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Efficacy of topical application of coumarin on incisional wound healing in BALB/c mice

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Background: Wound healing is one of the main problems faced by medical scientists. Nowadays, herbal compounds are used to accelerate the repairing process. Coumarin is a plant compound with anti-inflammatory and anti-oxidant effects. In the present study, the benefits of using coumarin in accelerating wound healing were investigated in mice.

Methods: Sixty male BALB/c mice were used. After making a linear wound on the dorsum of the animals, they were randomly divided into five equal groups: the first and second groups received topical cream of coumarin at concentrations of 1% and 2%; the third and fourth groups received nitrofurazone cream (positive control) and eucerin cream (negative control), respectively. The fifth group as the sham group was not treated. Then on days 4, 7, 10, and 14 of experiment, biopsies were performed on three mice from each group. Histological examination was performed using hematoxylin and eosin and Masson trichrome staining. Data were analyzed by ANOVA and Tukey tests.

Results: Inflammation significantly decreased in both experimental groups at days 4, 7, and 10, compared to the control groups. In the proliferation phase, fibroblast cells, granulation tissue formation, and epithelialization were significantly higher in both experimental groups than the control groups. In addition, collagen synthesis significantly increased in the experimental groups compared to the control groups.

Conclusion: Topical application of coumarin had beneficial effects on different phases of wound healing in the skin of BALB/c mice.

Keywords: coumarin, wound healing, skin, mice

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INTRODUCTION

Skin is a vital organ for humans because of preventing loss of water, bleeding, and invasion of microorganisms and regulation of body temperature [1]. Wound healing is a restorative process that involves hemostatic, inflammatory, proliferation, and remodeling phases. At the inflammatory phase, inflammatory response is organized by granulocyte and lymphocyte cells. The subsequent proliferation phase is formed by angiogenesis, epithelialization, formation and accumulation of fibroblasts and collagen synthesis of the extra cellular matrix (ECM). At the end of wound healing, remodeling phase is formed that involves fundamental changes in the collagen structure [2].

Various factors affect wound healing, including oxygenation (tissue perfusion), infection, age, stress, nutrition, sex hormones, ECM, proteases, and cytokines [1,2]. Increasing the rate of wound repairing leads to positive financial and health

results, which is the main aim of medical science in recent decades. One of the most important ways to achieve this goal is to reduce inflammation and wound infections [3].

The use of herbal medicines has grown enormously because of their low cost and side effects and also easier preparation compared to chemical drugs. Furthermore, many plants have anti-inflammatory and anti-infective properties. Hence, the plant-related medicine industries are expanding in the world [4,5].

Coumarin C9H6O2 belongs to a group of polyphenolic compounds which are benzopyrone derivatives, and in nature, there are two glycosylated and free forms. Benzopyrone is divided into alpha and gamabenzopyrone sub-types, and coumarin belongs to the alpha-banzopyrene subtype [6,7]. Coumarin has anti-cancer, anticonvulsant, anticoagulant, and immune regulator properties [6,8,9]. It also has anti-inflammatory, anti-oxidant, anti-viral, anti-bacterial, and anti-fungal effects [9,10]. Among the investigations conducted on these properties, there are some important studies which took the natural and synthetic coumarin derivatives with their anti-inflammatory/antioxidant activities into account. These studies indicated that coumarin can reduce tissue edema and inflammation. It also induces high degree of antioxidant activity. Not only coumarins have some effects on the formation and removal of reactive oxygen species (ROS), but also the processes involving free radical-mediated injuries have been affected. Furthermore, prostaglandin biosynthesis, which takes part in fatty acid hydroperoxy intermediates, is inhibited with coumarin and its 7-hydroxy-derivative [10]. Also, the results from the study of anti-inflammatory effect of MC13 (a new coumarin compound extracted from the leaf of Condiment Murraya extract) showed that this compound inhibits the production of lipopolysaccharide (LPS) that is produced by various inflammatory mediators such as nitrite oxide, IL6, and TNFα [11].

These studies consider the anti-inflammatory/anti-oxidant properties of coumarin, a good candidate to accelerate the wound healing process; however, there have been no precise studies on the therapeutic effects of this substance on the wound repairing process so far. Therefore, in this study, we set to investigate the usefulness of coumarin in accelerating wound healing in mice.

