Topical ozonated virgin coconut oil improves diabetic ulcer wound healing in diabetic mice model

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Abstract. Diabetic ulcers are open sores on the skin through into the dermis, which if not properly managed, can increase amputation cases. Over the past few decades, ozone generated using plasma medical technology has been investigated to have the ability as an agent that helps wound healing. This study aims to evaluate the effect of topical ozonated VCO on the diabetic wound healing in the diabetic mice model. This study was an experimental study with post-test control design. Ulcer wound model was made in 50 diabetic male Wistar mice. They are divided into 5 groups, the first group (control) was given conventional therapy and the other groups (treatment) were given conventional therapy and topical ozonated VCO with different flow durations (0 min, 90 min, 7 h, 14 h). Then, the characteristics of wound healing (macroscopic and wound lengths) were observed in day 1, 3, 5, 7, and 14. The results of this study showed that the reduction of wound length was proportionally related to the duration of ozone flow. Topical VCO with the longer duration of ozone flow would heal the wound more quickly and had the shortest wound length at the end of the observation. VCO with ozone flow for 14 hours (16837.10 µm) had the biggest reduction of wound length, following by VCO with ozone flow for 7 hours (14209.64 µm), 90 minutes (14071.96 µm), 0 minutes (8531.99 µm), and control group (6370.77 µm). Therefore, we concluded that topical ozonated VCO improved diabetic wound healing process in diabetic ulcer mice model and can be used as adjuvant therapy for diabetic ulcers.

1. Introduction
International Diabetes Federation estimated that by 2030, the total number of diabetic patients worldwide will reach 366 million people. From those diabetic patients, 15% of them will develop a diabetic ulcer. A diabetic ulcer is an open wound on the skin layer down into the dermis, which usually
occurs on the soles of the feet. If the diabetic ulcer is not managed properly, it can develop into gangrene, thus increasing the case of amputation. 12-24% of patients who have diabetic ulcers end up with amputation [1-4].

The management of diabetic ulcers needs a holistic approach, by considering systemic conditions, complications of neuropathy or atherosclerosis, and classification of injuries. Treatment of wounds in diabetic ulcers involves three stages, that consists of cleansing (washing), debridement, and dressing (using proper wound dressing). In addition, pressure control and infection control are also needed. Ulcers can become an area of bacterial growth that must be treated with antibiotics according to the results of bacterial culture [5,6,7].

Ozone is known to have a role in protecting the ecological balance of the earth and can interact at a basic level with industrial pollutants. Ozone also has a unique biological ability that has the potential to be used in the medical field, which became widely investigated in recent years. Christopher Frieddrich Schoonbein was the first people to unleash the potential of ozone to treat wounds and infections caused by anaerobic bacteria [8,9].

Previous studies found ozone acts as an agent that helps ulcer wound healing in patients with diabetes mellitus. Ozone interactions with skin tissue causes inactivation of bacteria, viruses and fungi, stimulates antioxidant production, reduce blood and plasma viscosity, increases erythrocyte membrane fluidity, loosens tissue, stimulates haemoglobin activity and increases oxygen absorption and release, improves blood circulation to tissues, induces tissue collagen formation, activates of granulation tissue, and accelerates epithelialization (growth of epidermal cells), as well as increasing phagocytic activity and activation of fibroblasts. Ozone also supports the therapy of diabetes mellitus through decreasing blood sugar levels and increasing oxygen supply into the tissues [9,10,11].

Currently, there are limited studies of ozone therapy in animal models of diabetic ulcers and lack of study in the form of clinical trials concerning the safety and efficacy of ozone treatment for ulcers in patients with diabetes mellitus, especially in Indonesia. We used virgin coconut oil (VCO) as the ozone carrier liquid in this study. This study aims to evaluate the effect of topical ozonated VCO on the wound in the diabetic mice model.

2. Materials and methods
The subjects of this study were Wistar male mice with a weight of 250 ± 50 grams, which fulfilled the inclusion criteria for healthy conditions (active moves). The mice included in this study exhibited no signs that met our exclusion criteria, behavioural changes (activities seemed weak and lazy). On the diabetic group, we induced type 1 diabetes in the mice by injecting 40mg/kg BW streptozotocin single dose, dissolved in the buffer solution of 50 mM citrate with pH of 4.5 to obtain a final concentration of 40 mg/ml. The diagnosis of type 1 diabetes is made if blood glucose levels reach more than 200 mg/dl (11.1 mmol/litre) after 10 days after streptozotocin injection, or if blood glucose levels reach twice the baseline blood sugar noted at the time of injection. Mice were kept at a constant room temperature of 28.0±2.0ºC with fluorescent lighting that is turned on for 12 hours per day between 9.00 AM to 9.00 PM, with adequate food supply.

