

Effect of topical Red grape seed hydroethanol extract on burn wound healing in rats

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Abstract: Grapeseed oil is a rich source of proanthocyanidins (procyanidins). Mixtures of procyanidins are referred to as oligomeric proanthocyanidin (OPC) complexes. The antioxidant properties of OPCs have made products containing these extracts candidate therapies for a wide range of human disease. The second degree burns are probably one of the most frequent occurrences in homes or countries that need improvement of treatment. In this study, we compare the effects of 1.5% and 3% grape seed hydroethanol extract creams with Silver sulfadiazine cream (SSD) for healing of burn wounds in rats. Seventy two adult male Wistar rat was divided into four groups. A full-thickness second-degree skin burns created by Brass rod with a circular end of 15mm diameter, on the backs of all rats. All rats were divided randomly into four groups of twelve rats each. Group 1, animals received combination Eucerin (75%) and Vaseline (25%) as a base formulation of the cream; Group 2, animals treated with Sulfadiazine cream (SSD) and 3rd and 4th group, applied respectively, with the 1.5g and 3g of red grape seed hydroethanolic extract mixed with a base (GSC). The percentage of burn wound healing was monitored on Days 3, 6, 9, 12, 15 and 18 and histologically evaluation was carried out on the samples on 3th, 9th and 18th days after surgery. The percentage wound size result showed that there were significant differences ($P < 0.05$) between SSD and GSC 1.5% groups compared to the control group. The histological results show that groups SSD and GSC 1.5% served to accelerate the wound healing process and specifically increased neovascularization, fibroblast migration and epithelialization in the treatment groups compared to the control group. Thus, this study demonstrates that topical use of grape seed hydroethanol extract special creams 1.5% may be effective in stimulating the enclosure of burn wounds.

Key word: wound healing, red grape seed, second degree burns, cream, Wistar rat.

Introduction

Grape is a type of fruits, which typically an ellipsoid or spherical shape is drawn, that has a long history. 6,000–8,000 years ago in the Near East, cultivation of the domesticated grape began (1). Grapes can be many different colors, including two main color red and white (2), and other colors yellow, crimson, orange, dark blue, black, green and pink. Anthocyanins are pigments in the skin of grapes; the different colors are red grapes (2). Nowadays, European grapes (*Vitis vinifera*), North American grapes (*Vitis labrusca* and *Vitis rotundifolia*) and French hybrids are the main species of grapes (3) and a type of grape that is cultivated in Iran, is Grape *Vitis vinifera*, is Vitaceae family. Grape seed oil contains high concentration of vitamin E, linoleic acid, oligomeric proanthocyanidin complexes (OPCs) and phenolic compounds such as flavonoids, phenolic acids and antioxidants (4) stilbenes and anthocyanins (5). These compounds have many biological activities and may

help prevent or relieve symptoms of certain conditions, include inhibiting some degenerative diseases such as cardiovascular diseases(6), certain types of cancers(7), anti-inflammation and wound healing (8), antimicrobial and Alzheimer's disease, age-related cognitive decline, and diabetes properties(3). Most evidence of activity derives from in vitro and animal studies for oligomeric proanthocyanidin complexes or grape seed oil; however, some clinical studies are also available. The stilbene Resveratrol (3, 4', 5 trihydroxystilbene) has also been the focus of much investigation and exhibits anti-inflammatory, antithrombotic, anti-carcinogenic and anti-bacterial activities, but it is uncertain whether significant amounts are present in the seeds and oil.

Compounds of silver, especially silver sulfadiazine cream (SSD) is one of the most widely used topical medications used to treat burn wounds due to the antibacterial effects that have been used for centuries. SSD cream, while being effective causes some systemic complications, including neutropenia, erythema multiform, crystal Luria and methemoglobinemia(9).

Now, according to two major factors causing impaired wound healing (free radicals and oxidative stress) (10) on the one hand, and the presence of phenolic compounds and antioxidant properties of grape seed extract, the aim of this study was to compare the effects of Grape seed hydroethanol extract cream dressing, silver sulfadiazine cream and Eucerin cream on clinical and histological healing rates of skin burn wounds in a rat model.