MATERIALS AND METHODS

Preparation of ointment

Coumarin (code: C85557-5G) was purchased from Sigma-Aldrich Company. 1% or 2% cream containing this substance with the basis of cold cream (eucerin) was prepared and used [12]. Nitrofurazone cream and other creams were purchased from the Behnam Company in Tehran.

Experimental animals

In this study, sixty mature (2.5 months of age) male BALB/C mice weighing 25 ± 5 gr were used. The animals were provided from the Experimental Medicine Research Center of Birjand, University of Medical Sciences (BUMS). Each mouse was kept individually in a clean cage. Environmental conditions were controlled by daily replacement of cages, 12:12 h light-dark cycle, 22 ± 1°C temperature, and an average air humidity of 40 to 45%. Mice had free access to water and food. The number of mice and method of this study were determined based on previous studies [13,14].

Surgical procedure

The mice were initially anesthetized with intraperitoneal (I.P.) injection of Xylasine 2% and ketamine 70 mg/kg, and the hair of dorsal thoracic skin was completely shaved. Then, the shaved area was disinfected with 10% iodine (Iran Drug Production Company) under sterile conditions (observing all the rules of surgery). A full-thickness incisional wound (2 cm length) was created on dorsal thoracic skin of the mice, using a ruler and a sterile blade [13].

Afterwards, the mice were divided into five groups: the first and second groups were treated with cream containing coumarin 1 and 2% and the third and fourth groups as the positive and negative controls which received nitrofurazone 0.02% and cold cream (eucerin), respectively. The fifth group was not treated (sham). The first to fourth groups were dressed twice daily at 8:00 am and 8:00pm for 14 days [13].

Histological study

On days 4, 7, 10, and 14 after the treatment, three

animals from each group were sacrificed with an overdose of anesthetics, and incisional wounds areas were removed for histopathological studies. The samples were embedded in paraffin after fixation in 5% formaldehyde solution, dehydrated in ethanol series with excessive concentration, and cleared in xylene. Then, 5 µm serial sections of specimens were prepared by rotary microtome (Leits, Italy) and stained with hematoxylin and eosin (H & E) and specific staining of Masson's trichrome. Finally, the slides assessed under light microscope (Olympus, BX54) in order to evaluate the epithelialization, granulation, and collagenization [15].

Statistical analysis

The quantitative data were reported as mean ± standard error of the mean. One-way ANOVA test was used to compare the groups, and Tukey test was used in case of significant results. Data were analyzed with SPSS statistical software (ver. 19). Results were considered statistically significant at $P < 0.05$. Image J software was used for quantitative data and numerical analysis of the number of fibroblast cells, epithelialization, and granulation in different stages of repair. Evaluation of inflammation and collagenization areas were estimated by semi-quantitatively score of - to +++, based on the following score system; - low, -/+ low to mild, +/- very mild, + mild, ++ moderate, and +++ severe.

Ethical considerations

The ethical standards of working with laboratory animals were designed based on the protocol of work with laboratory animals approved by Birjand University of Medical Sciences with the approved code (455171) (Ethics Code: ir.bums.REC.1396.81).

RESULTS

Study samples on the 4th day

The mean of the inflammatory cells decreased significantly in the treated groups with coumarin 1% and 2% and also nitrofurazone compared with the negative control and sham groups. This parameter also showed a slight decrease in the experimental groups compared to the positive control group ($P < 0.05$; Table 1, Figure 1).

Study samples on the 7th day

Inflammatory cells in the experimental groups showed significant decrease compared with the negative and sham control groups ($P < 0.05$; Table 1, Figure 1). Also, the total number of inflammatory cells in these groups decreased significantly compared to the nitrofurazone group ($P < 0.05$; Table 1, Figure 1).

The findings on the 7th day indicated that the mean of fibroblast cells also increased in the experimental groups compared to the negative and sham control groups ($P < 0.05$; Table 2, Figure 2). On the other hand, only the mean number of fibroblasts in the coumarin 2% treatment group was significantly higher than the nitrofurazone group ($P < 0.05$; Table 2, Figure 2).