The manufacture and testing of ozonated VCO were carried out at the Plasma Research Center (PRC), Diponegoro University, Indonesia. The tools used for making ozonated oil were ozone generators and magnetic stirrers. The ozone outlet is connected to an anti-oxidation hose with a diffuser which served to increase increases the effectiveness of ozone absorption in the oil. Magnetic stirrers were used to facilitate the ozone dissolving process into the oil. The oil used in this study is Virgin Coconut Oil (VCO). Ozone was dissolved into VCO with a volume of 100 cc in each cycle and oxygen flow rate of 0.1 litres/minute with an ozone concentration of 3360 ppm. We used variable duration of ozonation process to create several concentrations of ozonated oil used in this research. The duration of ozonation was carried out with a variation of 90 minutes, 7 hours, and 14 hours.

The research process begins by making the diabetic ulcer model on the Wistar mice in the diabetic group. We soaked cotton with 0.05ml of chloroform and put the cotton into a beaker glass containing a mouse. This allows us to do the aerosol administration of anaesthesia, which we applied for 2 minutes.
After hypoesthesia was achieved, the hair in the region of the spine at the top of the back (thoracolumbar area) was shaved, then we made four excisions, each with a diameter of 6 mm, separated by the median line. The full-thickness skin excision was done using punch biopsy with a depth of 1 mm.

The sample was then divided into 6 groups, where each group consists of 10 mice. These groups were negative control group (“Group C-“), a control group contains mice with negative diabetes mellitus status, which received conventional treatment such as washing the wound with normal saline and antibiotic ointment; positive control group (“Group C+”), a control group with positive diabetes mellitus status, which received the similar conventional treatment as the negative control group, such as washing the wound with normal saline and antibiotic ointment; the first treatment group (“Group P1”), which received normal saline wound wash and non-ozonated VCO treatment; the second treatment group (“Group P2”) which received normal saline wound wash and 90-minute ozonated VCO oil therapy; the third treatment group (“Group P3”) which received normal saline wound wash and 7-hours ozonated VCO oil therapy; and the fourth treatment group (“Group P4”) which received normal saline wound wash and 14-hours ozonated VCO oil therapy.

We applied the oils (ozonated VCO or regular VCO according to the group) to the wound once a day for fourteen consecutive days, thinly covering the entire wound surface of each treatment. The wound was left open and not covered with any kinds of covering. Before applying ozone oil, we measured the wound contraction length at the first day, third day, fifth day, seventh day and fourteenth day, and recorded it into a follow-up table for further analysis. Data analysis was done using the computerized system with SPSS version 16.0 software. We used one-way ANOVA test to compare means of wound contraction rate between the control group and the diabetic mice group. Data were considered as significant if p <0.05.

This study is an experimental study with post-test control design. This research was approved and declared ethically feasible by the Ethics Commission of the Faculty of Medicine, Diponegoro University/RSUP Dr. Kariadi Semarang.

3. Results
The measurements of wound length on day 1 to 14 (Table 1) as an indicator of wound healing shows a better wound healing process in the VCO with a longer duration of ozone flow. The reduction of wound length is directly proportional to the duration of ozone flow. The greatest to smallest wound length reduction is found in the VCO with ozone flow for 14 hours group, 7 hours, 90 minutes, non-ozonated VCO, and diabetic wound without VCO application respectively. The fourth therapeutic group, which received VCO with ozone flow for 14 hours shows the greatest reduction in wound length (1,683.710 µm). The smallest reduction in wound length was found in the positive diabetes control group without VCO application (6370.77 µm), as expected. The greatest difference of wound length between the first day and the fourteenth day are found in the fourth therapeutic group which received highest concentration (14-hours ozonation) virgin coconut oil treatment. The results can be seen in the table and figure below.
Table 1. Mean wound length comparison between study groups in day 1, 3, 5, 7 and 14.

| Day | Control- | Control+ | Non-Ozonated VCO (90 Minutes) | VCO 7 Hours | VCO 14 Hours |
|-----|----------|----------|-------------------------------|-------------|--------------|
| 1   | 22229.13 | 21496.31 | 20212.90                      | 22825.82    | 21486.2      |
|     |          |          |                               |             | 23079.7      |
| 3   | 21576.44 | 18243.73 | 16380.89                      | 16762.67    | 14925.4      |
|     |          |          |                               |             | 13126.5      |
| 5   | 21307.52 | 17760.51 | 14672.56                      | 15342.78    | 13666.5      |
|     |          |          |                               |             | 10939.5      |
| 7   | 15151.54 | 17590.09 | 11382.99                      | 13730.39    | 13466.6      |
|     |          |          |                               |             | 7940.71      |
| 14  | 14675.47 | 15125.54 | 11680.91                      | 8753.86     | 7276.65      |
|     |          |          |                               |             | 6242.61      |
| Delta day 1-14 | 7553.66 | 6370.77 | 8531.99                      | 14071.96    | 14209.6      |
|     |          |          |                               |             | 16837.1      |

Figure 1. Wound length comparison between between study groups in the 1\textsuperscript{st}, 3\textsuperscript{rd}, 5\textsuperscript{th}, 7\textsuperscript{th} and 14\textsuperscript{th} day.

