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Laser Doppler Evaluation to the Effect of Local Application of Eucalyptus Oil on Wound Healing: Histological Study on Male Rats

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ABSTRACT

Objective: *To examine the effect of local application of eucalyptus oil on wound healing in the skin of the rats, both histologically and by using laser Doppler flowmetry.* ***Methods:*** *Total 30 male rats used in this study were divided into 2 groups, the experimental group: 15 rats were treated locally with eucalyptus oil, the control group: 15 rats were treated locally with distilled water. All animals were subjected to dorsal skin incision (2 cm) the treatment was conducted every day until the animals were sacrificed to determine histologically the epithelial thickness and inflammatory cell counts. The wound healing was assessed clinically, and blood perfusion scan was performed with laser Doppler.* ***Results:*** *The results revealed that the wound size was significantly reduced in the eucalyptus treated rats, as compared with the control. Blood perfusion was significantly increased with eucalyptus group as compared with the controls. Hematoxylin and eosin (HE) staining revealed a significant reduction in the inflammatory reaction and accelerated epithelialization in the eucalyptus group as compared with the control. The result shows a high significant difference ($p \leq 0.01$) between experimental and control group regarding all result.* ***Conclusion:*** *The present study demonstrated that the local application of eucalyptus oil promoted remarkable neovascularization and skin regeneration and may lead to novel treatments for skin wounds.*

Keywords: Wound healing, Skin blood perfusion, Laser Doppler imager, Angiogenesis

INTRODUCTION

Skin is the largest organ in the body; it plays a vital role through the regulation of water and electrolyte balance, thermoregulation, and by acting as a barrier to external noxious agents and micro-organisms [1]. Skin health is influenced by various internal and external factors, these factors affect wound healing, and therefore to support the health of skin and the healing of wounds, clinicians need to appreciate, recognize and comprehends both the complex nature of skin and what lies beneath it [2].

A wound is defined as a break in the epithelial integrity of the skin; the disruption could extend to the dermis, subcutaneous fat, fascia, muscle or even the bone [1]. Wound healing in adult skin is a dynamic process that involves many cell types and processes, like epidermal, fibroblastic, and endothelial cell proliferation, cell migration, matrix synthesis, and wound contraction [3].

The major goals of the treatment of wounds are rapid wound closure and a functional and aesthetically satisfactory scar; recent advances in cellular and molecular biology have highly expanded our understanding of the biologic processes involved in wound repair and tissue regeneration [4].

As our understanding of wound healing progress, our therapeutic options expanded, and it becomes apparent that no single agent or modality will heal every wound [5]. Herbal therapy is becoming more and more accepted among patients and physicians, many herbal products are marketed to the public for skin and dermatologic uses, most patients are looking for herbal therapy because of their little toxicity, their wide acceptance by consumers, and their utilization for multiple purposes [6].

Eucalyptus essential oil is obtained from the leaves of eucalyptus tree, which firstly come from Australia and spread throughout the world because of its favorable prosperities like adaptability, easiness of cultivation and rapid growth, the essential oil is widely used in the medicine because of its antibacterial, anti-inflammatory, anti-fungal, and analgesic effect [7,8].

Laser Doppler is a non-invasive method that can be used in determining the relative amount of blood flow through superficial skin; these instruments are helpful in assessing blood flow in healing wounds, flaps, and grafts [9]. Laser Doppler perfusion imager is an instrument that maps the perfusion from an area by scanning the laser beam over the area of interest, its non-contact nature allows its uses in a clinical condition where contact with the tissue may be unwanted [10].

PATIENTS AND METHODS

All experimental procedures were carried out in accordance with the ethical principles of animal experimentation. A total of 30 male Albino rats were used (7 weeks old, weighing about 250-300 g). The animals were subjected to dorsal skin incision (2 cm) and divided randomly into 2 groups:

- Group A: Control group which include 15 rats treated with 1 drop of distilled water daily
- Group B: Experimental groups which include 15 rats treated with 1 drop of eucalyptus oil daily

The treatment was conducted every day until the animals were sacrificed to be studied histologically (5 rats for each period) at 3rd, 7th, and 10th day of treatment, the wound healing was assessed clinically, and the blood perfusion scan was performed with laser Doppler [11]. The rats were kept in an environment that had a constant temperature, and under a light/dark cycle (12/12 h), with free access to food and water.

