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The Effects of Olea Europaea Leaf Extract on Inflammatory Gene Expressions in Infected Wound Healing Process in Mice Model

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Abstract

Aims: The use of natural antimicrobial agents can decrease the risk of infection during wound healing. Olive (Olea europaea L.) is anti-inflammatory agent that can be used in this way. This study was conducted for the first time to evaluate the effects of ointments prepared from olive leaf extract (OLE) on inflammatory gene expressions in infected wound healing process in mice model.

Materials & Methods: We created two circular full-thickness wounds on the dorsal inter-scapular part of per mouse by a 5 mm biopsy punch and infected with Staphylococcus aureus and Pseudomonas aeruginosa. Following induction of infection, animals were treated with mupirocin (mupirocin group) and basal ointments containing 2.5% and 5% of the extract (2.5% & 5% OLE). A control group was also considered. We selected five mice per group and samples were collected at days 3, 7, and 14 for investigation of total bacterial count and gene expressions.

Findings: The results showed that control group significantly showed higher wound area, total bacterial count, and higher expressions of IL-1β and TNF-α ($P<0.05$) and lower expressions for IL-10 and TGF-β ($P<0.05$). The treatment with OLE could significantly decrease wound area, total bacterial count, expressions of IL-1β and TNF-α, and increase the expression of IL-10 and TGF-β ($P<0.05$).

Conclusion: In sum, ointments prepared from OLE could decrease total bacterial count, decrease inflammatory phase, and improve wound healing. We suggested to use the OLE for the treatment of wound healing in combination with synthetic agents.

Keywords : IL-1β; Inflammatory phase; Olive; TNF-α; Wound healing

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Introduction

One of challenges that people commonly face is wounds [1]. It is estimated that 6 million people suffer from chronic wounds all over the world [2]. A wound is defined as a damaged condition of tissue that is created through chemical, physical, microbial, or immunological disorders or typically related to loss of function [3]. Wounds are also defined as physical damages that cause opening or break of the skin that result in disturbance in the normal skin performance and anatomy [4]. Bacterial infection delays wound healing process [5]. The most usual and unavoidable obstruction for wound healing is induction of infection, especially in the chronic wounds [6]. Staphylococcus aureus, methicillin-resistant S. aureus (MRSA) and Pseudomonas aeruginosa are known as microbial strains that are commonly found in individuals with infected wounds. The wound healing process comprises several continuous and sequential steps including: hemostasis, inflammation, proliferation, and remodeling. Fault in these steps causes to impair the wound healing and increases healthiness and economic problems [7,8]. Inflammation occurs following hemostasis step. Hemostasis lasts for several hours following injury and inflammation lasts from 1 up to 3 days after injury [9]. In the inflammation step, following activation of the immune system, neutrophils and monocytes immediately move into the damaged skin. The inflammation occurs concurrent with hemostasis phase, and both are early steps of wound healing [10]. Excessive and prolonged inflammation delays wound healing and increases production of wound [9]. Shortening inflammatory phase can accelerate wound healing process [12].

The use of topical antimicrobial agents such as silver sulfadiazine can decrease the risk of infection during wound healing [11,12]. Olive (Olea europaea L.) has active compounds including oleuropein, rutin, luteolin, and apigenin, triterpenes, and chalcones [13].

It is known to have antioxidant and anti-inflammatory properties [14]. Seemingly, olive can improve wound healing due to its anti-inflammatory properties. This study was conducted for the first time to evaluate the effects of ointments prepared from olive leaf extract on inflammatory gene expressions in infected wound healing process in mice model.

Materials and Methods

Preparation of olive extract

Olea europaea L. leaves were prepared from south regions of Iran, washed and air dried in shade. We grounded dry leaves were ground into a fine powder and soaked in 80% ethanol for 24 h. After filtration, the solvent was evaporated at temperature not exceeding 45 °C by rotary evaporator as suggested by previous studies [15].

