Silymarin promotes wound healing through regulating epithelial-mesenchymal transition in rat model: Histopathological and immunohistochemical evidences

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Abstract

The wound is the disruption of the normal structure, integrity, and function of the skin and subcutaneous tissue. It is known that current wound management strategies applied in the treatment of acute and chronic wounds both cause an increase in health costs and do not achieve the desired level of success. Therefore, more effective and easily applicable treatment methods are needed. SM is an agent known to have hepatoprotective, anticancer, antidiabetic, cardioprotective, neuroprotective, antimicrobial and antioxidant effects. And it is also used in dermatological applications. However, the therapeutic effects of Silymarin (SM) on wound healing are still unknown. In this study, the effects of SM were investigated by comparing it with dexpanthenol (Dxp), whose favorable effects on wound healing are known. Sham, Dxp, and SM groups were formed. 18 animals were used for each group. Two circular full-thickness skin wounds were taken from the nape of the neck (1.5 cm) using a six-mm punch biopsy tool. SM and Dxp was applied once daily for 15 days, in sufficient amounts to cover the entire wound and the effects of the drugs were investigated immunohistochemically on the 5th, 10th, and 15th days in rats. Inflammation, collagenization and epithelialization were evaluated in histochemical H-E and Masson trichrome staining. E-cadherin, N-cadherin, Occludin, Vimentin, FGF-1 and MMP-9 expression levels were examined immunohistochemically. Based on histological and immunohistochemical results, SM and Dxp enhanced epithelialization and reduced inflammation more than the sham group. Furthermore, there was no significant difference in the effects on the epithelial-mesenchymal transition between SM and Dxp. Results indicated that SM is a useful therapeutic agent at least as much as Dxp in wound healing. The wound re-epithelization and anti-inflammatory effects of SM may be a new approach to the treatment of wound healing.

Keywords: Silymarin, wound healing, epithelial-mesenchymal transition, cadherin, NfκB

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Introduction

Our skin provides a variety of important hemostatic functions between the external environment and our body, from heat regulation to perception mechanisms [1]. More specifically, the skin functions as a defense against mechanical, chemical or phototic damage to the body [2]. The skin may undergo structural deformation or damage, which is defined as a wound, in the process of performing its task. It is known that the skin has effective and fast mechanisms to correct this unfavorably picture or to close the breaches. This process, known as wound healing, could be listed as hemostasis, inflammation, proliferation, and dermal remodeling [3]. Keratinocytes are the structures responsible for the formation and protection of the epidermis, which is the outer layer of the skin. These functions of keratinocytes are completed by a differentiation process that starts at the stratum basale and ends at the stratum corneum. Keratinocytes are polarized and show cohesion with the nearby epithelium. Therefore, keratinocytes are required to go through the partial epithelial-mesenchymal transition (EMT) to repair tissue damage [4]. EMT, also known as differentiation of epithelial cells into motile mesenchymal cells, is an integral part of wound healing and pathologically contributes to fibrosis and cancer progression [5].

When we evaluate the entire wound healing process at the molecular level, the major roles of many structural factors emerge. Vascular endothelial growth factor (VEGF) is a hemostatic agent responsible for initiating angiogenesis, which is necessary for repair of damaged tissue or organ [6]. Therefore, it has been shown in many studies that VEGF expression increases during the wound healing process [7,8]. With the rapid induction of angiogenesis, the initiation of the inflammatory process is regulated by nuclear factor kappa B (NfκB) and tumor necrosis factor-α (TNFα). The functional roles of E-Cadherin are known, especially in the migration of large epithelial layers of the skin [9]. It has been reported that the mechanism of action underlying cadherin’s remodeling of cell junctions is tyrosine phosphorylation [10]. In the phase of the proliferation, keratinocytes in the neoeipidermis release matrix metalloproteinases (MMPs) to aid their migration pathways [11]. MMPs, especially MMP-9 is vital for keratinocyte migration as they assist integrin receptor cleavage [4]. Also, at this phase, it has been shown that vimentin enables the union of focal adhesions formed in response to collagen binding by β1-integrin regulation [12]. Similarly, in another study, downregulation of E-Cadherin and Occludin-1 and overexpression of N-Cadherin and vimentin were shown by EMT induction [13]. In contrast, it has been shown that EMT, an important physiological process in wound healing, is suppressed by inhibition of the MAPK/ERK kinase pathway of Fibroblast growth factor-1 (FGF-1) [14].

