Journal of Biological Studies
ISSN 2209-2560

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JBS An Open Access Peer Reviewed Journal

Research Full-Text Paper

Efficacy of 10% *Calendula officinalis* extract gel for oral wound healing in rats

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Received December 25,2021; Accepted February 5,2022; Published online April 10,2022

Abstract

This study assessed the efficacy of 10% *Calendula officinalis* (C. officinalis) extract gel for oral wound healing in rats. Thirty-two male Wistar rats were randomized into test and control groups (n=16). The rats were anesthetized to create a wound in their buccal mucosa by a #3 puncher. Next, 10% C. officinalis gel and a placebo gel were applied over the wounds in the test and control groups, respectively. Each group was randomly divided into two subgroups for assessment at 7 and 14 days (duration of treatment). At each time point, a biopsy sample was obtained from the wound and type of inflammatory cells, severity of inflammation, type of connective tissue, and percentage of wound contraction were assessed by a pathologist. Data were analyzed by univariate ANOVA and Chi-square test. The mean percentage of wound contraction was 28.28% and 62.58% in the test and -15.98% and 7.08% in the control group at 7 and 14 days, respectively, indicating significantly greater wound contraction in the test group at both time points ($p<0.001$). The type of connective tissue was significantly different between the two groups at 14 days ($p<0.05$). The number of giant cells was significantly higher in the test group than the control group at 7 days ($p<0.05$). No other significant differences were noted.

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Application of 10% C. officinalis gel improved wound contraction and enhanced healing but had no significant effect on the severity of inflammation in traumatic oral ulcers in rats.

Keywords: Calendula officinalis; Inflammation; Wound healing; Gel; Oral ulcers

1 Introduction

Ulcer refers to the loss of tissue integrity, which is often associated with impaired function (Arun et al., 2016; Sabharwal et al., 2012). Traumatic ulcers have a high prevalence in the oral cavity, and are the most common type of oral wounds (Duarte et al., 2011). Wound contraction is often commenced 2-3 days after the wound formation and is accomplished within 14 days, reducing the size of wound by 80% (compared with its baseline size). Contraction enhances healing, and if prevented, healing is decelerated, leading to formation of a large unsightly scar (Ravishankar et al., 2014). Assessment of the wound healing process is often performed at 7 and 14 days in the literature (Mortazavi et al., 2020).

The process of wound healing involves a series of events including hemostasis and clot formation, inflammation, proliferation (granulation tissue formation and epithelialization), wound contraction, and tissue remodeling (Arun et al., 2016). These phases may overlap, and the duration of each phase is influenced by a number of different factors (Eming et al., 2007).

The important cells in the process of wound healing include neutrophils, lymphocytes, macrophages, and giant cells (Eming et al., 2007). Neutrophils are dominant in the acute phase of inflammation and initial stages, and prevent wound infection (Dovi et al., 2004). Lymphocytes play an important role in wound healing and scar tissue formation in the chronic phase of inflammation (Martin and Muir, 1990). Macrophages are involved in all phases of healing and serve both pro-inflammatory and anti-inflammatory roles in this process (Dovi et al., 2004). Giant cells are involved in the chronic phase of inflammation and foreign body reactions (Miron and Bosshardt, 2018).

Use of medicinal herbs in medicine and dentistry is on the rise since they often have fewer side effects than routine synthetic medications (Chaturvedi et al., 2013). *Calendula officinalis* (C. officinalis) has several therapeutic applications (Parente et al., 2012). Its antiviral, antimicrobial, antifungal, anticancer, and antioxidant (Aslani et al., 2013). Description of variables properties have been previously documented (Eghdampour et al., 2013) C. officinalis includes flavonoids, flavoxanthin, lutein, and polyphenols, with antioxidant properties. Helenalin is its effective constituent that inhibits the enzymatic activity of matrix metalloproteinases, and decreases inflammation by down-regulation of interleukin-1B, interleukin-6, and tumor necrosis factor alpha (Singh and Bagewadi, 2017). It has wound healing potential by inducing the proliferation and migration of fibroblasts through a phosphatidylinositol 3 kinase-dependent pathway, and reinforces collagen synthesis as such (Dinda et al., 2015). The C. officinalis extract decreases the level of C-reactive protein and cytokines in the human body and protects the cells against the adverse effects of free radicals, exerting antioxidant effects. The pharmaceutical constituents of this extract that are used in medicine include triterpenoid, flavonoids, coumarin, quinones,