The density of collagen fibers in the experimental groups was higher than the all control groups (Table 1, Figure 2). On this day the mean of granulation tissue formation was significantly increased in the experimental groups in comparison with negative and sham control groups and also nitrofurazone group ($P < 0.05$; Table 2, Figure 2).

Study samples on the 10th day

The parameters studied on this day included

Table 1. Efficacy of topical application of coumarin on inflammation phase and collagen density in the wound repair model.

Groups/Days	4		7		10		14	
Studied parameters	Inf. C	Col. D	Inf. C	Col. D	Inf. C	Col. D	Inf. C	Col. D
Coumarin1%	+	-	+/-	+	-	++	-	+++
Coumarin2%	+	-	+/-	+	-	++	-	+++
Nitrofurazone	++	-	++	+/-	+	+	-	+++
Eucerin	+++	-	++	-	+	+/-	-	+
Sham	+++	-	+++	-	+	+/-	-	+

Inflammatory cells (Inf. C) and collagen density (Col. D) were estimated by the following score system; - low, -/+ low to mild, +/- very mild, + mild, ++ moderate, +++ severe.

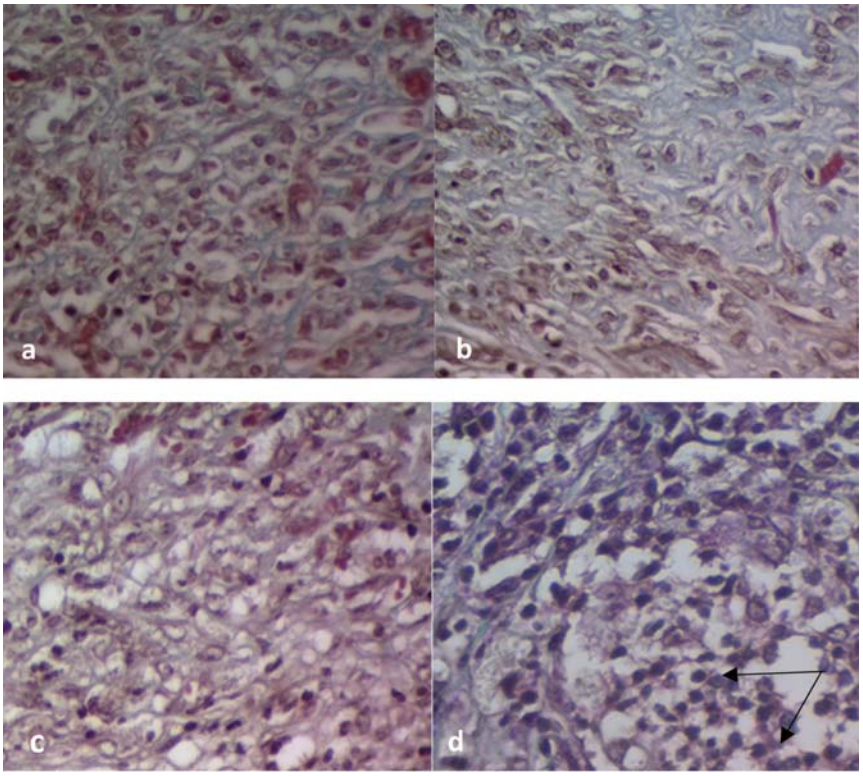

Figure 1. Inflammatory cell infiltration in (a) co2%, (b) co1%, (c) Nit, and (d) sham. Black arrows represent inflammatory cells; Masson's trichrome stain, ×400 on day 10. Coumarin, co; Nitrofurazone, Nit.

inflammatory and fibroblast cells, collagen density, granulation, and epithelization. The total number of inflammatory cells in the experimental groups decreased significantly in comparison with the control groups ($P < 0.05$; Table 1, Figure 1). The mean number of fibroblast cells in the experimental groups demonstrated a significant increase compared to the control groups ($P < 0.05$; Table 2, Figure 2).

Table 2. Efficacy of topical application of coumarin on fibroblast proliferation, epithelialization, and granulation tissue (μm2) in the incision wound repair model.

