Original Article

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Histopathological Evaluation of Hydroalcoholic Extraction of Capparisspinosa on the Oral Wound Healing in Rats

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ABSTRACT

BACKGROUND AND OBJECTIVE: Many factors affect the speed and quality of wound healing. One of the purposes of medical sciences is wound healing in a short time with reduced side effects, since the effect and mechanism of action of Capers extract is not known on oral wound healing, this study was conducted to investigate the effect of the extract of this plant for the first time in oral wound healing in mice.

METHODS: Thirty-six adult male wistar rats weighing approximately 150-200 g each were used in the present study. We have 2 main groups, first group sacrificed at 5 days and second group at 10 days. Each of the groups was randomly subdivided into three subgroups as following: 1.rats treated with 200 mg/kg dosage, 2.rats treated with 300 mg/kg dosage, 3. Control groups (treated with distilled water), and each group contains 6 rats. After anesthesia wound was placed on the right side of dorsal surface of the tongue and the time of sacrifice histopathological examination, morphometry and immunohistochemistry was performed.

FINDINGS: After 5 days; Histopathological studies showed inflammatory cells (mast cells) count in treatment group with 200 mg/kg extraction dosage and blood vessels count in treated groups with 300 mg/kg was significantly higher than control groups. Result showed new epithelium is thicker in treated groups is higher compare to control group but it was not statistically significant although papillae thickness was significantly higher and lamina properia was lower in treatment groups. Results from immunohistochemistry showed significant lower nitric oxide synthase in treated group with 200mg/kg. Results after 10 days: results showed no significant results.

CONCLUSION: Capers extract by increasing the thickness of the epithelium papillae, reducing the thickness of the lining, increasing the number of blood vessels, increasing the number of mast cells and reduced expression of Nitric oxide synthases (INOS) can be involved in wound healing, oral rats.

KEY WORDS: *Wound Healing, Capers Extract, Rats, Mast Cells, Eosinophil.*

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Introduction

Wound healing which is also called healing means rebuilding of structure and function of tissue after injury. Wounds healing institution stated more precise definition as "healing of anatomical and functional tissue injury" (1). Many factors are involved in the speed and quality of wound healing including the size of the wound, the blood supply, foreign bodies and microorganisms, age, nutritional status and health of the patient (2). Wound healing is a complex process that started in response to wounds and damaged tissue and continue to reform faster and occurs by a cascade of cellular and molecular events and includes hemostasis, inflammation, repair and regeneration. In the process of healing, acute and chronic inflammatory cells, blood vessels and lymph, cytokines, growth factors and producing collagen agents and matrix metalloproteinase and their inhibitors are involved in a cascade of cellular processes for wound healing (2). The most common mouth sores that we are always prone to ulcers can be caused by trauma and referred recurrent aphthous ulcers (3).

Because in oral ulcers after hamper in the integration of oral tissue and loss of surface epithelium, created pain and discomfort has a significant impact on daily life, the local application of drugs with anti-inflammatory, antibacterial and anesthetic drugs has extended to speed up treatment. In recent decades in many countries, particularly developed countries, the use of herbs and herbal products has been increased to treat and accelerate wound healing presses. Today, medicinal plants due to reduced time and health effects, and the drug has been of interest to people in the medical industry (4).

One of the purposes of Medical Sciences is wound healing in a short time with minimal side effects, therefore identification of naturally new compounds to accelerate wound healing is most important. Capers plant has many chemical compounds that the most important of which include quercetin, rutin, kaempferol, alkaloids, campestrol, beta sitosterol and stigmasterol (5, 6), which in total raised its role in wound healing because of quercetin (5).

Flavonoids are one of the strong antioxidants that have important effects on cell biology. They have anti-inflammatory, regenerative, cytotoxic, antioxidant and are effective in performance of enzymes (7). Quercetin is a flavonoid that were identified for the first time in oak. The flavonoids have anti-inflammatory, anticoagulant, antibacterial, antihypertensive and anti-atherogenic properties. Capers extract has the greatest amount of quercetin than other plants (8, 7). Several studies were done related to skin wound healing with plant extracts and by examining using hematoxylin - eosin staining (10, 9). But the effect of hydroalcoholic extract of capers on oral wound healing in mice have not been studied. Amiri et al. examined the restorative effect of capers extract on skin wound contaminated with bacteria. The results of their study indicated that the formation of granulation tissue and thickness of the epidermis in the treatment group compared to the control group (3).