Material and Methods

Plant material and oil preparation

The fresh Red grape was collected locally during July 2011 (summer season), in Gardens near the city of Urmia, West Azerbaijan province, Iran (latitude: 37° 34', longitude: 44° 58'); and identified by the Department of Botany Sciences, the Urmia researches Agricultural and Natural resource center. Grape seeds were separated from the pulp and dried in the shade at room temperature. The seeds then were finely powdered using a grinder. Then 100g of the mentioned plant powder was suspended in 300ml of hydroethanolic solution (50:50) for 72 h at room temperature. The mixture was filtered using a fine muslin cloth followed by filter paper (Whatman No 1). The filtrated extract was evaporated under vacuum below 40°C. Then a final yield (10.3g) was kept at 0 °C until it was used in the experiment(8).

Experimental animals

Animal studies were conducted after the obtaining ethical approval from the Islamic Azad University (Urmia branch) Research Committee. Healthy seventy two male Wistar rats weighing (185-200g) approximately nine weeks of age were obtained from the experimental animal house; veterinary colleges of Urmia University were used for the study. All rats were divided randomly into four groups of eighteen rats each. The animals were left in separate cages for five days at room conditions for acclimatization. Animal houses were in standard environmental conditions of temperature (22 ± 3°C), humidity (60 ± 5%), and a 12h light/dark cycle. The animals were maintained on a standard pellet diet and tap water. All rats were closely observed for any infection, and if they showed signs of infection were separated, excluded from the study and replaced.

Formulation of topical application forms

After creating of burned wound, all rats randomly were labeled by a non-toxic color and divided into three groups. Group 1, animals received combination Eucerin (75%) and Vaseline (25%) as a base formulation of the cream; Group 2, animals treated with silver sulfadiazine cream (SSD) (11) and 3rd and 4th group, applied respectively, with the 1.5g and 3g of red grape seed hydroethanolic extract mixed with base formulation as a cream (GSC). The cream (0.5g) was topically applied twice a day, starting from the day of operation, on the burned wound area until the wound healed completely. All rats were monitored for any wound fluid or any evidence of infection or other abnormalities, until complete epithelialization.

Burn wounds induction

The animals were anesthetized with an intra-peritoneal injection of Xylazine 2% (10 mg/kg/IP; Alfasan International, Woerden, Holland) and Ketamine 5% (70 mg/kg/IP; Alfasan International, Woerden, Holland) and the hair on the back was clipped with electric clippers. Animals were subjected to full-thickness second-degree skin burns by Brass rod with a circular end of 15mm diameter, was immersed in boiling water (95 °C) for 15s, until thermal equilibrium was reached, and immediately it was placed on the back of the rats for 6 Sec

without applying pressure(12, 13). After this, 0.2 ml saline solution was administered intraperitoneal injection to each animal, and placed in a separate cage for full recovery from anesthesia before being returned to holding rooms.

Burn wound area assessment

The wound area was measured by immediate placing of a transparent paper over the wound and tracing it out; area of this impression was calculated using the graph sheet. The wound healing percentage was calculated by Walker formula after measuring the wound size (14). The percentage of wound healing was computed at the beginning of experiments and on days 3, 6, 9, 12, 15 and 18 days post-test. Percentage of wound size = Wound area on day X / Wound area on day zero × 100.

Percentage of wound healing = 100 - Percentage of wound size.

Burn wounds histological assessment

In order to, specimens of skin were taken on the 5th, 10th and 18th days after surgery, with the rats under general anesthesia(13). Sample tissues, excised along with 1 to 2 mm surrounding normal skin and in a depth of approximately 3mm, were pinned on a flat cork surface and fixed in neutral-buffered formalin 10%. Then the sample tissues were routinely processed, paraffin wax embedded, sectioned at 5 µm, and stained with hematoxylin-eosin (H&E) stain. Stained sections were then microscopically (by the light microscope, Olympus CX31RBSF attached cameraman) evaluated to assess the predominant stage of wound healing. Three parallel sections were obtained from each specimen. Following factors such as poly morph nuclear cell (PMN) and mononuclear cells (MNC) migration, angiogenesis (the number of new blood vessels and capillary buds), Fibroblast migration and epithelialization qualitatively evaluated, scored and according to the criteria (Table 1).

