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Topical application of silymarin enhances cutaneous wound healing in rats

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ABSTRACT

Wound healing in a short period with minimum side effects is one of the major goals of medical sciences. Silymarin, an extract from *Silybum marianum*, has been shown to have antioxidant and anti-inflammatory properties. This study investigates the wound healing activity of silymarin topical formulation in an in experimental model.

A 875 mm^2 (25 × 35 mm) full-thickness excision was made on the abdominal region of each rat by a surgical blade and the day on which the wound was made considered as day 0. Each rat was treated two times each day. On days 1,4, 8 and 12, the wound area was measured using precise caliber and camera imaging. On day 12, blood samples were collected for the analysis of antioxidant, malondialdehyde and estradiol levels. After 12 days of treatment, rats were sacrificed and abdominal region tissues used for histological analyses.

The study showed that topical application of silymarin on wound in rats improved wound healing correlating with less redness, exudates and swelling. Furthermore, in serum of rats treated with silymarin ointment improved antioxidant and estradiol levels, while decreased malondialdehyde levels, a marker of oxidative stress. Histological analyses showed also an improve of novel blood vessels. This effect on angiogenesis correlated with improve nitric oxide synthase expression and epithelial cells after treatment with silymarin.

Silymarin ointment represents a promising therapeutic agent for the treatment of wounds through its antioxidant and anti-inflammatory properties.

1. Introduction

Skin has an intricate structure composed of the epidermis and dermis, including the subcutaneous fat (Ahmad et al., 2013; Takeo et al., 2015). Wounds are physical injuries that are caused by skin opening and detachment. Wound healing is a well-orchestrated process, where numerous factors are activated or inhibited in a sequence of steps (Kasuya and Tokura, 2014) and it is necessary to resolve anatomical and functional disturbances of the skin (Murthy et al., 2013). Wound healing is a conserved evolutionary process among species and encompasses spatially and temporally overlapping processes including inflammation, blood clotting, and cellular proliferation, contraction of collagen and rearrangement of extracellular matrix (Fisher et al., 2013; Seifert et al., 2012; Takeo et al., 2015). The initial step is an inflammatory change with activation of innate immunity, which is followed by proliferation phase, including fibroplasia, angiogenesis and re-epithelialization (Kasuya and Tokura, 2014). Many types of cells migrate in the wound to perform repair processes. Skin fibroblasts are absorbed into the wound site togheter with neutrophilis and monocytes to begin the proliferation phase, secreting collagen and glycosaminoglycan. A prolonged inflammatory and oxidative stress process as well as pathogen infection may disrupt or delay the process of wound healing (Gupta et al., 2002; Sharifi et al., 2013). In particular, reactive oxygen species (ROS) that are produced in response to cutaneous injury may impair the healing process (Sharifi et al., 2012). Regulation of inflammation and oxidation are important in the process of wound healing.

For these reasons a compound with an appropriate balance between antioxidant and anti-inflammatory property could represent a promising drug able to accelerate the wound healing process. Among the anti-inflammatory and antioxidant compounds, silymarin plays an emerging role (Sharifi et al., 2012; Varoglu et al., 2010).

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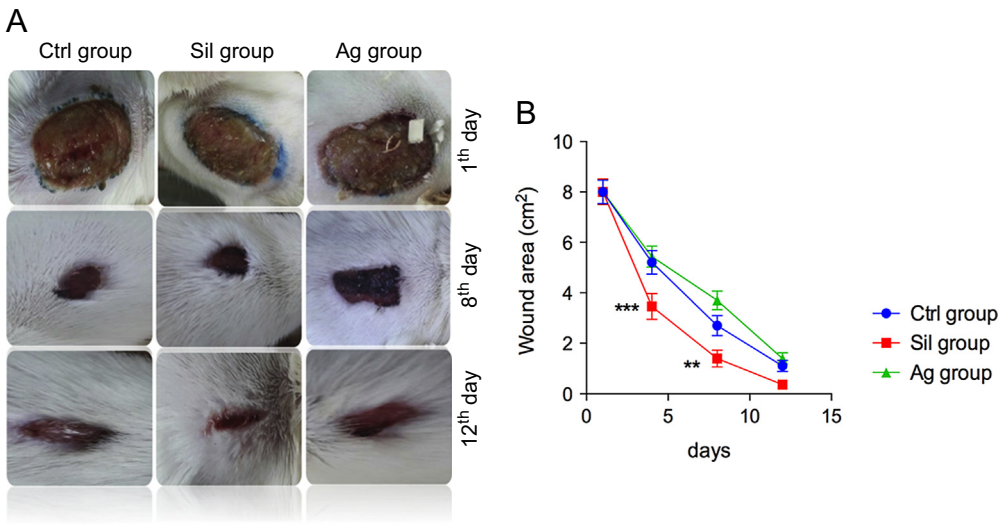

Fig. 1. Effect of silymarin oinment on wound healing. (A) Representative images of wound healing after 1,4, 8 and 12 days in the group treated with base of ointment (Ctrl group), silymarin ointment (Sil group) or silver ointment (Ag group). (B) Mean wound area (cm^2) for the different treatment groups applied for 12 days. The horizontal axis shows days (1–12) and the vertical axis shows the wound area (cm^2). Bars represent one standard deviation of the mean. Two-way ANOVA followed Dunnett'smultiple comparison post-test $^{**}p < .01$, $^{***}p < .001$.