The materials used in the present study were eucalyptus essential oil, anesthetic solution (ketamine+xylazine/kg B.W.), formalin 10%, ethanol, alcohol 96%, xylene, paraffin wax, and hematoxylin and eosin (H and E) stain. The surgical producer was performed under a sterilized condition; each rat has been weighted carefully to measure the dose of general anesthesia, the rats were anesthetized with xylazine (2%) and ketamine (HCL 50 mg) via intraperitoneal injection.

The dorsum of the rats was shaved and cleaned with topical povidone-iodine, and a (2 cm) line was drawn on the dorsal skin, the palpable hip joints were used as anatomical landmarks for defining the base of the wound defect, incision was made with a disposable scalpel blade along the marked dorsal line [12]. All incisions were made under sterile conditions, and included a full skin thickness.

The blood perfusion in the wound area on 3rd, 7th, and 10th day was measured using a scanning laser Doppler imager (Model LDI2-IR, Moor Instruments) with a near-infrared laser diode at 785 nm. The imaging machine uses a low power infrared laser beam to scan in the sequence of the wound area. The resulting signal linearly equal with tissue perfusion, it is defined as the product of the speed and concentration of blood cells, and called laser Doppler perfusion index (LDPI), the signal is represented as a 2-dimensional color image on the monitor, the colors produced show the spectrum of perfusion in the wound: dark blue illustrate the lowest level of perfusion and red the highest. Then, the mean LDPI value was calculated within the wound area, the scanner was placed 15 cm above each animal. About 3 consecutive scans were performed until blood flow measurements were stable.

The wound contraction rate was measured by using the (ruler technique); the palpable hip joints were used as anatomical landmarks for defining the base of the wound defect in all animal groups. The new full thickness skin layer that was regenerated at the wound area after wounding was removed by a surgical scalpel for histological analyses; samples were fixed in 10% formalin, embedded, and sectioned at 5 μm. The sections were stained with hematoxylin and eosin (H and E). Section images were captured to evaluate reepithelialization, granulation tissue, and inflammatory response.

RESULTS

Laser Doppler Finding

To better determine the functionality of the developing vasculature, we analyzed wounds on 3rd, 7th, and 10th day using laser Doppler to assess blood flow to the wound area; we found that on day 7, eucalyptus oil induced more blood flow to the wound area than did the control. For example, the blood flow with eucalyptus was 186.8 perfusion units, whereas the blood flow was only 125.8 perfusion units for the control group. On the 10th day, both groups returned to the normal levels.

The descriptive statistic for the number, mean, standard deviation, standard error, lower and upper bound, minimum and maximum values of blood perfusion was measured at different healing periods for all groups (Table 1).

Table 1 Descriptive statistics of blood perfusion (PU) with a time of healing for all groups

Period	Groups	N	Mean	SD	SE	95% Confidence interval for Mean		Min	Max
						Lower bound	Upper bound		
3 day	Control	5	96.80	1.300	0.580	9565.00	97.94	95.00	98
	Eucalyptus	5	136.40	1.810	0.810	134.80	137.99	134.91	138
7 days	Control	5	125.80	0.830	0.370	125.07	126.52	125.21	127
	Eucalyptus	5	186.80	1.300	0.580	185.65	187.94	185.95	188
10 days	Control	5	71.80	0.836	0.370	71.06	72.53	71.00	73
	Eucalyptus	5	96.60	1.510	0.677	95.27	97.92	95.00	99

Wound Contraction Finding

Every wound was monitored by the same observer from day 1 to 10 postoperatively, on the 7th day, eucalyptus seems to accelerate the wound contraction than did the control. For example, the wound contraction with eucalyptus was 1.02 cm, whereas wound contraction was 1.5 cm for the control group.

The descriptive statistic for the number, mean, standard deviation, standard error, lower and upper bound, minimum and maximum values of data was measured at different healing periods for all groups for contraction of the wound (Table 2).