Studied parameters	Groups/Days	7	10	14
Fibroblast proliferation (number)	Coumarin 1%	15.20 ± 2.52*#	38.40 ± 5.91*#	28.90 ± 5.85*#
	Coumarin 2%	19.50 ± 1.77*#	44.50 ± 3.17*#	29.40 ± 5.98*#
	Nitrofurazone	12.40 ± 3.23	27.60 ± 4.90*	18.70 ± 6.16*
	Eucerin	11.60 ± 2.71	13.10 ± 4.86	12.10 ± 4.86
	Sham	9.90 ± 1.79	10.90 ± 1.79	8.90 ± 1.79
Epithelialization (μm^2)	Coumarin 1%	-	17.16 ± 2.11*#	25.25 ± 3.11*#
	Coumarin 2%	-	23.65 ± 1.79*	26.57 ± 3.48*#
	Nitrofurazone	-	15.96 ± 1.92*	18.62 ± 1.88*
	Eucerin	-	11.17 ± 2.08	12.66 ± 1.78
	Sham	-	8.90 ± 0.97	9.10 ± 1.17
Granulation tissue (μm^2)	Coumarin 1%	12545.71 ± 4519.03*#	23770.23 ± 2397.99*#	22198.69 ± 3820.91*#
	Coumarin 2%	13394.35 ± 3514.45*#	24476.02 ± 7007.80*#	23196.29 ± 1959.68*#
	Nitrofurazone	9227.23 ± 985.91	16004.09 ± 2075.76*	13507.67 ± 3520.29
	Eucerin	6248.78 ± 1281.43	12547.70 ± 1510.85	11194.85 ± 2616.32
	Sham	2886.63 ± 957.33	8631.52 ± 1312.44	7094.19 ± 2120.01

Values are expressed as the Mean ± SEM. *P*-value: * < *0.05* compared with the negative and sham control groups. *P*-value: # < *0.05* compared with the positive control group.

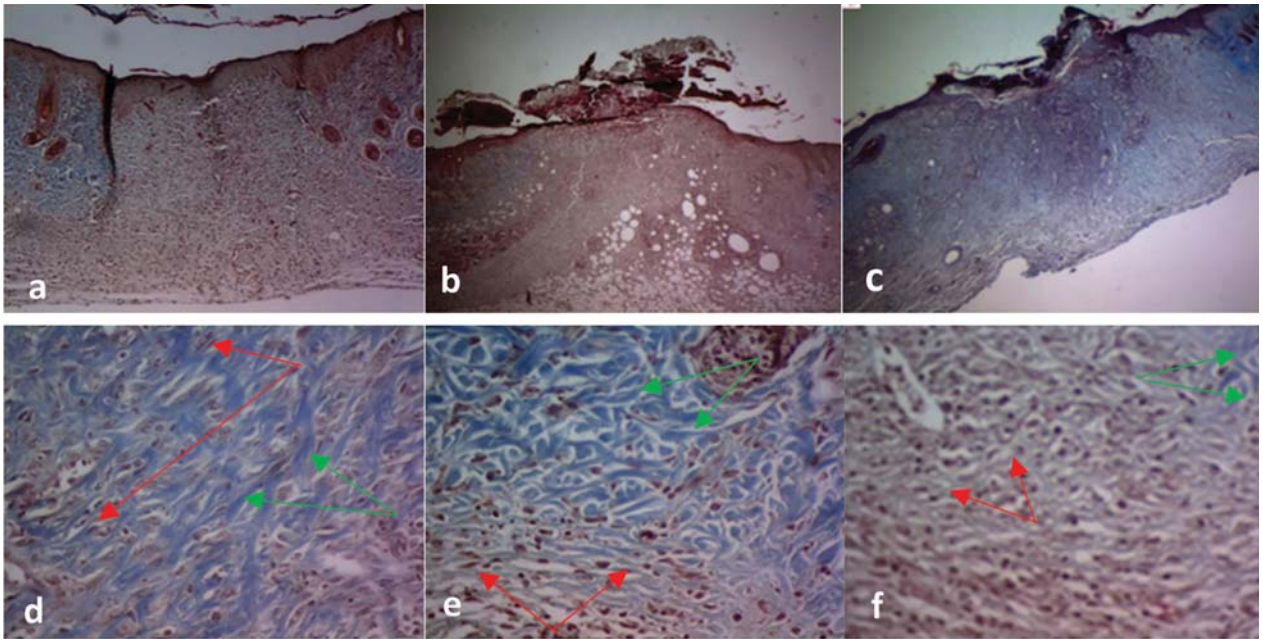

Figure 2. The area of granulation in (a) co2%, (b) co1%, (c) sham; Masson's trichrome stain, ×40 in 3204/12× 2401/34 μm^2 on the 10th day. Red and green arrows represent fibroblast cells and the density of collagen fibers, respectively, in (d) co2%, (e) co1%, (f) sham; Masson's trichrome stain, ×400 on the 10th day. Coumarin, co.