Table 1: Histological scoring criteria for burn wound.

Score	Poly morphonuclear cells	Mononuclear cells	New Vessels	Fibroblast migration	Epithelialization
-	Absence	Absence	Absence	Absence	Thickening of the cutting edge
+	Less (surrounding tissue)	Less (surrounding tissue)	Less (subcutaneous tissue)	Less (granulation tissue)	Cell migration is less than 50%
++	Mild (granulation tissue)	Mild (granulation tissue)	Mild (granulation tissue)	Mild (granulation tissue)	Cell migration is less than 75%
+++	Medium (granulation tissue)	Medium (granulation tissue)	Medium (granulation tissue)	Medium (granulation tissue)	Cell migration is 100%
++++	High (granulation tissue)	High (granulation tissue)	High (granulation tissue)	High (granulation tissue)	Complete epithelial cells Bridging
+++++	Very high (granulation tissue)	Very high (granulation tissue)	Very high (granulation tissue)	Very high (granulation tissue)	Corneous layer formation

Evaluation and measurement of wound healing

The measurements of the wound areas in animals of all groups were taken by using transparencies in days 3, 6, 9, 12, 15 and 18 of experiments. The wound areas of all groups were recorded and measured on graph paper. The percentage of wound healing was calculated by the Walker formula after measuring the wound area (14). Percentage of wound area = $\frac{1}{4}$ Wound areas in day X / Wound area in the first. Percentage of wound healing = $\frac{1}{4}$ 100% percentage of the wound area.

Statistical analysis

The means of wound area measurements, between test and control groups were compared using one way ANOVA descriptive texts. The Epithelialization period between the test and control groups were compared using independent t tests. Data were analyzed using SPSS (Version 18) and P value was set $p < 0.05$ for all analyses.

Results

Histopathological study

Following histopathological examination, the qualitative results of the sample tissue slide in 3th, 9th and 18th days, which stained with haematoxylin – eosin stain, evaluated, scored and presented in Table 3.

According to the findings contained in Table 3, the PMN scores increased on day three in all groups, especially the burned wounds in the control group, thereafter; it was decreased (Table, 3). The MNC score has increased in all groups, especially the burned wounds treated with the SSD and GSC 1.5%, of the day 3th, thereafter; it was decreased (Table, 3). According to results of microscopic slides, the new vessels, scores on days 3rd were increased in all treatment groups, especially the burned wounds treated with the SSD and 1.5% GSC creams, thereafter; it was decreased (table 3). The fibroblast migration score was increased until day nine in all burned wounds, markedly increased in the burned wounds treated with the SSD and GSC 1.5% creams (Table 3). The epithelialization and the epithelial bridging score increased on day nine in all wounds, and were markedly increased in eight days after burn induction, was higher scores observed in the burned wounds treated with the SSD and 1.5% GSC creams (Table 3).

Table 2: Comparison of percentage wound size in control group, SSD group and GSO group. Data are expressed as mean $\pm$ SEM. There are significant differences between the control group vs. SSD and GSO groups.

Groups	Wound area (mm ²) $\pm$ S.D.					
	3	6	9	12	15	18
Control	146.2 $\pm$ 8.2 (4%)	124.52 $\pm$ 10.1 (25%)	99.22 $\pm$ 7.3 (41%)	63.52 $\pm$ 4.5 (61%)	36.12 $\pm$ 5.3 (77%)	19.82 $\pm$ 1.3 (88%)
SSD	125.82 $\pm$ 10.8 (18%)**	98.82 $\pm$ 7.4 (41%)**	61.12 $\pm$ 4.3 (63%)**	38.92 $\pm$ 7.8 (75%)**	12.02 $\pm$ 1.3 (93%)**	0.72 $\pm$ 0.7 (100%)**
GSC 1.5%	130.52 $\pm$ 12.2 (16%)*	102.12 $\pm$ 7.7 (37%)*	67.92 $\pm$ 4.4 (60%)*	44.22 $\pm$ 5.1 (73%)*	16.92 $\pm$ 2.7 (90%)*	3.12 $\pm$ 0.8 (98%)*
GSC 3%	136.12 $\pm$ 11.1 (14%)	110.2 $\pm$ 5.2 (34%)	74.12 $\pm$ 5.7 (58%)*	53.92 $\pm$ 9.2 (68%)*	27.52 $\pm$ 5.6 (88%)*	8.12 $\pm$ 0.9 (95%)*

