycerine) in the left part of the incisions and dalethyne in the right part of the incisions. Every treatment consists of six samples.

The rats were kept at stable room temperature (± 25°C), with 12 hours of light/dark cycle. The rats received standard pellets for food and water ad libitum. During the treatment period, each rat was kept on an individual closed-system cage to prevent cross-infection.\textsuperscript{14}

Incision Procedure

Rats were put under anesthesia by intramuscular injection of ketamine (80 mg/kg BW) and xylazine (8 mg/kg BW). Afterward, the rat fur was shaved in the incision area. Three pairs of 1-cm incisions were made along the torso on both the left and right side with 1.5 cm distance for each incision using a biopsy punch. The gauze was then put to cover the incision.\textsuperscript{15}

Microbes Inoculation

Inoculation of the microbes was done after the incision. Before microbial application, the incision was irrigated using normal saline. Inoculation was done by topically applied 0.2 mL of a bacterial suspension of $4.12 \times 10^9$ CFU/mL on the site of the incision. Bacteria were obtained from the University of Pancasila. The normal group received phosphate buffer solution (PBS) instead of a microbe suspension. After inoculation, the incision was covered using sterile gauze.\textsuperscript{15}

Treatment Application

Twenty-four hours after inoculation, the incision was then irrigated using normal saline. Treatment was done by topically applying a placebo to the left side incisions and 30% dalethyne to the right-side incisions. Afterward, the incision was covered again using sterile gauze. The process was repeated every 24 hours for five days (Day 2 to 6).

Sample Collection

At the end of the study (Day 7), the rat was sacrificed to collect tissue samples. Rats were sacrificed under anesthesia injection of ketamine + xylazine.\textsuperscript{14} Necropsies were done after confirmation of death to collect tissues around the incision site. Tissue samples were then put into a 10% formaldehyde buffer to be processed for histopathological evaluation using Hematoxylin-Eosin staining.

Macroscopic Evaluation

A macroscopic evaluation was done daily by inspecting and documenting incision after irrigation but before the application of placebo or dalethyne. A blinded pathologist did the review. The assessment was conducted by measuring the diameter of the wound. The infected incision was defined by the wound surface that was covered by pus, spreading of necrotic tissue to
the surrounding area of the incision, lowered surface level of a wound, and lowered wound healing rate compared to the normal group.\textsuperscript{15}

**Microscopic Evaluation**

Microscopic evaluation was done by examining the histopathological sample. A blinded pathologist conducted the assessment. Evaluated characteristics include epithelialization, fibroplasia, neovascularization, and wound closure.\textsuperscript{15}

**Statistical Analysis**

The normality test was conducted using a One-sample Kolmogorov Smirnov test with 95% significance, and data was considered normal if $p \leq 0.05$. The homogeneity test was then conducted with 95% significance, and data were deemed to be homogenous if $p \leq 0.05$. Statistical analysis for parametric data was done using ANOVA with Tukey posthoc. Non-parametric data were analyzed using the Kruskal-Wallis test, followed by Mann-Whitney U.

**Results**

Observation and calculation conducted in this study have produced the result in the form of macroscopic and microscopic evaluation. The macroscopic evaluation was obtained by inspecting wound closure, as shown in Figure 1. Calculation of the wound closure rate percentages was done based on the difference of wound size from day one to the last day of the study (Figure 2).

Dalethyne tends to increase the wound closure rate, though not statistically significant, for uninfected incision and infected incision caused by *Staphylococcus aureus*, *Staphylococcus epidermidis* and *E.coli*. Dalethyne was found to increase the wound closure rate significantly for the incisions infected by *Pseudomonas aeruginosa*. We found a worsening in wound closure in incisions infected with *E.coli*, while in dalethyne groups, we found a small improvement in wound closure rate (Figure 2).

The macroscopic wound closure results were confirmed by microscopic evaluation done by examining Hematoxylin-eosin (HE) stained histopathological samples. A summary analysis of each group is given in Table 1, while the histopathological assessment is presented in Figure 3. We did not find differences in the degree of dermatitis in the normal group treated with vehicle versus dalethyne. No specific abnormality or minimal necrosis was observed in *Escherichia coli* and *Staphylococcus epidermidis*-infected skin treated with vehicle and dalethyne.

![Figure 1. Macroscopic evaluation of wound closure. Photos were taken on Day 1 and Day 6.](image1)

![Figure 2. Wound closure rate of incision infected with bacteria when treated with vehicle only (Glycerine) or Dalethyne. Results are given in mean ± SEM. *: p<0.05.](image2)
In wounds infected with *Staphylococcus aureus*, both groups, vehicles, and dalethyne showed dermal necrosis. In wounds infected with *Pseudomonas aeruginosa*, there were signs of cutaneous necrosis in the vehicle group, while in the dalethyne group, there were only signs of mild dermatitis.

**Discussion**

The present study was aimed to evaluate the efficacy of dalethyne in the healing process of skin wounds infected with bacteria. Dalethyne is an ozonated olive oil with promising results in *in silico* study, and *methicillin-resistant staphylococcus aureus* (MRSA) wound infections. The ozonization of the olive oil might partly contribute higher wound closure rate in the dalethyne group. Ozonization of the olive oil was aimed to stabilize the double bonds of the monosaturated fatty acids in the olive oil. However, the ozone itself has been widely recognized to have bactericidal and fungicidal activity. Ozonides is known to penetrate the skin efficiently, therefore aiding wound healing and repair of the skin. Ozone has also been widely recognized as bactericidal and antifungal. It also has been suggested that the bond between O₃ and polyunsaturated fatty acids leads to the activation of transcription factors such as NF-κB that induce the synthesis of growth factors, therefore, increase the rate of wound healing.