Since the mechanism of action of capers extract is not exactly known, in this study the effect of hydroalcoholic extract of capers on oral wound healing in mice was examined for the first time with immunohistochemical and histological methods. Perhaps the results of this study be used for future research and clinical trial to expedite human oral wound healing.

Methods

This experimental study approved by the Research Ethics Committee of Babol University of Medical Sciences. A total of 6 groups of male wistar rats in the weight range of 150-200 g were independently separated into two categories five days and ten days, and each category contains three independent groups as follows:

Experimental Group 1: Includes wounds treated with doses of 200 mg/kg capers extract

Experimental group 2: consists of wounds treated with doses of 300 mg/kg capers extract

Experimental group 3: Includes wounds treated with distilled water (negative control)

In each group, 6 rats for molecular and histological evaluations were used.

Animal and puncture methods: In this experimental study, 36 male mice were used. Animals were kept in conditions of 2 ± 23 °C, humidity of 50% and light-dark cycle of 12 hours with unlimited nutrition and municipal water in separate cages. Mice were anesthetized using pharmaceutical composition containing ketamine, xylene. After sterilization of the area, a wound with length of 3 ± 1 and depth of 4±1 was created using forceps, scissors and scalpel on the right side of tongue mucosa of each mouse (removal of muscle tissue underlying the epithelium). In the treatment group, capers extract was daily applied for

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10 days once 200 and 300 mg per body weight on the oral wounds as oral gavage.

Capers hydro-alcoholic extract preparation: in order to prepare extract, the stem and leaves of capers were isolated and dried in shade and at 25 °C. In the preparation of alcoholic extract in order to extract, the dried stem and leaf of Capers were powdered by shredder and 100 grams of capers plant powder was poured into one liter flask and 75% ethyl alcohol was added as maintaining the level of powder covered. After 72 hours, the solution was filtrated and then the filtrated solution was concentrated by vacuum distillation device at 50 ° C and rotation speed of 70 rpm to 3.1 original volume (11).

Sampling and histopathological evaluation: histopathological studies were performed on three occasions, first in terms of percentage of some involved cells in the inflammatory process, (Mast cells, and eosinophils), the second, immunohistochemical study and third, histomorphometry evaluation of thickness of the epithelium, blood vessels and the severity of inflammatory infiltrate was determined. After 5 and 10 days mice were sacrificed for molecular and histology and immunohistochemistry studies and samples were taken from each group (4).

In histological analysis, the oral mucosa samples in each group were immediately placed in formalin 10% and after being fixed for 48 hours, were sectioned in the longitudinal direction and the greatest cross section and then molded and paraffin blocks with 4-5 micron-thick slices were sectioned using microtome device. Prepared sections were stained with hematoxylin-eosin, toluidine blue (identification of mast cells), Congo red (identification of eosinophils), immunohistochemistry (Anti iNOS) (free radical assessment nitrogen) at the wound site to compare the histological changes.

To evaluate mast cells and eosinophils the slides were observed by 10x microscopy and areas with the highest density of mast cells with 40x in 5 microscopic fields (12) and to evaluate eosinophils in 4 microscopic fields with x40 and average was considered as a number. Using hematoxylin - eosin staining and histomorphometry, the thickness of epithelium (distance from the basal layer to the surface keratin) was investigated. The images were taken at 10 x from the epithelium and images were transferred to Motic plus2 software [Micro optic Industrial Group co] and in three areas of the basal layer to the surface creatine were measured and the average was considered as thickness of epithelium. To evaluate iNOS, in case of non-staining cytoplasm of epithelial cells or if they are less than 25% color were negative and more than 25% was considered positive (20).

Breast ductal carcinoma was positive control and negative control was the primary antibody elimination. After collecting the data, the results were analyzed. Data were analyzed using statistical software SPSS (18) and T-Test and One Way ANOVA tests and $p<0.05$ was considered significant.

Results

The findings of staining with toluidine Blue: The evaluation of fifth day groups showed that mast cells density had a significant increase in the first group than the second and third groups ($p<0.001$). Most of mast cells density was observed in capers extract at dose 200 mg/kg. There were significant differences between the groups in groups of ten days with one and two control groups ($p<0.001$) but there was no significant difference between one and two groups. Most of mast cells density was observed in the control group (Table 1) (Fig 1,2).