Silymarin is a flavonoid obtained from *Silybum marianum (L.) Gaertn.* (Milk thistle plant) (Dixit et al., 2007; Luper, 1998). The main role of this compound is liver protection. Silymarin improves hepatocytes rearrangement, prevents lipid peroxidation, and through its antioxidant property, also helps to detoxify the liver (Abdel-Moneim et al., 2015). Moreover, silymarin decreases inflammatory processes and inhibits the synthesis of leukotrienes and prostaglandines (Surai, 2015). Silymarin have a positive anti-inflammatory and antioxidant effect on burns and wound healing (Toklu et al., 2007). Studies have shown that silymarin plays an important role in the treatment of inflammation by controlling the spread of cytokines and the infiltration of neutrophils (Delfan et al., 2014; Mady et al., 2016).

The aim of this study is to evaluate the effect of silymarin ointment in *in vivo* model of wound.

2. Materials and methods

2.1. Chemicals and reagents

Sodium carbonate, ascorbic acid, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 4, and 6-Tris (1-pyridyl)-5-triazin (TPTZ) were purchased from Merck Chemicals (Darmstadt, Germany). Methanol, Acetonitrile and other solvents used for the analyses were purchased from Daejung, Korea. Silymarin were purchased from Merck (City, Nation), and submitted to exhaustive HPLC analysis prior to use to verify their purity. DMEM, paraformaldehyde and hematoxylin were purchased from Sigma (City, Nationality).

2.2. Wound healing animal model

This study was performed on male Wistar rats, weighing 100–110 g and obtained from animal house of Babol University, Medical Sciences (Babol, Iran). The animals were kept under light–dark cycle (12 h − 12 h) and under controlled temperature of 24 ± 3 °C and moisture 50 ± 5%. Animals had free access to tap water and food. The experimental procedures were performed in accordance with the guidelines for care and use of laboratory animals and approved by Ethics Committee of Babol University of Medical Sciences, Number of Ethics Committee: (MUBABOL.HRI.REC.1396.129).

The animals were anesthetized with intraperitoneal injection of 7 mg/kg xylazine hydrochloride (Alfasan, Woerden, Holand) and 70 mg/kg ketamine hydrochloride (Alfasan, Woerden, Holand). Their abdominal region (back of the body) were shaved and cleaned with 70% ethanol. A 875 mm^2 (25 × 35 mm) full-thickness excision was made on the abdominal region of each rat by a surgical blade and the day on which the wound was made considered as day 0. A total of 18 rats were randomly divided into three groups of equal number (vehicle, treatment and positive control groups). Rats in group I (n = 6) were treated with base of ointment (eucerin). Rats in group II (n = 6) were treated with silymarin ointment. Silymarin ointment (2%) was prepared by dissolving 500 mg silymarin in 10 g eucerin. Rats in group III (n = 6) were treated with silver ointment (1%). Each rat was treated two times each day. On days 1,4, 8 and 12, the wound area was measured using precise caliber and camera imaging. On days 12, blood samples were collected for the analysis of antioxidant factors using ferric reducing/antioxidant power assay (FRAP), malondialdehyde and estradiol levels. After 12 days of treatment, the rats were sacrificed with giyotin according to the guidelines of the Committee on Animal Research and Ethics then abdominal region tissues used for histological analyses.

2.3. Determination of ferric reducing/antioxidant power assay (FRAP) in serum

FRAP test was performed suing TPTZ (2, 4, 6-tripyridyl-s-triazine). This method is based on the measures of antioxidant potential in samples through the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). The FRAP solution contains 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl and 20 mM iron chloride solution (III) with ratios of 1–1–10. The FRAP solution must be prepared freshly always. In this method, 100 μl diluted serum was mixed with 4.1 ml FRAP solution and the absorbance was read at a wavelength of 593 nm after 20 min.

2.4. Malondialdehyde content in serum

Malondialdehyde (MDA), a marker for oxidative stress (Casini et al., 1986) in serum was measured by the method of Casini (Casini et al., 1986) in serum. The serum sample was added with 10% ice-cold trichloroacetic acid in test tubes.This mixture was vortexed and centrifuged for 15 min at 3000 rpm. Then 1.5 mL of the supernatant was centrifuged 10 min at room temperature at 3000 rpm. 100 μl of supernatant was added with equal volume of thiobarbituric acid 0.67% (*w*/*v*). The absorbance of each sample was read at 535 nm by spectrophotometric method (Kazemi et al., 2012).