Induction of wound and the treatment

Infected wound was induced as reported by previous studies [12]. In the current study, a total number 60 BALB/c mice with initial weight of 30 ± 5 g were purchased from Pasture Institute and grouped into four groups. The mice were divided into four groups. A group was selected as negative control that considered as infected group and without any the treatment. Three other groups were considered as infected and treated with mupirocin ointment as control standard and ointments prepared from olive extract. To induce wound, animals were anesthetized, the dorsum back hairs were shaved and skin scrubbed. We created two circular full-thickness wounds on the dorsal interscapular part of the mouse by a 5 mm biopsy punch. Following creation of wound, 50 μl of suspension containing 25 × 107 cells Gram-positive of species S. aureus (strain ATCC 25923) and Gram-negative species P. aeruginosa (strain ATCC 27853) were inoculated on per wound [12]. Animals were treated with mupirocin (mupirocin group) and basal ointments containing 2.5% and 5% of the extract (2.5% & 5% OLE). The animals were treated with ointments 24 h after creating wound. In the current study, five mice per group were selected and samples were collected at days 3, 7, and 14 for investigation of total bacterial count and gene expressions. We measured wound area by a graph sheet as described by Farahpour et al [12].

Investigation of total bacterial count

We collected the samples and 0.1 g of sample was crushed and homogenized in the sterile mortar containing 10 mL of sterile saline, serially diluted in the tube containing 9 mL of sterile saline. The samples were cultured on plate count agar (Merck KGaA, Darmstadt, Germany) and superficially duplicated. Following incubation, all colonies were counted and reported as CFU/g of granulation tissue [12].

RNA extraction and quantitative real-time PCR

Following creation of wound, samples were collected for evaluating the genes expression profile on days 3, 7, and 14. In the current study, 3–5 g of wound tissues were transferred into tubes containing RNasesolution (Qiagen, Germany) and rapidly transferred to the lab. Following homogenization, RNA was extracted by Trizol method (Roche, Germany). We synthesized cDNA using the Exiqon cDNA Synthesis Kit on the basis of manufacturer's instructions. Samples were rapidly incubated at 25 °C for 5 min, then by 42 °C for 60 min; the reaction was finally terminated by heating at 70 °C for 5 min. To assess the mRNA expression, light Cycler 96 Roche was used and primers were used for genes of IL-10, IL-1β, TNF-α, and TGF-β1. The primers sequences were as follows: IL-10, forward (5′-CCA TCA TGC CTG GCT CAG CAC-3′) and

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reverse (5′-TGT ACT GGC CCC TGC TGA TCC-3′); IL-1β, forward (5′-AAC AAA CCC TGC AGT GGT TCG-3′) and reverse (5′- AGC TGC TTC AGA CAC TTG CAC-3′); TNF-α, forward (5′-GAA GCT CCC TCA GCG AGG ACA-3′) and reverse (5′-TTG GGC CAG TGA GTG AAA GGG-3′); TGF-β1, forward (5′-CTG AAC CAA GGA GAC GGA AT-3′) and reverse (5′-GGT TCA TGT CAT GGA TGG TG-3′).

Data analysis

The data were analyzed by SPSS software and the data were reported as the mean ± standard deviation (SD). ANOVA procedure was used for analysis of the data and Tukey test was used for comparison of the data. Changes in the fold number were evaluated using the 2-ΔΔCt method.

Findings

Wound area

The results for wound area are shown in Figure 1. The results showed that wound area was not influenced by experimental treatments in day 3 ($P>0.05$). The results also showed that wound area was significantly higher in control group compared with other groups in days 7 and 14 ($P<0.05$). The treatment with mupirocin and OLE in the both levels significantly decreased wound area and the best responses were observed in 5% OLE treatment ($P<0.05$).

Total bacterial count

The results for total bacterial count are illustrated in Figure 2. The results showed that total bacterial count was not affected by the treatments at day 3 ($P>0.05$). The results showed that total bacterial count was significantly higher in control mice compared to other mice at days 7 and 14 ($P<0.05$). The results show that the treatment with OLE and mupirocin significantly decreased total bacterial count and the best responses were observed in the treatment of 5% OLE.