In the treatment of the wound healing process, shortening the treatment period or accelerating the process is of great importance in terms of increasing the quality of life and patient compliance. In vitro and clinical studies have provided evidence that topically applied dexpanthenol (Dxp) promotes superficial and post-procedural wound healing [15]. On the other hand, recent findings confirmed that Dxp up-regulates genes critical to the healing process [16,17]. Although there are agents or adjuvants used in wound healing, the search for more effective and/or safe products continues. Silymarin (SM) is a polyphenolic flavonoid isolated from milk thistle (Silybum marianum (L) Gaertn) seeds [18]. The main component of SM, silybinin, is generally considered very safe [19]. Within this scope, in this study, we investigated the effects and possible effects of Silymarin (SM) obtained from Silybum marianum plant on
wound healing by comparing it with Dxp [20]. Anti-diabetic, cardioprotective, neuroprotective, antimicrobial, and antioxidant effects of SM have been demonstrated to date [21–26]. With its strong free radical scavenging and antioxidant properties, silymarin has been shown to significantly reduce burn-induced oxidative skin damage in rats [27]. Sharifi et al. reported that silymarin increased epithelialization and reduced inflammation in full thickness wounds. It has also been shown that it can significantly stimulate epithelialization and reduce inflammation [28]. However, although the UVB protective property of SM has been demonstrated, there are not enough studies on its effects on wound healing [29].

**Materials and Methods**

**Experimental Model and Protocols**

This study was performed after approval of the Ethical Animal Research Committee of Afyon Kocatepe University (AKUHADYEK-49533702/97). Four-week-old male *Wistar* rats were housed under temperature and humidity-controlled rooms (20–22°C) with a 12-h light-dark cycle. The animals were fed with a standard rodent chow diet that composed of starch, protein, fat, cellulose, standard vitamins, and salt mixture and water. After acclimation for one week, the rats (n=54) were randomly divided into three groups: Sham (n=18), Dexpantehol (Dxp; n=18), and Silymarin (SM; n=18). Backs of the rats were shaved and cleaned with 70% ethanol; a six mm punch biopsy instrument was used; two circular full-thickness skin wounds were taken from the back of the neck (1.5 cm) [30]. The day the wound was made is considered day 0. On days 5, 10, and 15, six animals in each group were randomly selected [28]. At the end of follow-up period, the rats were anesthetized with a mixture of ketamine–xylazine (100 and 10 mg/kg, respectively, *i.p.*) and scar tissues were taken all around from biopsy sites.

Silymarin has been supplied from Sigma-Aldrich, Germany and SM cream was prepared by dissolving excipients (15 g stearic acid, 5 g glycerin, 0.72 g potassium hydroxide, 79 g water, 0.1% sodium benzoate and 1% Tween 80°). SM was applied once daily for 15 days, in sufficient amounts to cover the entire wound. Dxp was applied as Bephanthol® cream which includes lanolin and Dxp (%5) from Bayer-Germany. Rats were treated with an equal volume of Dxp once daily for 15 days.

**Histopathological Evaluation**

Tissue samples for histopathological analysis were separately fixed in 10% formalin and embedded in paraffin blocks, which were processed histologically. Five μm thick samples were taken on classic slides and then deparaffinized. After standard Hematoxylin–Eosin (H-E) and Masson’s trichrome (MT) staining, the slices were examined with light microscopy (Nikon, Eclipse E600, Tokyo, Japan). HE staining was applied for routine histopathological evaluation. MT staining was used to determine degree of collagenization. Inflammation and collagen deposition were graded as: 0 (none), 1 (scant), 2 (moderate), and 3 (abundant). Epithelization was graded as either: 0 (none), 1 (partial), 2 (complete, but immature or thin), and 3 (complete and mature) [31]. Scored for immunohistochemical staining as follows: 0 for staining <1%, 1 for 1 to 25%, 2 for 26 to 50%, 3 for 51 to 75%, and 4 for >75% of the examined cells. Staining intensity was graded as follows: 0, negative staining; 1, weak staining; 2, moderate staining; 3, strong staining. The histological score (H-score) for each specimen was computed by the formula: H-score = proportion score × intensity score A total score of 0 to 12 was calculated and graded as negative (−, score: 0), weak (+, score: 1 to 4), moderate (+++, score: 5 to 8) or strong (+++, score: 9 to 12) [32].

**Immunohistochemistry**

The samples mounted on poly-L-lysine coated slides were deparaffinized, and then rehydrated in descending concentrations of ethanol. For antigen retrieval, the sections were incubated in citrate buffer (pH 6) at high temperature in microwave for 20 min and then soaked with 3% H₂O₂ and methanol mixture to eliminate peroxidase activity in tissue and finally incubated with primary antibodies for N-Cadherin (ab18203,1/200), E-Cadherin (ab76055,1/200) Vimentin (ab8978,1/200), FGF-1 (sc7910, 1/100), Occludin (ab216327,1/200), Claudin-1 (RB-
9209-RT), VEGF-A (ab52917,1/200), NfkB (ab16502,1/200), TNF (ab34674,1/200), and MMP-9 (ab76003, 1/200) at 4°C overnight. The next day, the slides were incubated with HRP secondary antibody at room temperature. The slides were developed with 3-amino-9- ethylcarbazole and counter stained with Mayer’s Hematoxylin. At the end, slides were mounted with water-based mounting medium. All the chemicals were purchased from Labvision Corp. (Fremont, CA, USA). The immunoreactivity of the antibodies was evaluated under light microscopy (Nikon, Eclipse E600, Tokyo, Japan).