Table 2 Descriptive statistics of wound contraction in cm with a time of healing for all groups

Period	Groups	N	Mean	SD	SE	95% Confidence interval for Mean		Min	Max
						Lower bound	Upper bound		
3 day	Control	5	1.88	0.044	0.019	1.84	1.91	1.8	1.9
	Eucalyptus	5	1.58	0.083	0.037	1.50	1.65	1.5	1.7
7 days	Control	5	1.56	0.054	0.024	1.51	1.602	1.5	1.6
	Eucalyptus	5	1.02	0.083	0.037	0.94	1.09	0.9	1.1
10 days	Control	5	1.36	0.054	0.024	1.31	1.40	1.3	1.4
	Eucalyptus	5	0.52	0.083	0.037	0.44	0.59	0.4	0.6

Inflammatory Cells Count Finding

To determine the anti-inflammatory effect of eucalyptus oil, we analyzed the wounds on 3, 7, and 10 days, on day 7, eucalyptus seem to have a great anti-inflammatory effect than did the control. For example, the inflammatory cells count with eucalyptus was 31.2, whereas inflammatory cells count was 51.6 for the control group.

The descriptive statistic was the number, mean, standard deviation, standard error, lower and upper bound, minimum and maximum values of inflammatory cells count measured at different healing periods for all the groups (Table 3).

Table 3 Descriptive statistics of inflammatory cells count with a time of healing for all groups

Period	Groups	N	Mean	SD	SE	95% Confidence interval for Mean		Min	Max
						Lower bound	Upper bound		
3 day	Control	5	62.8	3.5	1.62	58.2	61.02	58.2	62.5
	Eucalyptus	5	55.2	6.2	3.10	43.5	55.30	42.6	55.6
7 days	Control	5	51.6	2.6	1.32	52.6	55.20	52.3	56.2
	Eucalyptus	5	31.2	2.4	1.05	30.5	35.20	31.2	34.8
10 days	Control	5	46.6	1.4	0.69	43.5	47.30	41.3	46.2
	Eucalyptus	5	21.1	1.4	0.66	20.2	22.90	20.6	24.5

Epithelial Thickness Finding

The process of wound surface epithelization was enhanced and accelerated by topical application of eucalyptus oil, on day 7th, the epithelial thickness of the eucalyptus group was 654.4, whereas epithelial thickness was 618.5 for the control group.

The descriptive statistic was the number, mean, standard deviation, standard error, lower and upper bound, minimum and maximum values of epithelial thickness measured at different healing periods for all the groups (Table 4).

Table 4 Descriptive statistics of epithelial thickness with a time of healing for all groups

Period	Groups	N	Mean	SD	SE	95%Confidence interval for Mean		Min	Max
						Lower bound	Upper bound		
3 day	Control	5	506.8	18.2	8.090	502.74	504.85	503	506
	Eucalyptus	5	598.6	4.3	1.050	574.16	581.83	574	595
7 days	Control	5	618.5	2.2	1.020	596.03	599.96	595	611
	Eucalyptus	5	654.4	15.7	7.044	645.59	656.20	651	655
10 days	Control	5	685.2	15.7	7.070	661.33	679.06	670	684
	Eucalyptus	5	799.4	14.6	6.570	766.50	792.23	789	797

DISCUSSION

Wound healing is a process in which the body tissue repairs itself through harmonicas action of intra and extracellular events [13]. As our understanding of wound healing progress, our therapeutic options expanded, and it becomes apparent that no single agent or modality will heal every wound [5]. Herbal therapy is becoming more and more accepted among patients and physicians, many herbal products were marketed to the public for skin and dermatologic uses [6]. Herbal preparations were only one component of alternative medicine, which includes a wide range of styles, a large number of herbal therapies were available for wound care [14].

There are different mechanisms of action of each essential oil; one of the means of action is the aroma of these oils, another mechanism is absorption through the skin, the molecules of the oils are small enough to penetrate through the skin barrier, the molecules will be absorbed simply into the skin within 20 to 40 min [15]. In order to analyze the events of the normal wound healing process, it is necessary to use animal and cellular models to help in understanding the underling pathological processes [16].

The skin of rats represents one of the most frequent models used in experimental studies regarding the skin wound healing; it is a useful model because it is possible to study the healing of epidermis, dermis and striated muscle [17].