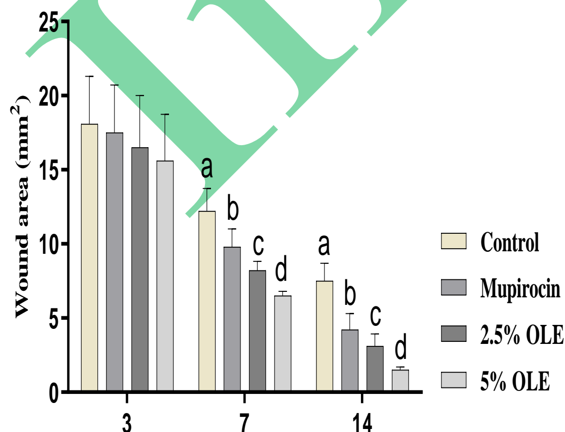


Figure 1) The effects of experimental treatments on wound area. The results showed that OLE and mupirocin significantly decreased wound area. Superscripts (a–d) show significant differences among groups at $P<0.05$.

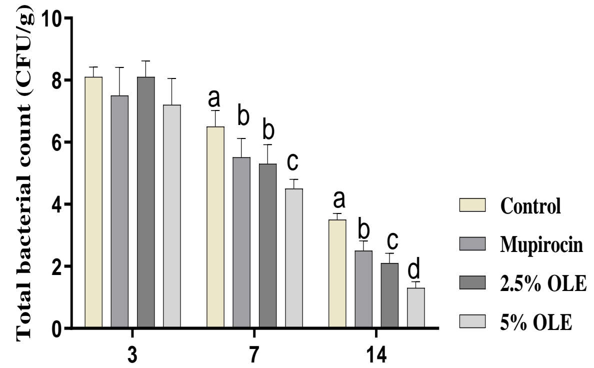
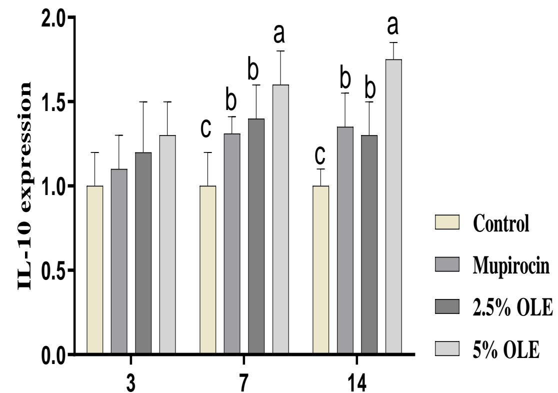
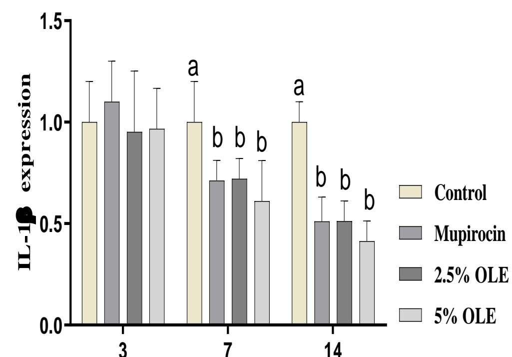


Figure 2) The effects of experimental treatments on total bacterial count. The results showed that OLE and mupirocin significantly decreased total bacterial count. Superscripts (a–d) show significant differences among groups at $P<0.05$.

Inflammatory gene expressions

The results for inflammatory genes are shown in Figure 3. The results showed that inflammatory genes are shown in Figure 3. The results showed that gene expressions were not influenced by experimental treatments in day 3 ($P>0.05$). The results showed that OLE and mupirocin significantly decreased the expression of IL-1β and TNF-α and increased the expression of TGF-β and IL-10 compared to control group at days 7 and 14 ($P<0.05$). The results showed that higher expression was observed for TGF-β and IL-10 in days 7 and 14 ($P<0.05$).