Valued are expressed as mean $\pm$ S.D. for 6 rats in each group. ** P <0.01 and * P <0.05 vs Control. Percentages of contraction record in parentheses.

Table 3: Comparison of epithelialization process, as you see above control group has significant differences with both SSD and GSO treated groups, also there are other significant differences between SSD treated group and GSO treated group.

Group	The parameters					
	Days	PMN	MNC	NC	Fm	RE
Control	3	+++++	++	++	+	-
	9	+++	+++	+++	+++	+
	18	+	+	+	+	+++
SSD	3	+++	+++++	+++++	+	-
	9	+	++	++	+++++	++
	18	-	-	+	++	+++++
GSO 1.5%	3	+++	+++	+++	++	-
	9	++	++	++	+++++	++
	18	-	-	+	+	+++++
GSO 3%	3	+++++	+++	+++	++	-
	9	++	++	++	+++++	++
	18	-	-	+	+	+++++

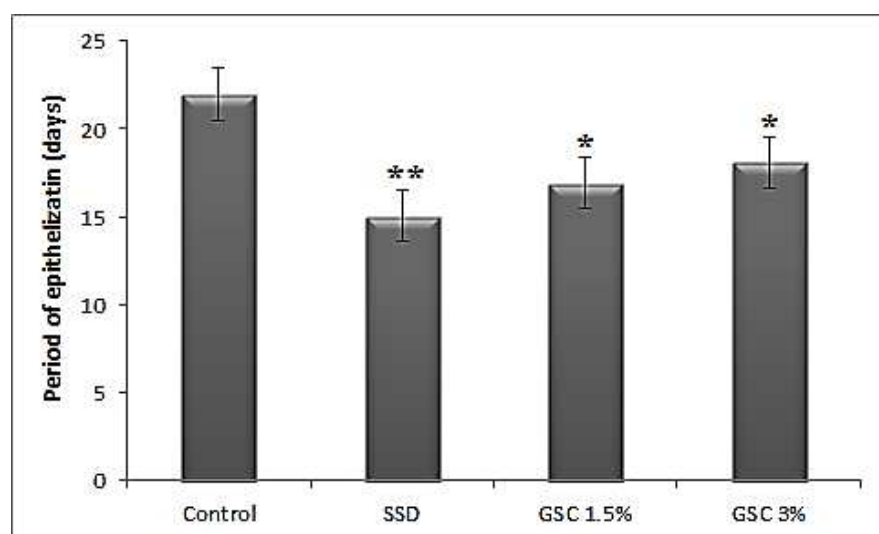
Table 3: Histological evaluation of Red grape seed extract cream on wound healing process in different groups of treatment in rat. Hematoxylin and eosin stained sections were scored as absent (-), present (+), mild (++), moderate (+++), severe (++++), and very severe (+++++) for PMN: Poly morphonuclear cells, MNC: Mononuclear cells, NC: New capillary buds, Fm: Fibroblast migration, RE: re-epithelialization.

Burned wound measurement

The wound area measured every third day after burn wound induction in all groups, and shown in table 1. Significantly higher reduce of wound size, was observed in the group treated with silver sulfadiazine cream ($p < 0.01$), and follow it, in treated groups with Red grape seed extract, especially in lower dose (1.5%), compared with control group ($p < 0.05$). In comparison, for example wound area on day 18 after burn wound induction, showed in control group $19.82 \pm 1.3(88\%)$, SSD group $0.72 \pm 0.7(100\%)$, 1.5%GSC $3.12 \pm 0.8(98\%)$, 3%GSC group $8.12 \pm 0.9(95\%)$. Overall, the contraction of the wound was in the order of SSD group-treated $>$ 1.5%GSC-treated $>$ 3%GSC -treated $>$ control.

