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Histological effect of traditional rose ointment application in the excisional wound model

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ABSTRACT

The aim of our study was to evaluate the histological effects of traditional rose ointment application on artificially induced skin wound treatments. 18 adult BALB/c mice were artificially wounded by the formation of a puncture-generated exhaled wound model. Wound tissues of mice were analyzed histologically with light microscopy after hematoxylin eosin and Masson trichrome stainings were performed. Vascular endothelial growth factor (VEGF) expressions were evaluated by immunohistochemical analysis in order to demonstrate the angiogenesis throughout the tissue. Tissue regeneration rate was significantly increased in traditional rose ointment treated group although there was no significant difference in granulation and angiogenesis between the groups. Traditional rose ointment treatment seems to have a positive effect in the treatment of skin wound by inducing the regeneration capacity in the tissue. Further studies are needed to confirm this finding and to evaluate its potential to be used in wound healing.

Key words: Phytotherapy, Wound Healing, *Rosa damascena,* Histology, Rats.

INTRODUCTION

Phytotherapy includes the treatment methods in which herbal compounds are used to assist medical treatment. Fragrant roses are widely used in phytotherapy and the rose oil is one of the most widely used among the rose deriva-

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tives[1]. *Rosa damascena* Mill. (Damask rose, Oil-bearing rose, Pink rose) is the most important species, producing a high-value aromatic oil, which is used in the pharmaceutical, flavourings and fragrance industries[2]. Turkey is one of the countries leading the production of *Rosa damascena* in the world with a production capacity of approximately 10.000 tonnes of rose flowers and rose oil annually[3].

The process of wound healing is a dynamic, complex interplay of cytokines, involving many different cell types. The skin has important immune and protective characteristics and has an amazing ability to heal, invariably with scarring. Scarring is quite variable and is based on many factors, dependent on patient characteristics and overall health (intrinsic) as well as the healing environment (extrinsic). All epithelial tissues in the body, except for bone, heal by scar formation rather than regeneration. The skin is not spared by this. It is important to identify wound-healing problems early to minimize scarring. To understand the effects of injury and potential for scarring, one must first look at the layered histology and physiology of the largest organ in the body. The skin is separated into an epidermis, dermis, and hypodermis. The epidermis itself has 5 layers or strata from superficial to deep: corneum, lucidum, granulosum, spinosum and basale [4,5,6]. The skin has two tissue layers: a keratinized stratified epidermis and an underlying thick layer of collagen-rich dermal connective tissue providing support and nourishment. Adjunct like glands and hairs are derived from and linked to, the epidermis but project deep into the dermal layer. As the skin serves as a protective barrier against the outside, any break in it must be immediately and efficiently repaired. A temporary mend is achieved in the form of a clot that plugs the defect, and over following days steps to regenerate the missing parts are began. Inflammatory cells and then fibroblasts and capillaries invade the clot to form a contractile granulation tissue that draws the wound margins together; meanwhile, the cut epidermal edges migrate forward to cover the denuded wound surface[7].

We aimed to investigate the efficacy of the Rosa damascena ointment treatment by comparing the outcome with Madecassol® (Bayer, 00001199) which is used as a pharmacologically reference drug on the studies dealing with excisional wound healing which contains 1% *Centella asiatica* extract[8].

METHODOLOGY

Traditional *Rose damascena ointment* was prepared by Ayten Altıntaş as following: beewax (1g) + rose solution (rose water + pure olive oil) (3 g) + 50µl of rose oil. *Rosa damascena* were obtained from Isparta, Turkey.

Experimental Model

Healthy adult male BALB/c mice (25-30 g, n = 18) obtained from Istanbul Medipol University Medical Research Center (Istanbul, Turkey) were fed ad libitum and kept in a controlled room at 24°C temperature and humidity under 12 h long light/dark period.

The mices were anesthetized intraperitoneally (i.p.) with ketamine (10 mg/kg) and xylazine (80-100 mg/kg), both anterior-dorsal side of each mouse was shaved and washed with povidone-iodine solution. Two full thickness excisional skin wounds were created 5 mm in diameter by punch biopsy as described previously[9].