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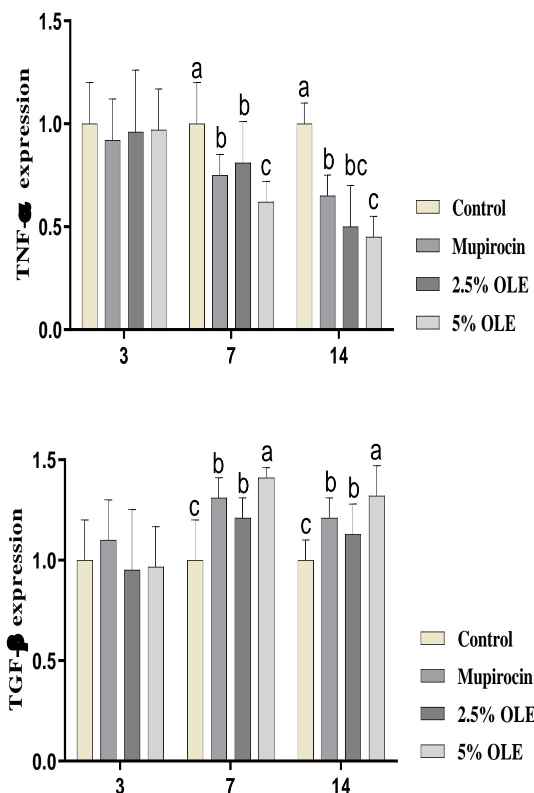

Figure 3) The effects of experimental treatments on total inflammatory genes expression. The results showed that OLE and mupirocin significantly decreased the expression of IL-1β and TNF-α and increased the expression of TGF-β and IL-10. Superscripts (a–c) show significant differences among groups at P<0.05.

Discussion

This study was conducted to evaluate the effects of ointments prepared from OLE. The results showed that ointments prepared from OLE decreased inflammatory phase, total bacterial count, and wound healing. It is well accepted that inflammatory reaction cascades play important roles in wound healing process. Inflammation is commonly initiated by harm to living tissues due to injury caused by infections induced by live organisms such as bacteria and/or physical agents; and faulted immune response. The basal purpose of inflammatory response is to remove the foreign agents and injured tissue parts for improving healing of the influenced tissues, organs, or system [16,17]. Activated host immune system increases fight infection, whereas overproduced inflammation causes to produce the tissue damage or even multiple organ failure. Overproduction of pro-inflammatory cytokines delays wound healing process. It is known that innate immune system is involved in the defense system through activating the infiltration of the immune cells, including neutrophils, macrophages, and dendritic cells for phagocytosis of infectious pathogens to the site of injury when the elementary stages of the process of wound healing [18]. The results showed that OLE could decrease total bacterial count. Decreasing microbial load of the wound and lowering the tissue inflammation induce the second stage of wound healing [19]. Decreased total bacterial count could be attributed to antibacterial properties of OLE that significantly decrease total bacterial count. The results show that OLE significantly decreases total bacterial count and promotes wound healing process and proliferative phase. Bacteria are found in all the open dermal wounds and a contact between bacteria and some hosts from contamination by colonization on local infection increases infection by cellulitis and/or septicemia [20]. Bacterial infections usually complicate wound healing process that keeps successive influx of neutrophils and macrophages and delays wound healing process [20]. Decreased inflammatory phase cause the migration of fibroblasts to the wound site, collagen production, and increase the epithelial cells from the edge to the wound site [12]. The results show that OLE improves wound healing by modulation in inflammatory phase genes. In following, we will discuss the genes.

TNF-*α* is a pleiotropic cytokine that is produced by a group of cells such as keratinocytes, macrophages, and mast cells. It is involved in some mechanisms such as leukocyte recruitment mainly neutrophils, inducing control of molecular adhesion, production of chemokines, and metalloproteinases matrix and also tissue preventors of metalloproteinases. The TNF-α is known inflammatory cytokine and have important roles in the first stage the inflammation [12]. The IL-1β is known as an inflammatory molecule that induces the aggregate of neutrophils to the site of infection [21]. The TNF-⍰ and IL-1β prolongs inflammatory stage inflammatory phase during wound healing and delay wound healing [22]. TGF-β1 activates the fibroblasts and begins the proliferative phase [23]. It was reported the role of TGF-β1 for participating in angiogenesis, regulating the granulation tissue formation [24] and re-epithelialization [25]. In addition, increase in the expression of IL-10 increases activity of macrophages in the wound site and promotes wound healing [26, 27]. In addition, increased IL-10 enhances activity of macrophages in the wound site and improves wound healing [26, 27].

Conclusion

In sum, ointments prepared from OLE decreased inflammatory phase and bacterial count and hastens wound healing. We suggest to use it for the treatment of infected wounds in combination with synthetic agents.

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