When the wound is covered with epithelial tissue, refers to epithelialization time. Wounds treated with SSD, GSC1.5%, GSC 3% and control groups, found epithelial tissue at day 15.1, 16.9, 17.1 and 21.97 after burn induction respectively (**figure 1**).

Figure 1: Effect of extracts on days of epithelialization



Valued are expressed as mean $\pm$ S.D. for 6 rats in each group. ** $P < 0.01$ and * $P < 0.05$ vs Control.

Discussion

The skin is one of the largest organs in the body, which injures inescapably during the life; these often arise due to physical or chemical injury or microbial infections. Following burn injury the organ, and the entry of pathogens into the body, it is prone to infection. To prevent this from happening, the skin wound healing process begins immediately (15). Herbal medicine has become an integral part of standard health care, based on a combination of time honored traditional usage and ongoing scientific research. Medicinal plants are coming into prominence because of the overuse of conventional medicines such as antibiotics, which has resulted in the development of resistance in many infectious organisms. Thus, herbal preparations can be more effective than conventional medicines and their non-toxic nature means that they can be administered over long periods(15, 16).

In the process of wound healing, inflammation is an important part of the acute response, results in a coordinated recruitment of PMN cells (neutrophils) at the wound site(16). These cells are able to produce active oxygen radical, which is critical for defense against bacteria and other invasive pathogens(17). Macrophages are also involved in the production of oxidants at the wound site which builds up a long-term response to injured cells following the acute response(18). Wound-related non-phagocytic NAD (p) H oxidase or Nox dependent mechanism. Superoxide anion radical is rapidly converted to membrane permeable form, H_2O_2 , by superoxide dismutase activity or even spontaneously. Release of H_2O_2 may promote formation of other oxidant species, including hypochloric acid, chloramines, aldehydes, etc. Taken together, this suggests that the wound site is rich in both oxygen- and nitrogen -centered reactive species along with their derivatives (e.g. H_2O_2 , $O^{\cdot-}$, $^{\cdot}NO$, peroxynitrite, HOCl and chloramine) mostly contribute by neutrophils and macrophages(17). Grape seed extract, contains proanthocyanidins or condensed tannins. These are a group of biologically active polyphenolic bioflavonoids that are synthesized by many plants(3, 8). Proanthocyanidins and other tannins are known to facilitate wound healing (19, 20). The mode of action, however, remained unclear. The proanthocyanidinthe

existing at grape seed extract has been reported to have various clinically relevant redox-active properties(21, 22)In previous reports, shown that other types of inflammation such as silica or bleomycin-induced pulmonary fibrosis and rat paw edema may be prevented by GSE(23, 24).Oxidants present at the wound site arethought to support wound repair (25). A few studies suggest that GSE treatment was associated with enhanced tissue oxidation at thewound site. While it is known that under certain conditions GSE may demonstrate potentantioxidant properties, it must be consideredthat oxidative modification of antioxidants mayresult in the formation of potent oxidants. Tannins and tannic acid are an integral component of grape seed (26). Tannic acid is known to be capable of generating hydroxyl radicals. The radical structures obtained after oxidation of flavon (ol) s and proanthocyanidins have been characterized(27). The current study supports the idea thatthe topical application of GSC represents afeasible and productive approach to enhancedermal wound healing. We found that topical use of grape seed hydroethanolic extract could increase the cell mitosis and proliferation, therefore, cause earlier epithelialization in comparison with the control group. Also, topical use of grapes seed hydroethanolic extractcauseddecreases in healing period and according to Table 2.We found significant differences between grape seed hydroethanolic extract cream 1.5% and SSD groups vs. Control group.

In conclusion, the current study revealed that burn wounds treated with grape seed hydroethanolic extract, as a topical applicationto burn wounds, can significantly accelerate the wound healing process.

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