Statistical Analysis
All data is represented as mean ± standard error of the mean (SEM) throughout the study. The data were analyzed with Kruskal-Wallis analysis and Dunn’s multiple comparison tests. Graphics were drawn with Graphpad Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA). Values of p<0.05 were considered as significant.

Results
Histopathological Results
As shown in Table 1 according to the Image 1; results of histopathological examination showed that, there were no epithelization findings on 5th day. However, epithelization accelerated in all groups at day 10 and day 15. Although this difference was more severe in Dxp and SM groups compared to the sham group, there was no significant difference between Dxp and SM treatment types. It was observed that inflammation developed on the 5th, 10th, and 15th days in all groups due to wound formation, and the inflammation decreased as time progressed. This reduction in inflammation during the process was higher in the Dxp and SM groups than in the sham group while no significant difference between Dxp and SM treatment types. Collagenization for tissue remodeling was observed to increase over time in all groups on the 5th, 10th, and 15th days. On the other hand, it is seen that this recovery process is faster in the Dxp and SM groups, and there is no difference between the treatment effects of the Dxp and SM groups.

Immunohistochemical Results
As shown in Figs.1a and b, E-Cadherin and Occludin HScores could not be calculated because there was no staining. However, it was observed that both protein levels increased significantly with Dxp and SM treatments on the 10th and 15th days compared to the sham (Image 2). In all time periods, Dxp and SM treatments were found to significantly reduce N-Cadherin and Vimentin HScores compared to Sham (Figs 1c and d). On the other hand, N-Cadherin values

<table>
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increased on the 10\textsuperscript{th} day compared to the 5\textsuperscript{th} day and normalized on the 15\textsuperscript{th} day (Image 2). While Vimentin levels did not change between conjugates in the process, HScore values of only the SM group decreased significantly. As shown in Fig. 1e, no differences between all groups on the 5\textsuperscript{th} day of FGF1 values. On the 10\textsuperscript{th} day, FGF1 HScore of Sham and Dxp groups were increased compared to 5\textsuperscript{th} day, significantly; but SM did not change. Moreover, while MMP-9 values in the Sham group did not change depend on the time, the Dxp and SM groups increased significantly on the 10\textsuperscript{th} and 15\textsuperscript{th} days (Image 2). However, no significant difference was found between Dxp and SM groups (Fig. 1f). No staining Claudin-1 on the 5\textsuperscript{th} day, therefore HScores did not calculate. But, on the 10\textsuperscript{th} day, Dxp and SM treatment were enhanced the Claudin-1 values compared to sham; there is no differences between all groups on the 15\textsuperscript{th} day.

![Image 1](image1.png)

**Image 1.** Histopathological features of the wound sections from Sham, Dxp, and SM groups on the 5\textsuperscript{th}, 10\textsuperscript{th}, and 15\textsuperscript{th} days. H-E staining (×200) shows epithelization and inflammation whereas MT (×40) staining shows collagenization of the wounds of the tissues.
VEGF-A values of the SM treated rats were increased significantly time dependent and compared to Sham, but no differences were found in all the groups (Fig. 1g). Additionally, in terms of NfκB values, no significant change was observed between groups and depending on the time (Fig. 1i). However, TNFα values were significantly decreased on the 15th day in SM group compared to Sham and 10th day (Fig. 1j).