Mices were randomly divided into 3 different groups as, untreated control group (n: 6), Topical Madecassol applied group (n: 6), and Topical Rose ointment group (n:6). Madecassol and rose ointment was applied once a day topically onto the wounds sufficient to cover the surface of the wounds completely until the day rats were sacrificed at day 7th. All procedures were performed aseptically on dorsal sides of the mice.

Histological and Immunohistochemistry Studies

All animals were sacrificed by decapitation and the skin of the back including the wound area was removed at 7th day. Full-thickness biopsy samples extended from the outside margin to the center of the treated area were collected. The skin covering the wounds was fixed in 10% neutral buffered formalin solution for histological and immunohistochemistry analysis. All wound tissue parts were embedded into paraffin and 3 mm thick sections were sliced with microtome (Thermo, Microm HM 340E). Sections were stained with hematoxylin and eosin (H&E) and Masson trichrome staining for histologic evaluation and and vascular endothelial growth factor (VEGF) expressions were evaluated by immunohistochemistry to demonstrate the angiogenesis throughout the tissue.

Hematoxylin Eosin and Masson Trichrome Stainings

The first step in histological staining is deparaffinization. Paraffin sections were deparaffinized with toluene for 30 minutes. Then tissue sections were rehydrated through descending grades of alcohol (100%, 90 %, 70 %) to water for 5 minutes. The sections were stained with hematoxylin and eosin and Masson trichrome staining for histologic evaluations.

For hematoxylin and eosin staining; tissue sections were incubated in Mayer hematoxylin staining solution for ten minutes and then in running tap water for 30 minutes. After rinsing twice with distilled water, tissue sections were put into eosin staining solution for 30 seconds and were again rinsed with distilled water twice.

For Masson trichrome staining; tissue sections were differentiated with 1% acid alcohol and washed well in tap water. Then the sections were stained in acid fuchsin solution for 5 minutes, rinsed in distilled water and treated with phosphomolybdic acid solution b for 5 minutes. After drain, the sections were stained with methyl blue solution c for 4 minutes and rinsed in distilled water. Finally, the sections were treated with 1% acetic acid for 2 minutes and dehydrated through ascending grades of alcohol.

All tissue sections were dehydrated in alcohols and clarified with toluene and subsequently coverslipped in mounting medium.

Wound healing for each group was evaluated using the scoring system described by Geleano et al. for epidermal and dermal regeneration: 0 represents the absence of epithelial proliferation in 70%; 1 represents poor epidermal structure in ≥ 60%; 2 represents deficient epidermal structure in ≥ 40%; 3 represents limited epithelial proliferation ≥ 60%; 4 represents full epidermal remodeling in ≥ 80% of the tissue. For thickness of the granulation tissue scoring was; 0 represents immature and inflammatory tissue in ≥ 70%; 1 represents thin granulation layer; 2 represents limited granulation layer 3 represents thick granulation layer; 4 represents very thick granulation layer. For the evaluation of angiogenesis, intact vessels were counted and identified by the presence of erythrocytes in the lumen. To distinguish well-formed from poorly formed capillary vessels, we evaluated the presence or absence of edema, congestion, hemorrhage, thrombosis and intravascular or intervascular fibrin formation as: 1 represents altered angiogenesis (few vessels/site) characterized by high degree of edema, hemorrhage, occasional congestion and thrombosis; 2 represents few newly formed capillary vessels (3–4/site), moderate edema and hemorrhage, occasional congestion, intravascular fibrin deposition and absence of thrombosis; 3 represents recently formed capillary vessels (5–6/site); 4 represents recently formed and normal visible capillary vessels (>7/site).

The results obtained from histological observation of skin tissue sections were evaluated by analyzing epidermal and dermal regeneration, granulation tissue thickness and angiogenesis, as shown in Figures 1-3. Increasing epidermal and dermal regeneration is the first concern used for measuring the effect of treatment on wounds studies. Epidermal regeneration is characterized by well-structured epithelial layers with no evidence of crusting or intra-epithelial inflammatory cells [10].