sertraline, and amino-acids. Triterpenoids exert anti-inflammatory and anti-edema effects and stimulate fibroblasts; these effects lead to inhibition of 5-lipoxygenase (inhibiting leukotrienes), cyclooxygenase (inhibiting prostaglandins) and C3 convertase (inhibiting the resolution of inflammation). In addition, flavonoids have lipoxygenase activity, exerting antioxidant effects (Givol et al., 2019).

Mucoadhesion refers to the adhesion of a mucosal surface to a material. This property can be used for adhesion of drug delivery systems to a mucosal membrane (Komati et al., 2019) Use of different forms of mucoadhesive agents has gained increasing popularity in treatment of oral diseases (Fini et al., 2011).

The drug absorption rate depends on the duration of exposure of mucosa to the respective medication. Thus, new drug formulations with optimal adhesion and continuous drug release potential for a suitable period of time are required (Hamishehkar et al., 2015). The mucoadhesive agents have different forms, such as gel type (Aslani et al., 2013). The gel form has the advantage of optimal and durable surface coverage, patient comfort, high diffusion rate, easy preparation, and enabling controlled drug release (Fini et al., 2011; Hamishehkar et al., 2015). Several studies have addressed the anti-inflammatory and wound healing properties of C. officinalis in the skin and mucosa, but not in the oral cavity (Tanideh et al., 2016; Saffari et al., 2017; Shafeie et al., 2015; Carvalho et al., 2016). Considering the gap of information regarding the effect of C. officinalis gel on oral ulcers, this study aimed to assess the efficacy of 10% C. officinalis extract gel for oral wound healing in rats.

2 Materials and Methods

2. 1. Animals

This animal study was conducted on 32 male Wistar rats weighing 250-300 g purchased from the animal room of Shahid Beheshti University of Medical Sciences and were kept under similar environmental and nutritional conditions. The rats were kept in polycarbonate cages under standard laboratory conditions (24 h light/dark cycle, 22±2°C temperature, *ad libitum* access to food and water) for the purpose of acclimation. All the possible confounding factors such as weight, age, storage conditions, access to food and water, and method of wound induction were the same in all rats.

The study was conducted in accordance with the guidelines for the care and use of laboratory animals and approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.DRC.1398.016). The sample size was calculated to be 16 rats in each group assuming alpha=5%, beta=10%. The inclusion criteria were rats with approximately similar weight, and age between 10-12 weeks, no sign/symptom of disease and no infection (Rabiei et al., 2014).

2. 2. Preparation of herbal extract and the placebo gel

One kilogram of C. officinalis flowers was purchased and placed in a maceration machine; 70% ethanol was added to the flowers. The obtained extract was kept under the hood to achieve the desired consistency. After 7 days, 70% ethanol was added again to the flowers and the

process of maceration was continued for 14 days until the final extract was achieved.

The required gel was synthesized by the experts at the School of Pharmacy of Zanjan University of Medical Sciences. Distilled water, hydroxyl propyl cellulose polymer powder, carbopol polymer powder, and sodium hydroxide were used for this purpose. A total of 500 mL of medicinal gel and 500 mL of placebo gel were required for this study. A standard NaOH solution was first prepared. To prepare 100 g of gel, 100 mL of distilled water was added to a 500-mL beaker. The beaker was placed on a magnetic stirrer operating at 800 rpm; 40 g of carbopol was gradually added to distilled water through a filter paper (to enhance the solubility of powder). Next, 4 g of hydroxyl propyl cellulose was added to the solution as explained earlier. The stirrer was then turned off and 3-4 drops of NaOH were added for pH adjustment, and the solution was slowly stirred with a spatula to reach the desired consistency. Eventually, 100 mL of gel was obtained. The same method was used to prepare 500 mL of medicinal gel and 500 mL of placebo gel. To prepare the gel containing 10% C. officinalis extract, 50 g of the extract was gradually dissolved in 500 mL of the gel. The medicinal and the placebo gels were kept refrigerated in screw-top containers until use.