Based on Masson's trichrome staining, the density of collagen fibers in the two experimental groups was significantly higher than the control groups (Table 1, Figure 2). In addition, the mean area of granulation significantly increased in both experimental groups in comparison with the control groups ($P < 0.05$; Table 2, Figure 2). Besides, the thickness of epithelialization significantly increased in the experimental groups compared to the control groups ($P < 0.05$, Table 2).

Study samples on the 14th day

It was indicated that the mean number of fibroblast cells significantly increased in the experimental groups compared to the control groups ($P < 0.05$; Table 2, Figure 2). The density of collagen fibers in the two coumarin experimental groups was significantly higher than the negative and sham control groups. However, there was no difference between these experimental groups and the positive control group (Table 1, Figure 2). Furthermore, the mean area of granulation significantly increased in the treatment groups with 1% and 2% coumarin compared to the control groups ($P < 0.05$; Table 2, Figure 2). There was a significant increase in the mean of epithelialization in the two coumarin experimental groups in comparison to the control groups ($P < 0.05$; Table 2)

DISCUSSION

Wound is defined as a structural and functional breakdown of skin normal continuity and integrity, resulting in damage from physical, chemical, and biological factors [16]. Wound healing is a natural restorative response to injury. This process is performed based on collaboration of cellular and molecular events that involve the infiltration of cells into the wound, cell proliferation, and also synthesis and accumulation of new ECM [17,18]. These events occur during the three phases of skin repair including hemostatic-inflammation, proliferation, and remodeling [2].

The results showed that coumarin had positive effects during various phases of wound healing, especially inflammatory and proliferative phases. One of the most important events that occurs at the beginning of wound healing is the phenomenon of inflammation. The inflammatory process involves cell and hormonal parts. Neutrophils, macrophages, and lymphocytes are the most important inflammatory cells that appear at the wound area at this stage [18,19]. Our study revealed

that inflammation and infiltration of related cells from the beginning of the study on the fourth day were less in the coumarin-treated groups compared to all other control groups, and this trend was also observed in the following days.

Coumarins with a fused benzene and α-pyrone rings are found in plants and constitute a major group of phenolic derivatives. Over 1300 secondary metabolites of this compound have been identified, chiefly in green plants, fungi. and bacteria [10]. Studies have shown that coumarin, as a phenolic compound, has anti-inflammatory, anti-oxidant, and antibacterial properties [9]. Among these effects of coumarin, its anti-inflammatory properties have been confirmed in numerous studies as following: Iranshahi *et al.* conducted a study in which antioxidant, anti-inflammatory, and lipoxygenase inhibitory activities of the prenylated coumarin, umbelliprenin were evaluated. It was revealed that umbelliprenin exhibited antioxidant and lipoxygenase inhibitory properties [20]. In a study conducted by kafaj *et al.* on therapeutic effects of leaf extract of *Plantago lanceolata* on infected burn-wound in mice, the beneficial effects of this extract on wound healing process could be due to the presence of phenolic compounds, such as coumarin, which has anti-inflammatory properties and consequently accelerate the process of wound repairing [21].

Another experimental study also demonstrated that the substance (S) - (+) - decursin, a biological compound of coumarin (extracted from the *glacial* plant), reduced IgE levels of inflammatory cytokines in comparison with the control group, concluding that it may be valuable as a therapeutic drug for the treatment of atopic dermatitis [22]. Additionally, Luchini *et al.* noted that coumarin and 4-hydroxycoumarin prevent the glutathione depletion that occurs as a consequence of the colonic inflammation and intestinal oxidative stress and may be effective in the treatment of ulcerative colitis in rats [23]. Similarly, the present study confirmed the anti-inflammatory effects of coumarin during wound healing and acceleration of the wound healing process due to this positive feature.