Figure 2 shows the macroscopic wound morphology comparison of wound healing between the study groups. As shown on the picture, the wounds in the treatment groups healed much faster compared to the positive control group without any treatment. The greatest wound healing process is found on the fourth treatment group which almost closed completely at the 14\textsuperscript{th} day, where at the same time the wound on the positive control group still barely healed. The third therapeutic group also has almost similar wound healing result, although the wound length is still greater than the fourth therapeutic group, signifying that the fourth therapeutic group achieved the best wound healing result compared to all other therapeutic group. Therefore, we can conclude that topical application of ozonated virgin coconut oil can improve the wound healing in diabetic mice as shown by the improved wound contraction length compared to diabetic positive control group without any treatment.
4. Discussion

Virgin coconut oil (VCO) can be produced from fresh coconut or coconut milk which is rich in medium-chain triglycerides (TG) and lauric acid. Lauric acid is a precursor of monolaurin which can modulate immune cell proliferation. If a wound occurs, the inflammation process occurs and immune cell activity increases \([12,13]\). A previous research found that VCO caused a significant reduction in ear edema, claw edema and granuloma formation in Sprague Dawley mice \([14]\). VCO has been shown to have significant antioxidant effects including increased levels of the superoxide dismutase enzyme in the wound tissue of normal mice \([15,16]\). In the case of chronic human skin conditions such as xerosis and atopic dermatitis, VCO shows a significant healing effect \([17]\). VCO is also efficacious to be a therapy for wound healing and angiogenesis. VCO showed a significant effect on the wound healing of diabetic mice through increased WCR and total protein content and increased collagen synthesis and re-epithelialization. VCO proved to be significantly better than silver sulphadiazine cream in healing diabetic wounds \([18]\).

Our study shows that the application of ozone helps speed up the healing process of diabetic ulcer. These results were similar to several previous studies. Izadi et al. found that ozone therapy shortened time needed for diabetic wounds to heal \([19]\). A systematic review of several studies reported that ozone therapy reduces the ulcer area and shortens the length of hospitalization compared to control therapies. Ozone therapy was associated with a greater reduction of ulcer area compared to the antibiotics therapy \([20]\).

Reis et al. reported that ozone provided by a high-frequency device might potentially be useful in the treatment of ulcers, thus, contributing to the healing process \([21]\). This might be promoted by the
decrease in bacterial infection, fibroplasia activation and keratinocyte proliferation. Ozone is a powerful oxidant; if ozone contacts with body fluids, it will result in the formation of reactive oxygen molecules and several biochemical events, which influences cellular metabolism and provides tissue repair and antimicrobial effects. Ozone has the potential to promote the activation of transcription factor NF-kB, regulating inflammatory responses, and the release from platelets of platelet-derived growth factor (PDGF) and transforming growth factor β1 [22-24].

The previous study conducted by Kim et al. found an increased intensity of collagen fibres and a greater number of fibroblast in the ozone group of an acute cutaneous wound [25]. Furthermore, during a systematic review of the therapeutic use of ozone in wounds, the authors discovered that most of the studies analyzed in their research found stimulation of the healing process (62.2%), followed by improvement in wound appearance (43.5%) and a decrease in pain (17.4%). The mechanism on how ozone improved wound healing might be related to its effect to the growth factors, activation of antioxidant system and activation of superoxide dismutase [26,27]. Besides its effect to the wound healing process, ozone also improves the diabetic condition itself by improving glycemic control, prevented oxidative stress, normalizes organic peroxides, aside from activation of superoxide dismutase as explained above [26]. This condition might be beneficial to the wound healing process as the condition is more favourable to the wound healing due to the improved condition of the diabetes itself. The authors concluded that ozone could be an important treatment option for wounds and may bring numerous benefits to patients.

We identified that there were some limitations in the current study. First, we only measured macroscopic wound contraction length without analyzing the histological and biochemistry aspect of the wounds itself. The comparison of wound healing histological events and biochemistry profiles between diabetic control mice and therapeutic group mice might enable further explanation on the mechanistic basis on how ozone improved wound healing in diabetic wounds. This could be considered to seek better understanding on how ozone improved the wound healing process.

5. Conclusion
Ozonated virgin coconut oil increases wound healing in diabetic mice with improvement of wound healing process proportional to the duration of ozone flow in virgin coconut oil. This study shows that ozone application can be used as adjuvant therapy to improve the wound healing of diabetic ulcers. The authors expect further research to determine the effects of ozone application on diabetic ulcer wounds in humans.

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