2. 3. Intervention

The rats were randomly divided into two groups of 16 to receive the medicinal gel (test group) and the placebo gel (control group). Each group was then divided into two subgroups for microscopic and clinical assessment of the results at 7 and 14 days. All rats then underwent general anesthesia with intramuscular injection of 10% ketamine hydrochloride (90 mg/kg) and 2% xylazine hydrochloride (3 mg/kg). After ensuring adequate depth of anesthesia, the oral cavity of the rats remained open by a mouth gag, and a wound was created by a surgeon in the buccal mucosa of the rats using a #3 puncher (Kai Medical, Japan) without removing the muscle.

In case of bleeding, the wound would be rinsed with saline and dried with a sterile gauze. The medication (medicinal gel or placebo gel) was then applied over the wound.

The medicinal and the placebo gels were applied over the wounds from the first day and this process was continued twice a day, one in the morning and once in the evening with a 12-h interval. Each time, gel with an approximate thickness of 0.5 mL was applied over the wound site with an applicator. Biopsy samples were obtained at the end of day 7 and day 14 from the wound site (Figure 1). The specimens were fixed in 10% formalin with a pH of 7 and were sent for pathological assessment. The type of inflammatory cells, severity of inflammation, type of connective tissue, and percentage of wound contraction were assessed by a calibrated pathologist with an intraobserver agreement of 1.

Figure 1. Taking a biopsy sample from the wound

2. 4. Wound inflammation scoring

Wound inflammation was scored as follows:

Score zero: Normal epithelium, no vasodilation in the connective tissue, no hemorrhagic areas, ulceration or abscess.

Score 1: Mild vascular swelling, areas of re-epithelialization, mild inflammation with infiltration of mononuclear cells, absence of bleeding, edema, ulceration, or abscess.

Score 2: Moderate vascular swelling, areas of epithelial hydropic degeneration, infiltration of neutrophils, presence of hemorrhagic areas, edema, extensive ulceration, and abscess (Martins et al., 2009).

Type of connective tissue was categorized as:

Granulation tissue: Abundant blood vessels in the newly formed connective tissue

Fibrous tissue: High amounts of collagen in a dense connective tissue

Necrotic tissue: Necrosis of connective tissue cells due to trauma (Preethi and Kuttan, 2009).

The mean wound size was approximately 3 mm in both groups on day 1 (corresponding to the diameter of the puncher). To measure the percentage of wound contraction, the change in size of wounds was assessed at the respective time points. For this purpose, the wound area was outlined by a copying pencil and then a transparent paper was placed over the wound site. The outline of the wound was transferred on the paper (Figure 2) and then the paper was scanned and the size of ulcers was precisely measured by the caliper feature of Excel software.

Percentage of wound contraction: (wound diameter on day 1-wound diameter on day n) / (wound diameter on day 1) × 100

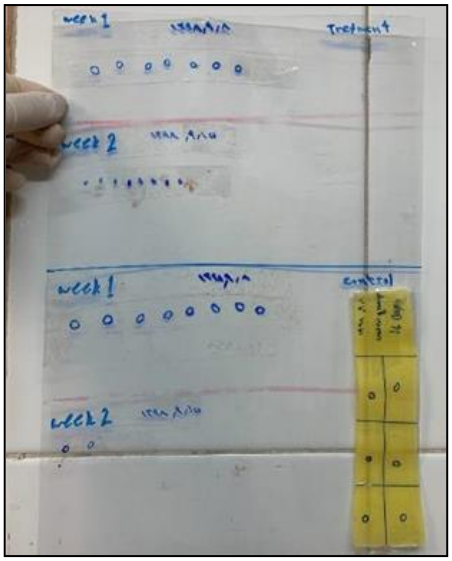

Figure 2. Calculation of percentage of wound contraction

2. 5. Statistical analysis

Data were analyzed using SPSS version 22. Univariate ANOVA was used to compare the two groups regarding wound contraction, which was assessed clinically. The Chi-square test was used to compare the two groups regarding other variables other than wound contraction.