In the proliferative phase which consists of the activation and migration of fibroblasts and production of glycosaminoglycan, proteoglycan, and collagen fibers, the granulation tissue is formed [24,25]. This phase of repair is very important and sensitive. Moreover, in this stage, epithelialization is accelerated due to the formation of granulation tissue [19]. So, any compound that can affect the rate and extent of the granulation tissue formation can also change the rate of wound healing. Our findings showed that coumarin could increase the area of granulation tissue formation and the epithelialization rate on days 7, 10, and 14 in the experimental groups compared to the control groups. Hence, these properties can accelerate wound healing.

Kiran *et al.* evaluated *Sesamum indicum L.* seed and sebum on wound healing activity in rats and demonstrated that this plant, due to coumarin content as one of the most effective ingredients, had better repairing effects on increasing granulation tissue and wound closure and also significantly reduced the epithelialization period compared to treated groups with *Aloe vera* as a standard control [26].

Ilango *et al.* carried out a study on wound healing. They found that the fruit pulp of *Limonia acidissima L.* (*Rutaceae*) had anti-oxidant activities in rats due to the content of flavanoids, tannins, glycosides, coumarin, and saponins. They demonstrated a significant increase in the number of fibroblasts, collagenization, granulation tissue weight, wound contraction, wound breaking strength, and decreasing of epithelialization period in the experimental group compared with the control group [27].

The *Plantago lanceolata* leaf ointment has been reported to be effective for enhanced repair in burn-wound by increasing the angiogenesis and granulation tissue formation as well as reducing the epithelialization period due to the content of specific compounds such as coumarin, glycoside, and phenol [21].

Kim *et al.* explored the anti-inflammatory and ulcerative healing effects of *Stellera chamaejasme L.* This herb is used as a medicinal plant in China. Most of its compounds include flavonoids, lichen, and coumarin. This herbal extract was proved to affect the acceleration of wound healing process via increasing the expression of TGF_β1 [28].

One of the most important components of the granulation tissue is fibroblasts which are involved in the production of collagen and ECM of granulation tissue. We also focused on these cells and manifested that the number of fibroblast cells

on days 7, 10, and 14 increased considerably in the two coumarin experimental groups compared to the control groups. Concentrations 2% of this compound demonstrated better effects in comparison to two different percentages of coumarin.

There is also limited evidence to confirm the positive effects of coumarin-based herbal extracts on fibroblast cells and collagen fibers' synthesis. Lee *et al.* conducted a study to promote the synthesis of collagen fibers using the root of *Angelica dahurica*, showing that the three types of coumarin derivatives isolated from this plant stimulated collagen biosynthesis in human fibroblasts [29]. Shiraviet *al.* investigated the effect of henna leaves' extract (*Lawsonia inermis*) on skin wound healing and revealed that the extract of this plant with chemical compounds such as multiple phenolic glucosides, coumarin, beta-cytosterol, and alkaloids, as the medicinal plant parts, could produce more collagen fibers, higher density of fibroblasts, and blood vessels and could also reduce healing periods [3].

Some studies also point to the mechanism of possible effects of coumarin on the increased the number of fibroblasts. Martino *et al.* evaluated the effect of *Hibiscus syriacus* extract (rich in flavonoids and coumarins) on skin wound healing and demonstrated that this extract could increase the closure rate of wound area via increase of neoepidermis and stimulation of the expression of wound-accelerated markers (TGF β). Moreover, this extract increased the expression of genes involved in hydration, homeostasis, and production of collagen and also increased keratinocytes and fibroblasts in the cell group treated with this extract [30].

Research on coumarin confirms that this substance causes vasodilatation and increases blood supply, which affects the applied capacity of fibroblasts and increases collagenization [31].

CONCLUSION

The results of this study confirmed that the coumarin compound plant has a highly positive effect on different stages of wound healing processes, including inflammatory and proliferative phases. The evidence indicates that coumarin would be a good candidate for wound healing. Hence, more supplementary studies are suggested to investigate the mechanism of coumarin effect during the process of skin wound healing.

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