Clinical Observations

All rats in this study were recovered well after surgery, and there was no sign of tissue reaction or bacterial infection (redness or discoloration, swelling, pain, and pus drainage) noted at the incision site in any animals during the whole healing period.

Laser Doppler perfusion imager finding: Recording of tissue perfusion is important in assessing the effect of environmental conditions, physical manipulations, diseases, and treatments on the microcirculation; laser Doppler perfusion imager is a technique that can evaluate non-invasively and in real time the tissue perfusion [18].

On the 3rd day, vasodilation of blood vessels at the periphery of the wound results in increased perfusion, this agrees with Gnyawali, et al., who found that the perfusion increased along the wound edge during early stages of wound healing, coinciding with the inflammatory phase of wound healing [19].

The highest mean values of perfusion in experimental group was on 7th day, which reflects the ability of eucalyptus oil in inducing angiogenesis, neovascularization, and promoting the wound healing, this agrees with Gurtner, et al., who found that neovascularization reach its peak during the tissue formation phase of wound healing [19]. On the 10th day, the perfusion of experimental and control group return to the normal level, this agrees with Gnyawali, et al., who found that as tissue remodeling occurs, perfusion in the wound area regresses and returns to normal levels similar to that of the adjacent normal skin.

Wound contraction finding: Wound contraction begins about 4th to 5th day after injury and continues for about 2-weeks, in an open wound, the contraction is obvious because wound edges become closer to each other [21].

The migration of fibroblasts into the extracellular matrix during the initial phase of wound healing appears to be an important part of wound contraction [22].

In this study, eucalyptus oil seemed to have a great tendency to accelerate epithelial migration, and wound contraction; it has the ability to increase the local capillary density, the local oxygen concentration, and regulate collagen synthesis, this agrees with Buemi, et al., who found that angiogenesis is essential for granulation tissue formation because the newly formed vessels will supply oxygen and nutrients to the developing tissue, and oxygen is vital for fibroblastic activity and collagen synthesis and maturation [23].

Inflammatory cells count finding: It should be well known that inflammation represents the earliest stage of wound healing; the inflammatory cells provide protection from infection and release chemotactic and mitogenic factors and this phase regress with the progression of proliferative and maturation phases of healing [24]. Eucalyptus oil affects the cellular events by its immune-stimulatory effect, it improves wound healing by its ability to promote morphological and functional activation of monocyte derived macrophages and stimulating the phagocytic process [25].

The result shows that the highest mean values of inflammatory cells were recorded at the 3rd day for control and experimental groups and this result in agreement with a study done by Al-Zamely, Z, who improved that there was high evidence of inflammatory cells in first 5 days on rat skin wound [26]. On day 7, there was a marked decrease in the number of inflammatory cells that infiltrated into the wound sites in the experimental and control group, this agrees with Carney, et al., who observed a decrease in the inflammation on day 7, in incisions treated with thrombin receptor activating peptide (TRAP-508), suggesting that the inflammatory phase has already occurred and the wounds progressed into a proliferation or maturation phase [24].

Epithelial thickness finding: The process of re-epithelization occurs soon after injury, it depends on the migration of epithelial cells from the wound margins and any remaining adnexal structures in the dermis, like hair follicles, sebaceous glands, and sweat glands, epithelial migration, and proliferation will continue until the wound is covered by an intact epithelial barrier [27].

During wound healing, the thickness of the neoepidermis is associated with the re-epithelization process, which involves increased keratinocyte proliferation and the resultant epidermis thickening and keratinization [28]. The results of the control group showed a gradual increase in epithelization, while for the experimental group; the process of wound surface epithelization was enhanced and accelerated by topical application of eucalyptus oil.

The local application of eucalyptus oil has kept the wound moist; this helped in re-epithelization, and agree with Enoch, et al., who suggest that if the wound is kept moist, the rate of epithelial coverage will increase. The results of epithelial thickness for both control and experimental groups reach its peak at 10 days, and this agrees with Al-Zamely, Z, who found that epithelization reaches its peak at 10th day.

CONCLUSION

The present study demonstrated that the local application of eucalyptus oil promoted remarkable neovascularization and skin regeneration and may lead to novel treatments for skin wounds.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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