<table>
<thead>
<tr>
<th>Group</th>
<th>Results</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal glycerine</td>
<td>Minimal dermal necrosis</td>
<td>Epithelial necrosis on dermis, hemorrhage, congestion, and mononuclear infiltration (lymphocyte).</td>
</tr>
<tr>
<td>Normal dalethyne</td>
<td>Minimal dermal necrosis</td>
<td>Cutaneous epithelial necrosis, cellular debris, mineralization, neutrophil and mononuclear infiltration (lymphocyte and macrophage), hypercellular, fibrosis, hyalinization, congestion, hemorrhage, and hair follicle epithelialization.</td>
</tr>
<tr>
<td>S. aureus glycerine</td>
<td>Minimal dermal necrosis</td>
<td>Epidermal epithelial necrosis, hair follicle growth, skeletal muscle necrosis, mononuclear infiltration (lymphocyte and macrophage), the fat layer is thinner than epidermis and dermis, hyalinization, congestion, and fibrosis.</td>
</tr>
<tr>
<td>S. aureus dalethyne</td>
<td>Dermal necrosis</td>
<td>Epidermis and dermis epithelial necrosis, muscle fiber fragmentation, and congestion.</td>
</tr>
<tr>
<td>S. epidermis glycerine</td>
<td>Minimal subcutaneous</td>
<td>No abnormality in epidermis and dermis, neutrophil and mononuclear infiltration (lymphocyte and macrophage) in subcutaneous tissue.</td>
</tr>
<tr>
<td>S. epidermis dalethyne</td>
<td>Minimal subcutaneous</td>
<td>No abnormality in epidermis and dermis, neutrophil and mononuclear infiltration (lymphocyte and macrophage) in subcutaneous tissue and muscular fiber degeneration.</td>
</tr>
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**Table 1. Microscopic Evaluation for All Treatment Groups.**
Olive oils, known to be rich in unsaturated fatty acids, known to have potent antibacterial activities. Phenolic compounds in olive oils such as aliphatic aldehydes can delay the growth of a wide range of bacteria.7,12

Out of the four types, bacterial skin infections studied, dalethyne gave the best activity against Pseudomonas aeruginosa. Pseudomonas aeruginosa is a drug-resistant strain of bacteria with a limited choice of antibiotics. Therefore, new treatment options to treat Pseudomonas spp. infection will be beneficial. In a previous study of Pseudomonas aeruginosa skin-infected rats, dalethyne was shown to reduce the inflammatory mediator (IL-1β) in fibroblast cells on day four after treatments, which reduces the inflammatory phase of the wound healing process.11

In treatment groups inoculated with E. coli, we observed worsening in placebo groups while in dalethyne groups, a small improvement was found. E. coli is a gram-negative bacteria that can cause debilitating skin and soft tissue infections in humans. E-coli produces non-specific exotoxin that disrupts intracellular signaling leads to cell deaths.19 The bacteria might have spread to adjacent areas, and cause the wound to widen. Dalethyne was shown to prevent the worsening of wound infection caused by E. coli and the spreading of the bacteria.

Overall, our microscopic findings confirmed what we found on the visible observation. No apparent differences microscopically in groups given vehicle (glycerine) or dalethyne in all groups. In the vehicle group, we found dermal necrosis, while in the dalethyne group, we found dermatitis, which is marked with neutrophil and mononuclear infiltration. In the wound healing process, neutrophils will be involved in the formation of the extracellular matrix. This process can be accelerated by administering wound care products.20,22

It was known that immediately after injury, local immune cells are activated, then release to the sites of the wound, proinflammatory mediators are released, neutrophils as the dominant white cells infiltrate quickly to the location of the injury.23 Whereas mononuclear cells, along with cytokines, chemokines, and growth factors, actively participate in providing healing process.20,24 Circulating lymphocytes migrate to the wound area during the first 24 hours and will decline after a week. The release of neutrophil and mononuclear cells marks the acute phase of wound healing, while in the later stage, which includes tissue remodeling and formation of scar or fibrotic tissues, which can last weeks or months.14,20,23-24

In the dalethyne group, the majority of the microscopic findings include neutrophil and mononuclear infiltration, which showed that the healing process was still in its early phase. In the placebo group, we observed more dermal necrosis along with congestion and hemorrhage. However, we also observed epidermis and dermis epithelial necrosis in dalethyne groups. In our experiment, our observation only lasts for seven days, which are still early for complete wound healing. Therefore, for a comprehensive wound healing representation, it is best to have more extended observation to demonstrate the whole reepithelization process.

Conclusions

Taken together, we conclude the activity of dalethyne as an antibacterial in open wound skin infections. Dalethyne provides faster wound healing as compared to vehicle, in particular against Pseudomonas aeruginosa infected wounds. Dalethyne tends to show a higher wound healing rate compared to the vehicle against Staphylococcus aureus, Staphylococcus epidermidis, and E. coli. However, due to the short period of observations in the present study, more prolonged effects of dalethyne is still needed to be investigated.

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Declaration of Interest

The authors declare no conflict of interest.

References