**Discussion**

In a clinical study, SM was shown to accelerate the wound healing process by reducing inflammation in thermal injuries [35]. In another study on rats, SM was shown to modulate skin inflammation [36]. According to the general histopathological evaluations of this study, it is seen that SM contributes positively to wound healing by reducing inflammation, increasing epithelialization and collagenization. At this stage, the wound healing effect of SM is largely parallel to that of Dxp treatment. Favorable preclinical and clinical outcomes have been reported for FGF-1 in healing of dermal injuries and ulcers in both diabetic animal models and diabetic human patients [37]. Growth factor-based therapies can potentially accelerate the overall healing process compared to more passive-based therapies, and FGF-1 is an effective single therapeutic agent for dermal healing [14]. In our study, FGF-1 expressions increased at 10th day and tended to decrease at 15th day. It is thought that the reason for this is that the amount of FGF-1 increases during the wound healing period and starts to decrease after epithelialization is achieved. The low level of FGF1 in both Dxp and SM groups compared to the Sham group may be related to the acceleration of EMT process by Dxp and SM. Similarly, while VEGF-A is expected to induce angiogenesis necessary for the initiation of the hemostatic process [6,7]. This study results show that VEGF-A HScore values are hyperactivated by Dxp and SM. Vimentin is a type III intermediate filament found in mesenchymal cells of various tissue types during developmental stages and preserves cell and tissue integrity [12]. Vimentin
directly coordinates four cellular activities important in wound healing control; fibroblast proliferation, collagen deposition, keratinocyte differentiation and re-epithelialization. Loss of vimentin disrupts this coordination, leading to slow, weak and incomplete wound healing. Vimentin intermediate filaments induce changes in cell shape, adhesion, migration and signaling in EMT [5,13]. In our study, the increase in Vimentin expression on the 10th day seems to contribute to wound healing, and its decrease in the later period of wound healing seems to be related to the shortening of the EMT process. However, MMP-9, one of the most studied MMPs, is a type IV collagenase that is expressed by keratinocytes at the anterior end of the wound and is known to promote cell migration and re-epithelialization [16]. In normal tissue,

![Figure 1. Immunostaining HScore results of the E-Cadherin (a), Occludin (b), N-Cadherin (c), Vimentin (d), FGF1 (e), MMP-9 (f), Claudin-1 (g), VEGF-A (h), NfκB (i), and TNFα (j) proteins from Sham, Dxp, and SM groups on the 5th, 10th, and 15th days. δ shows significantly difference from the sham in the same time period (p<0.05); * shows significantly difference in the conjugates from the 5th day (p<0.05); # shows significantly difference in the conjugates from the 10th day (p<0.05).]
MMP-9 is expressed at basal levels but is rapidly up-regulated following injury. MMP-9 decreases as wounds heal [38]. If normal MMP-9 levels are suppressed, epithelization is delayed. If there is a persistent excess of MMP-9 in chronic wounds, it leads to impaired healing. Therefore, the balance of this bimodal MMP-9 action is critical to the epithelialization process [39]. In our study, while MMP-9 expressions were found to be higher on the 10th day of wound healing, a decrease was detected in the following days in the both Dxp and SM groups compared to Sham. These data indicate that our treatment agents accelerate epithelization.

It has been shown that silibinin as a natural polyphenolic flavonoid, enhanced in the breast cancer MDA-MB-231 cells E-Cadherin expression, while significantly reducing the levels of mesenchymal biomarkers such as N-Cadherin and Vimentin; as well as decreased expressions of migration-related proteins MMP-2 and MMP-9 [40]. Another study showed that silibinin counters radiation-induced invasive and migratory phenotype of prostate cancer cells and leading to inhibition of N-Cadherin and also reverses E-Cadherin downregulation [41]. In our study, it has been shown that SM contributes favorably to wound healing by reducing inflammation and increasing epithelialization and collagenization. In addition, our study results indicate that SM treatment shortens the EMT process with E-Cadherin induction and N-Cadherin suppression, in line with the literature. In guinea pigs, effects of SM were investigated on the alcohol-induced endotoxemia and results indicated that SM increased TNFα, Occludin, and Claudin mRNA expressions whereas it did not change the NfkB and levels [42]. Similarly, our findings showed that Occludin and Claudin-1 HScore values were enhanced. The dramatic increase in Occludin levels on the 15th day compared to the 10th day is that Occludin has an active role especially in the collagenization process. On the other hand, according to our study results, NfkB and TNFα levels did not change. These differences indicate that SM may have affected these parameters, especially while suppressing the inflammatory process. When the therapeutic effects of Dxp and SM are evaluated among all findings, it is seen that SM mimics the effects of Dxp to a great extent, and that SM is partially more effective than Dxp, especially in the processes of induction of epithelialization and suppression of inflammation.

**Conclusion**

EMT is an important process in wound healing. Prolongation of this process may also lead to extend the wound healing process and scar formation. Dxp-based creams, which are frequently used in daily life, contribute to wound healing processes. As a result, the cream prepared with SM kept the EMT process within certain limits and gave results similar to Dxp, which is used continuously in wound healing. This shows that the therapeutic potential of SM is promising today, when herbal medicines are being used frequently. In this context, more comprehensive studies in vitro and in vivo are needed. Longer study periods, larger sample sizes, and studies with different animal models will allow us to obtain clearer ideas about the effect of silymarin on wound healing.

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**Conflict of interest**

The authors declare no competing interests.

**Data availability statement**

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.
References