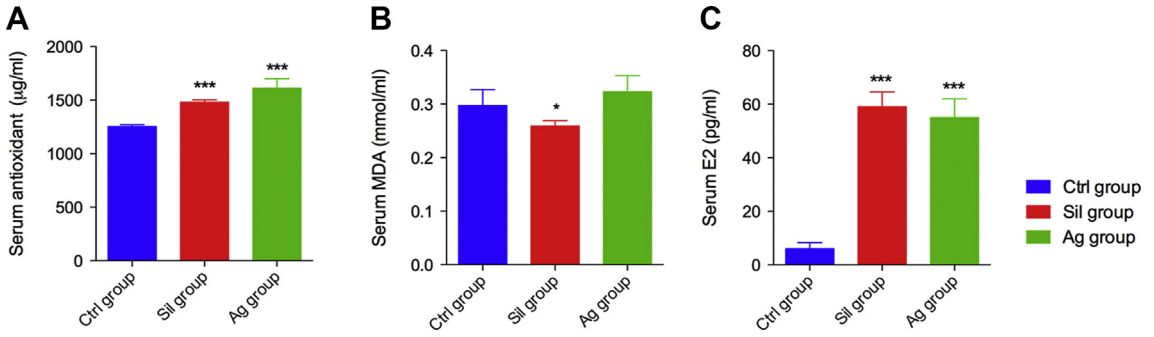

Fig. 2. Effect of silymarin oinment on serum markers. (A) Mean of antioxidant levels in serum of each group of animals. (B) Mean of MDA levels in serum of each group of animals. (C) Mean of estradiol E2 levels in serum of each group of animals, One-way ANOVA followed Dunnett'smultiple comparison post-test $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$.

2.5. Estradiol content in serum

Estradiol (E2) was measured by Elisa Microplate (Thermo Scientific, USA) following the manufacturer's instructions in serum of all animals at day 12. The absorbance was read at 450 nm by microplate reader (Bio Tek, Modular ELX808IU, USA).

2.6. Histological and immunohistochemical analyses

Tissue samples for histopathological analysis were collected from the wound-healing site when the rats were sacrificed (12 days after treatment). The samples were fixed with 10% formalin, washed in alcohol several times. Serial sections of 5 µm were prepared, and stained with Hematoxylin and Eosin (H&E) for routine histopathological evaluation. Furthermore, the sections were evaluated in terms of nitrogen free radicals using immunohistochemistry (Anti-iNOS) test to compare the histological changes of the stained sections. in the sections were also evaluated nitric oxide synthase (NOS) expression (Tajik et al., 2016).

2.7. Statistical analysis

Data were presented as mean ± standard deviation. The results were analyzed using Two-way or one-way ANOVA followed Dunnett's multiple comparison post-test. *p*-Values of less than 0.05 were considered statistically significant ($^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$).

3. Results

3.1. Effect of silymarin on wound healing

The macroscopic wound assessment was performed 1, 4, 8 and 12 days with caliber (Fig. 1A). On the first day of the experiment, all wounds were open. After 8 days, the wounds were still open with a slight contraction of all wounds and the group treated with silymarin showed an improve wound healing. After 8 and 12 days, the group treated with silymarin showed a statistically significant ($^{***}p < .001$ and $^{**}p < .01$, respectively) improved of wound healing correlated with less redness, exudates and swelling compared to control group (Fig. 1B).

3.2. Effect of silymarin on serum oxidative stress marker and estradiol

As shown in Fig. 2, when the rats were treated with silymarin, antioxidant levels improved in serum (Fig. 2A) while MDA levels, a marker of oxidative stress, decreased (Fig. 2B) both in statistically significantly ($p < .05$) way compared to control group.

Furthermore, we evaluated in serum of all groups of animal's estradiol (E2) levels as index of re-epithelialization. As shown in Fig. 2C, estradiol levels were significantly improved ($p < .001$) in serum of rats treated with Silymarin for 12 days compared to control group.

3.3. Effect of silymarin on blood vessel formation

H&E-stained sections of collected tissue on day 12 were examined after treatment in terms of new blood vessel formation and new epithelial cells. The images revealed that animals treated with silymarin showed more density of blood vessels compared to control group (Fig. 3A). The improve of new blood vessel was observed also in rats treated with silver ointment. Moreover, rats treated with silymarin showed an improved number of epithelial cells compared to control (Fig. 3B).

Finally, we evaluated the expression of iNOS, as index of angiogenesis, in the slide section of all group of animals. As shown in Fig. 3C, when rats were treated with silymarin, iNOS levels improved in cytoplasm of stained cells compared to control group Table 1.