3 Results and Discussion

3. 1. Percentage of wound contraction

Table 1 compares the percentage of wound contraction between the test and control groups at 7 and 14 days. As shown, the mean percentage of wound contraction in the test group was significantly greater than that in the control group at both 7 ($p<0.001$) and 14 days ($p<0.001$). On day 1, one out of 8 rats in the test group did not recover from anesthesia, and was excluded.

3. 2. Severity of inflammation

Table 2 represents the severity of inflammation in the two groups at 7 and 14 days. As indicated, the two groups were not significantly different regarding the severity of inflammation at 7 or 14 days ($P>0.05$).

3. 3. Type of connective tissue

Table 3 compares the frequency of different types of connective tissue in the two groups at 7 and 14 days. The difference between the two groups was not significant regarding the frequency of different types of connective tissue at 7 days ($p=0.412>0.05$). However, the difference in this respect was significant at 14 days ($p=0.021<0.05$) such that all specimens showed fibrous tissue in the test group while half of the specimens showed granulation tissue and the other half showed fibrous tissue in the control group.

3. 4. Inflammatory cells

Lymphocytes were observed in all specimens with no significant difference between the two groups.

At 7 days, 4 specimens in the test and 1 specimen in the control group showed the presence of neutrophils. At 14 days, neutrophils were not seen in any specimen. The difference was not significant in this regard between the two groups at any time point.

At 7 days, macrophages were found in four specimens from the test and four specimens from the control group. At 14 days, only three specimens in the placebo group showed macrophages. The difference was not significant in this regard between the two groups at any time point.

At 7 days, giant cells were found in only three specimens in the test group. At 14 days, giant cells were observed in only three specimens from the control group. The difference between the two groups was significant in this regard at 7 days (P=0.038<0.05) but not at 14 days.

Table 1. Comparison of the percentage of wound contraction between the test and control groups at 7 and 14 days (univariate ANOVA)

Group	Mean percentage of wound contraction at 7 days	Mean percentage of wound contraction at 14 days
Test group	28.28	62.58
Control group	-15.98	7.08
P-value	<0.001	<0.001

Table 2. Severity of inflammation in the two groups at 7 and 14 days (Chi-square test)

Day	Groups		Degree of inflammation				Total	P-value
			Normal (<10%)	Mild (10-30%)	Moderate (30-50%)	Severe (50%<)		
7	Test	Number	3	1	1	2	7	0.862
		Percentage	42.9%	14.3%	14.3%	28.6%	100%	
	Control	Number	4	1	2	1	8	
		Percentage	50%	12.5%	25%	12.5%	100%	
14	Test	Number	8	0	0	0	8	0.131
		Percentage	100%	0%	0%	0%	100%	
	Control	Number	6	2	0	0	8	
		Percentage	75%	25%	0%	0%	100%	

Table 3. Frequency of different types of connective tissue in the two groups at 7 and 14 days

Day	Groups		Connective tissue type			Total	P-value
			Fibrous tissue	Granulation tissue	Necrotic tissue		
7	Test	Number	3	1	1	7	0.412
		Percentage	42.9%	14.3%	14.3%	100%	
	Control	Number	4	1	2	8	
		Percentage	50%	12.5%	25%	100%	
14	Test	Number	8	0	0	8	0.021
		Percentage	100%	0%	0%	100%	
	Control	Number	6	2	0	8	
		Percentage	75%	25%	0%	100%	