Statistical analysis

All statistical analyzes were performed using GraphPad Instat for Windows

(Ver. 3.06) program. The results were expressed as means ± SD. For histological wound healing assessment, the differences between groups were analyzed by ANOVA followed by Tukey analysis of variance. Values for $p \leq 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Histological results

All groups showed complete wound healing closure. Re-epithelialization and granulation tissue organization were significantly better in Rose ointment group. There were also significantly higher dermal and epidermal regeneration in the Rose ointment ($p<0.01$) compared with the control and Madecassol groups, (Figure 1).

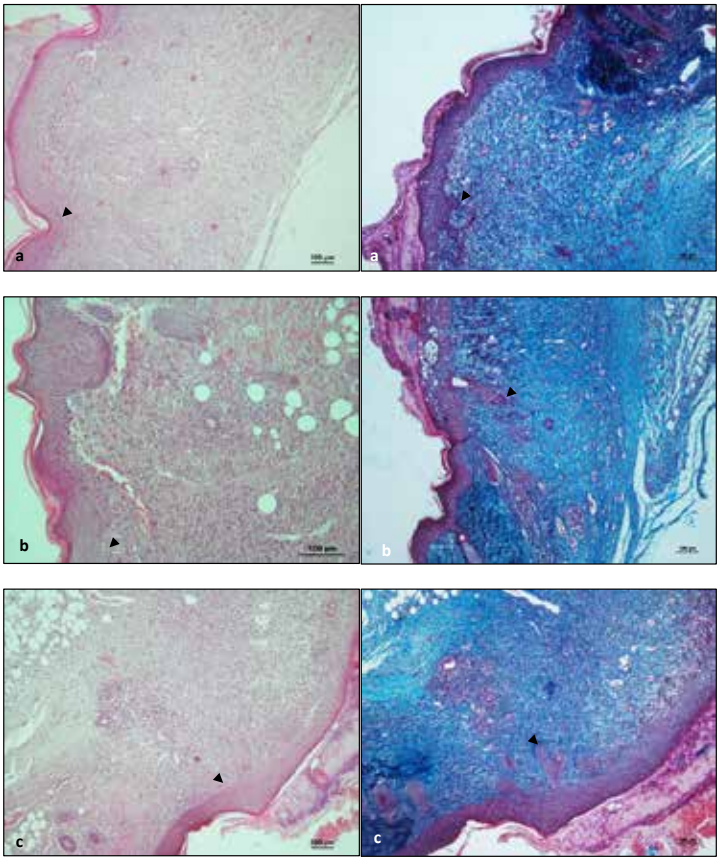

Figure 1. Light micrographs of wound healing area. Control (a), Madecassol (b), Rose ointment(c) groups. Hematoxylin eosin and Masson trichrome staining; bar = 100 mm (a, b, c). Arrowheads represent re-epithelialization.

Immunostaining results

An increase VEGF expression were demonstrated in the wound area in rose and Madecassol groups compared with the control group, although the difference was not statistically significant (Figure 2).

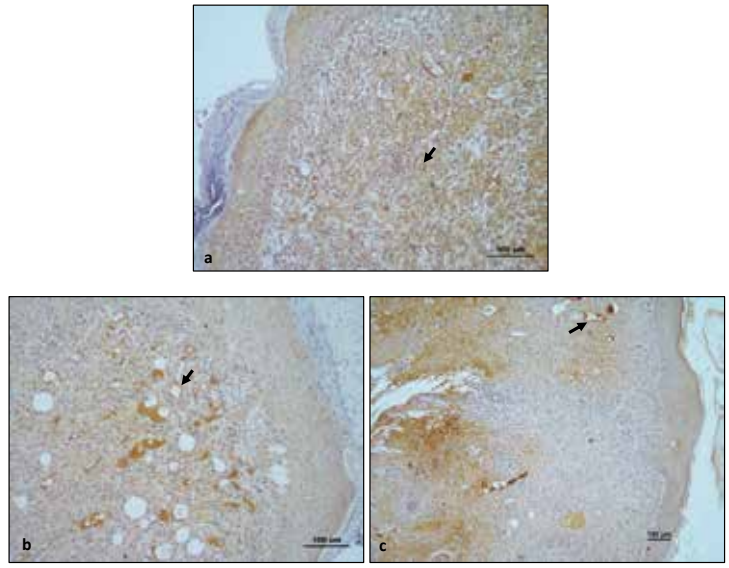

Figure 2. Light micrographs of wound healing area. Control (a), Madecassol (b), Rose ointment (c) groups. Vascular endothelial growth factor (VEGF) immunohistochemistry; bar = 100 mm (a, b, c). Arrows indicates endothelial cells positive for VEGF (VEGF+).