4. Discussion

Wound healing in a short period with minimum side effects is one of the major goals of medical sciences. Nowadays, research individuated a

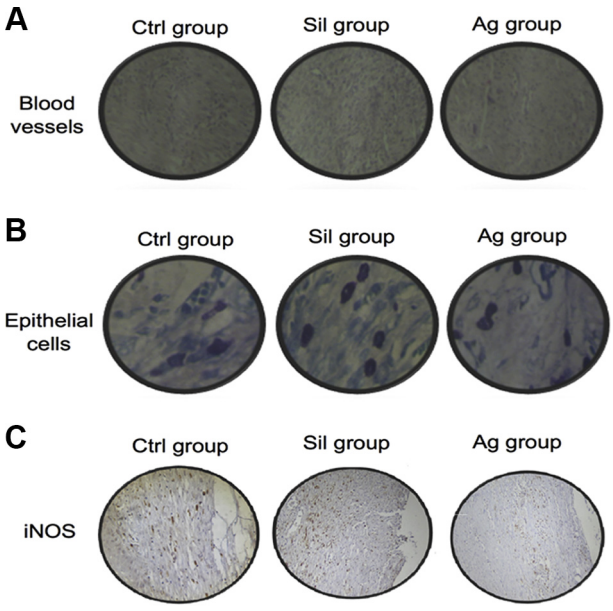

Fig. 3. Effect of silymarin on blood vessel formation on day 12 after treatmentwith base of ointment (Ctrl group), silymarin ointment (Sil group) or silver ointment (Ag group). (A) Representative images of new blood vessel formation. (B) Representative images of new epithelial vessels. (C) Representative images of NOS levels in cytoplasm of stained cells.

Table 1
Percentage of cytoplasm staining of epithelium cells in mice.

Group	<0.25%	>0.25%
A	4	2
B	3	3
C	1	5

A = Ctrl, B = Silver, C = Silymarin.

large number of wound healing drugs, most of which were natural compounds, but none of them represents the ideal drug (Zielins et al., 2015).

Regulation of inflammation and oxidation are important in the process of wound healing. Production of free radicals in response to cutaneous injury, may delay the healing process through the destruction of lipids, proteins and extracellular matrix elements (Russo et al., 2002) as well as inflammatory process.

Antioxidant and anti-inflammatory activity of silymarin was previously demonstrated *in vitro* and *in vivo* research (Surai, 2015) and we showed that treatment with silymarin improves wound healing in an experimental model.

In this study, silymarin was used as an ointment formulation for wound healing in an *in vivo* model for the first time. Topical application of silymarin in rats improved wound healing correlating with less redness, exudates and swelling. Furthermore, in serum of rats treated with silymarin ointment antioxidant and estradiol levels improved, while MDA levels, a marker of oxidative stress, decreased. Histological analyses showed also an improve of novel blood vessel correlated with an improved NOS expression and epithelial cells after treatment with silymarin.

The effects of wound treatment with silymarin ointment is in line with data reported by Mady and collaborators (2016) on eczema in human patients after topical application (Mady et al., 2016).

In a study entitled "Modulation of cutaneous ulcer treatment by silymarin" by A.Oryan on 85 albino mice in 2012, it was concluded that low doses of oral silymarin reduces lymphocytes and increases the number of fibrocytes in the early stages of wound healing, and high doses of silymarin reduce lymphocytes and macrophages and increase the number of fibrocytes in the secondary stages of wound healing. Therefore, topical application of silymarin may improve morphology, and biochemical and biomechanical properties of the wound (Oryan et al., 2012).

Also topical application of a *Silybum marianum* extract on cutaneous ulcer in albino mice improved epithelialization and decreased inflammation in deep wounds in mice (Sharifi et al., 2012). Moreover, our data are in agreement with the healing effect by silymarin on acetic acid induced large intestine ulcer and inflammation in Balb/C lab mice (Takhshid et al., 2011).

The levels of estradiol were significantly higher in the group treated with silymarin ointment than in the control group. This improve corroborates with the evidence that high levels of estradiol lead to the production and release of heparin-binding-epidermal growth factor (HB-EGF) by keratinocytes, that contributes to wound closure increasing re-epithelialization in skin ulcers (Luper, 1998). Estradiol may help wound healing by inducing also the production of multiple growth factors in various cell types, including neural growth factor and vascular endothelial growth factor in macrophages (Kanda and Watanabe, 2002; Kanda and Watanabe, 2003).

5. Conclusion

Silymarin not only increment dermal thickness and epidermal, but also increment the size and the number of restores collagen fiber size and hair follicles. In conclusion, silymarin ointment represents a promising therapeutic agent for the topical treatment of wounds through its antioxidation and anti-inflammation propriety.

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Declarations of Competing Interest

None.

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