This study assessed the efficacy of 10% C. officinalis gel for oral wound healing in rats. The results showed that the test group had significantly greater percentage of wound contraction at 7 and 14 days. Also, the test group had significantly higher number of giant cells at 7 days, and the type of connective tissue was entirely fibrous (which is favorable) in this group at 14 days, and had a significant difference with the control group in this respect. No other significant differences were found between the two groups. The results supported the superior efficacy of the medicinal gel compared with the placebo gel in wound contraction, which may be attributed to the antioxidant activity of C. officinalis extract and its flavonoid content. It also enhances angiogenesis, collagen deposition, and metabolism of glycoproteins, and improves the local blood circulation and formation of granulation tissue (Dinda et al., 2015). Dinda et al. (Dinda et al., 2015) evaluated the biocompatibility and would contraction potential of the alcoholic extract of C. officinalis and found that it affected the proliferation and migration of fibroblasts through a phosphatidylinositol 3 kinase-mediated pathway. Although their study had an in vitro design and was conducted on fibroblasts, their findings were in agreement with ours. Shafeie et al. (Shafeie et al., 2015) compared the therapeutic efficacy of C. officinalis gel with 5%, 7% and 10% concentrations for treatment of skin ulcers. Their methodology was similar to that of the present study with the difference that they created the wounds on the back of each rat's neck, and evaluated the wound size at 14 and 21 days. They noticed a reduction in wound size at 14 days in all groups but the reduction was significantly greater in the test group. In a similar study, Preethi and Kuttan (Preethi and Kuttan, 2009) reported 90% mean reduction in wound size in C. officinalis group versus 51.1% in the control group at 8 days. The trend of reduction in wound size continued at 12, 16 and 20 days, but with greater reduction in the test group. Their results were in agreement with our findings.

Assessment of the severity of inflammation in the present study showed no significant difference between the two groups at 7 and 14 days. This result was in contrast to the previous findings (Fini et al., 2011) which may be due to the protective effect of the placebo gel in the control group, because it protects the wound and subsequently decreases inflammation (Fini et al., 2011).

Regarding the type of inflammatory cells, lymphocytes were noted in both groups at both 7

and 14 days with no significant difference between the two groups. The difference between the two groups was not significant regarding neutrophils and macrophages at any time point either. The number of giant cells was significantly higher in the test group at 7 days but not at 14 days. Shafeie et al. (Shafeie et al., 2015) reported that the number of macrophages in 10% gel group was lower than that in the placebo gel group at 14 days. Macrophages are seen in the chronic phase of inflammation, and this difference in number of macrophages was also noted in the present study but did not reach statistical significance. Lymphocytes are seen in the chronic phase of inflammation, and their presence in the first week indicates acceleration of the healing process. The healing process was also enhanced in the placebo group due to the use of placebo gel and its protective effect. The present results regarding the number of lymphocytes were also in agreement with those of Shafeie et al. (Shafeie et al., 2015) Neutrophils are present in the acute phase of inflammation. The difference in the number of neutrophils was not significant at 7 days, and no neutrophils were seen at 14 days. Shafeie et al. (Shafeie et al., 2015) did not report the number of neutrophils at 7 days. However, similar to the present study, they found no neutrophils at 14 days. Giant cells are present in the chronic phase of inflammation. In the present study, higher number of giant cells in the test group at 7 days indicates acceleration of healing in this group. However, the difference was no longer significant at 14 days. No similar study was found in this respect to compare our results with.

Since the healing process is not completed within 7 days, no significant difference was found regarding the connective tissue type between the test and control groups at 7 days. However, at 14 days, the difference was significant, which is due to the completion of the healing process in the test group. The connective tissue was entirely fibrous in all specimens in the test group, which indicates enhanced healing in this group. According to Krafts (Krafts, 2010), the proliferation phase follows the inflammation phase in the wound healing process, which starts from the epithelium and then affects the connective tissue. The connective tissue is in the form of granulation tissue in early phases and then changes to fibrous tissue and then normal connective tissue, as the inflammation subsides. Accordingly, the present results indicated acceleration of wound healing process in the test group due to the effect of extract (Krafts, 2010). Also, Shafeie et al. (Shafeie et al., 2015) found higher number of fibroblasts at the wound site in rats treated with 10% extract of C. officinalis compared with the control group. They noticed higher areas of inflamed disorganized tissue in the control group. Fonseca et al. (Fonseca et al., 2010) reported that concentrations < 27% of C. officinalis extract induced the proliferation of fibroblasts in rats; however, increasing the concentration of extract decreased the viability of cells. The results of the abovementioned studies were in line with the present findings. Future studies are required to precisely assess the efficacy of C. officinalis mouthwash and mucosal patch.

4 Conclusion

Application of 10% C. officinalis gel improves wound contraction and enhanced healing but has no effect on the severity of inflammation in traumatic oral ulcers in rats.

Conflict of interests

The authors declare that there are no competing interests.

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