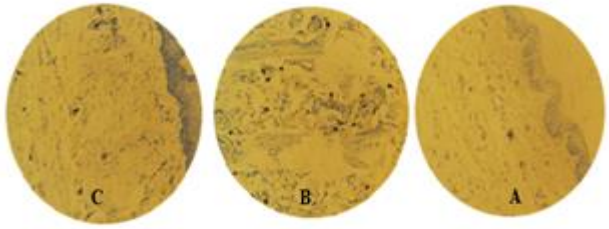

Figure 1. Section of rat oral mucosa tissue, mast cell density, the fifth day, stained with toluidine blue (10×). A: group treated with capers 200 mg . ml, B: Group treated with capers 300 mg . ml, C: control

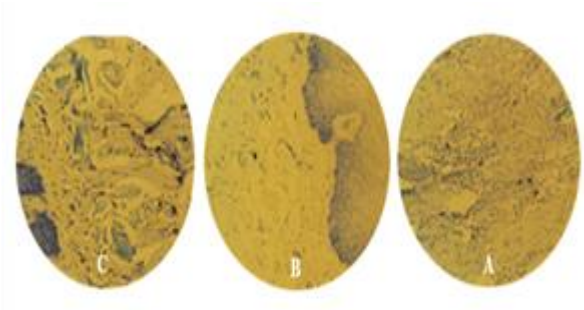

Figure 2. Section of rat oral mucosa tissue, mast cell density, the 10th day, stained with toluidine blue (10×). A: group treated with capers 200 mg/ml, B: Group treated with capers 300 mg/ml, C: control

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Haematoxylin -eosin staining results: In terms of density of blood vessels: in a five-day study groups the highest density of vessels was seen in second group. Significant differences were observed between the groups I, II and density of blood vessels in group II, capers at dose 300 mg/kg was observed compared to the first group. In groups of ten days, no significant difference has been achieved (Table 1) (Fig4).

Congo red staining results: Microscopic evaluation was carried out for eosinophils density measurements. Only on the fifth day 2-3 eosinophil cells was observed in per slide and in other slides were not seen (Fig 3).

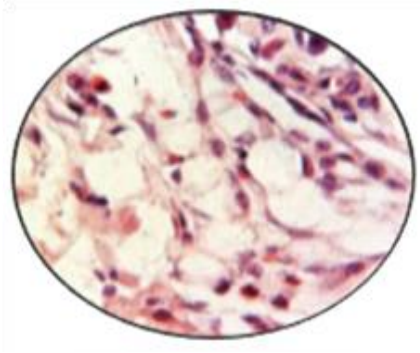

Figure 3. Rats oral mucosal tissue sections, the density of eosinophils with Congo red staining on fifth day (40×)

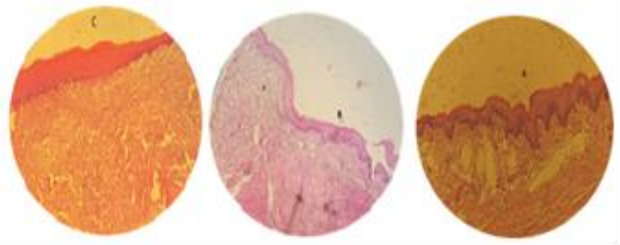

Figure 4. Section of rat oral mucosa tissue, density blood vessels, the 5th day, stained with *haematoxylin and eosin stain* (40 ×)

Evaluation of the thickness of the epithelium: The papilla on the fifth day of the first group treated with capers 200 mg/kg showed significant increase compared with the second and third groups ($p<0.001$). While no significant difference was observed between the groups on the tenth day. In the control group, the papilla was significantly different between the fifth and tenth days $p<.001$ but no significant difference was observed in the first and second groups $p>0.05$. In tissue repair lining (connective tissue of epithelium) in the third group (control) compared to the first and second groups on the fifth day of the lining of depth is very high and this difference was statistically significant ($p<0.001$).