Tissue regeneration was found to be statistically significant in rose ointment group, although there was no significant difference in granulation and angiogenesis between the groups (Figure 3).

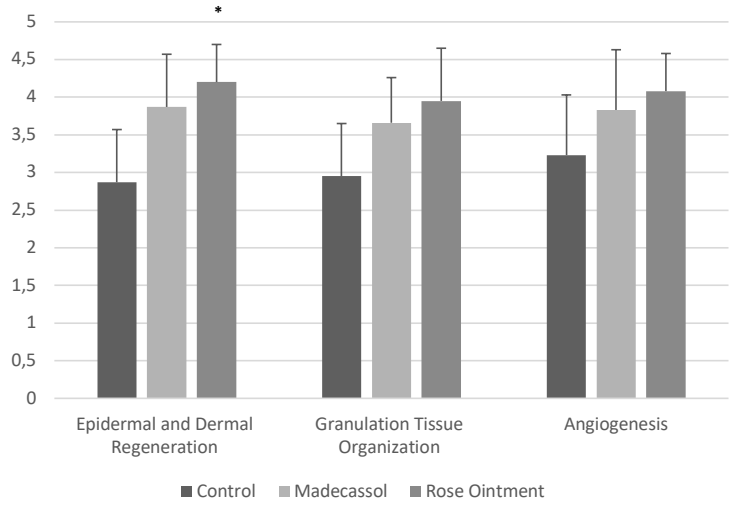

Figure 3. Histologic scores in wounds from mice given control, Madecassol, Rose ointment groups. Each point represents the mean SD of experiments. *p<0.01 vs. control.

Rose has been an important plant used traditionally to treat many diseases for many years. It is reported to contain carboxylic acid, myrcene, vitamin C, kaempferol and quercetin and its flowers also contain a bitter principle, tanning matter, fatty oil and organic acids [11, 12, 13, 14, 15]. It has been reported to be a promising plant for anti-HIV, antibacterial, antioxidant, hyptonic, anti-aging effect [16, 17, 18, 19, 20]. Rose oil is reported to be used in many different diseases including depression, grief, nervous stress and tension. It is also reported to help reduction of thirst, healing old cough, wound healing, and skin health. Vapor therapy of rose oil is helpful for some allergies, headaches, and migraine [21, 22]. Besides all these known effects, we showed the beneficial effects of *Rosa damascena* ointment on wound healing for the first time. Previous studies in the literature reported the effects of other natural products sequestered from medicinal plants for skin regeneration. In present study an in vivo animal wound healing model was employed to represent the effects of rose ointment.

Rose treated group showed significantly increased Re-epithelialization and granulation tissue organization. There were also significantly higher dermal and epidermal regeneration in the Rose ointment compared with the control and Madecassol groups indicating the positive effect of the Rose on wound healing. In addition, although statistically insignificant, an increase in VEGF expression were demonstrated in the wound area in Rose treated group indicating the increased angiogenesis which is an important parameter in wound healing.

According to the recent results it may be concluded that Rose damascana positively affect tissue regeneration during wound healing and may be used as a supportive treatment strategy for medical treatment in cases of serious wound treatment. Further studies with different doses and different applications are needed to confirm the findings.

STATEMENT OF ETHICS

Our study was approved by Medipol University local ethics committee with the number 38828770-604.01.01-E.3412 and all experiments were carried out according to the internationally accepted principles for laboratory, animal use and care as found in European Community Guidelines.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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There are no any funding sources.

AUTHOR CONTRIBUTIONS

These authors contributed equally.

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