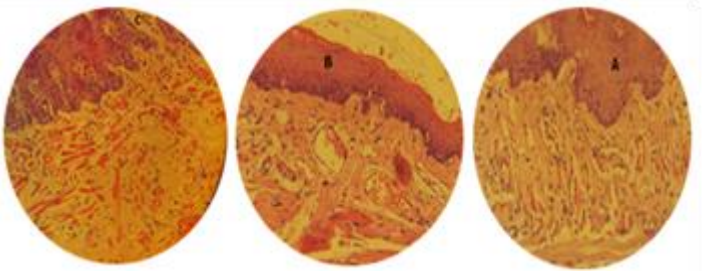

Figure 5. Rats oral mucosal tissue sections, the density of blood vessels, the tenth day, color

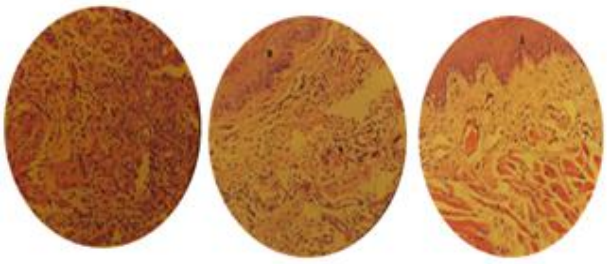

Figure 6. Section of rat oral mucosa tissue, density blood vessels, the 5th day, stained with *haematoxylin and eosin stain* (40×). A: group treated with capers 200 mg . ml, B: Group treated with capers 300 mg . ml, C: control

Table 1. Average density of mast cells, the density and thickness of blood vessels epithelium, papilla, connective tissue (micrometers)

Day	Group 1	Group 2	Control
Mast cell (day)			
5	*26.5±7.5	14.9±4.4	19.2±5.8
10	12.5±4.1	13±4.1	22.9±9.6
Density and thickness of blood vessels (consumption)			
5	5.3±2.3	*7.5±2.6	6.6±1.8
10	6.2±2	5.9±2.0	6.9±1
Thickness papilla(day)			
5	**373.31±6.08	210.54±4.9	110.10±3.84
10	333.47±3.99	201.45±9.43	250.79±3.15
Connective tissue (day)			
5	**1958.55±10.63	**2642.25±24.51	4017.48±35.07
10	5731.60±151.35	3495.90±45.45	2115.25±11.44

***p<0.05, **p<0.001**

The results of INOS staining: A statistically significant difference was observed in the percentage of cytoplasm staining cells in the first group and group

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3(p <0.001). While no significant difference among the ten days groups (Table 2) (Fig 7).

Figure 7. Rat oral mucosa tissue sections, immunostaining expression (inos) on the fifth day (10 ×). A: group treated with capers 200 mg . ml, B: Group treated with capers 300 mg . ml, C: negative control, D: positive control

Table 2. Percentage of cytoplasm staining of epithelium cells in mice.

Group	0.25%>	0.25%<
I*	4	2
II	1	5
III*	-	6

Weak (-), moderate to severe (+)

Discussion

The results of clinical and pathological study seems to pride extract at a dose 200 mg/kg can be effective in the oral wound healing of mice. This is the first study investigated the oral wound healing in mice and mechanism of capers histopathological effects have not been studied in any study (3).

Various substances were prepared and are presented for wound healing that most of these materials are plant compounds and chemicals, but none could be recommended as an effective drug. Nowadays, there is a tendency to use medicinal plants due to the lack of side effects, a variety of effective compounds found in plants, development of related industries to cultivate medicinal plants and now also their raw materials are available and used in the pharmaceutical industry (3).

Inflammatory phase begins immediately after the injury, which aims to provide a safety barrier against the influx of microorganisms. Various inflammatory cells are involved in this stage. Although the major role in wound healing of mouth is the role of macrophages, mechanism of action of mast cells and eosinophils is not known. According to results of this study, it seems that on the fifth day mast cells secreted factors that can contribute to faster wound healing and can be stimulator of macrophage function. In other words, in the fifth day granulation tissue formation is faster than other tissues, but in the tenth day, capers led to reduction of mast cell density and wound healing process goes toward further collagen production.

In evaluation of Congo red staining on the fifth day in two samples of the first group the eosinophils were very few. This suggests that eosinophils has no effect in the normal course of oral wound healing by capers extract. In other words, their main function was in hypersensitivity reactions and parasitic infections. Based on the results of this study, it seems that mast cells and the mediators released from mast cells have more active role in oral wound healing.

The results of this study also showed mast cells inflammatory cells on the fifth day was significantly higher than the control group and resulted in faster restoration phase started during the first days of recuperation. Then the density of inflammatory cells decreased in the treated group with capers extract, but the control group had still high cell density. Mast cells are known as a major source of inflammatory mediators and cytokines. These mediators lead to inflammatory and vascular changes (13).

Tellechea and colleagues showed that mast cells are responsible for the secretion of histamine which are effective in migration of fibroblasts and the cell proliferation process (14). In another study, Csaba and colleagues examined the process of wound healing in mice lacking mast cells using dimetidine as a H1 receptor antagonist. They found that this material by preventing the release of histamine can interfere with wound healing process.

As a result, they stated that activity of mast cells and release of histamine in the normal process of wound healing was essential to (15). The results obtained in this study to evaluate the density of inflammatory cells are consistent with the results of these researchers and the increase in the number of mast cells in samples treated on the fifth day after the repair and then reduced their density in the tenth day can be the evidence on the positive effects of this plant in the process of healing and effective in the inflammatory phase of wound healing. Since capers comprises ingredients such as, quercetin, rutin, kaempferol and alkaloids, the obtained results were not unexpected. Flavonoids are strong anti-oxidants, which has important effects in cell biology and has anti-inflammatory properties (14). Dweck showed that

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plants with anti-inflammatory properties have a high level of flavonoids (16). Quercetin is a flavonoids, a capers plant has the greatest amount of quercetin than other plants (8).

Azaizeh and colleagues in a study showed that quercetin (flavonoid) has anti-inflammatory activity and inhibits the release of histamine and other inflammatory mediators (17) Ruiz and colleagues showed that quercetin is a type of flavonoids inhibiting inflammatory cytokine expression and therefore weakening the inflammation (18). Another line of defense is the sub mucosal vascular bed bicarbonate that provide bicarbonate ions nutrients and oxygen for mucosal cells to clean up toxic metabolic products. The mucosal vascular network is effective in a quick recovery of mucosal wounds.

The results of this study showed an increase in blood vessels in the group treated with capers at dose of 300 mg/kg on the fifth day that this result probably shows the effective role of capers extract in wound healing process. Asadbe and colleagues stated that increase blood flow and oxygen to the wound through the dilated artery was another contributing factor in wound healing (10) and the results of our research are in line with the finding of these researchers.

Increased angiogenesis increases the speed of wound healing. Because following the increase of angiogenesis granulation tissue is increased and the depth of granulation tissue showed a large increase in the proliferative phase of wound healing (19). The results of this study also indicate this. Pathological investigations showed an increase in thickness in the restoration of the experimental samples compared to control samples.

Though this difference is not significant, but it may indicate that the positive effects of the extract on regeneration of wound. Contraction and formation of epithelial are major factors in wound healing and the first goal in wound healing is fast wound closing. Increased epithelium thickness due to the increased proliferation of these cells is the pride taken by the extract. In terms of formation of Papilla in microscopic evaluation, samples treated in 5th day groups showed more rapidly than other samples. Kumar and colleagues showed that flavonoids and terpenoids increases the wound contraction and epithelialization (20). In the present study, it can be said that the obtained results in the area of epithelialization is in line with the findings of the study of Kumar that probably is due to the presence of flavonoids. Gunes Bilgili and colleagues in the study of skin wound healing in rats indicated a significant increased collagen synthesis in wound and increased epithelialization in the experimental group compared to the control group. However, fibroblasts also had significant increase in the experimental groups. The study on Lawsonia plant also showed that ethanol extract of Lawsonia containing compounds such as alkaloids and beta sitosterol that have a significant impact on the healing process and leads to a high rate of wound contraction, decreases epithelialization period, high strength integration of tissue and significant increases in weight of granulation tissue that alkaloid with strong physiological effects was known as the major factor on wound healing property of Lawsonia (19).

The results obtained from this study show that the restoration of the mucosal lining tissues (connective tissue below the epithelium), depth of mucosal lining tissues in the control group was higher compared to the group treated with the capers extract on the fifth day and the difference was significant.

This indicates that the muscles in these regions in groups treated with capers extract were fully restored and has drawn near the surface. The compounds in this plant increased fibroblast growth and provided the context for accelerated healing process. The results of this study demonstrated that capers extract with an increase in the number of mast cells, increasing the number of blood vessels, increasing the thickness of the papilla to reduce the thickness of the lining, reduction of nitric oxide synthase can be effective in oral wound healing in mice and dose of 200 mg/kg of capers extract was more effective than dose of 300 nmg/kg.

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