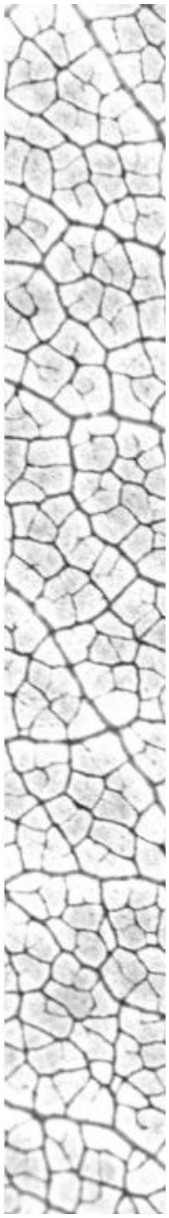

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Effect of topical curcumin on the healing of major oral mucosal ulceration

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Abstract

Background: A mucosal oral ulceration is epithelium damage that occurs anywhere inside the oral cavity. It is deep breakdown in the continuity of oral epithelium occur due to molecular necrosis associated with pain, redness and tenderness of affected site Curcumin was a turmeric extract, its multifaceted biological effects such as, anti-inflammatory, anti-oxidant, anti-carcinogenic and anti-infectious effects. The aim of the present study is to evaluate the effect of topical application of curcumin on mucosal oral ulcer healing.

Materials and methods: Thirty-six male white New Zealand rabbits age between (2-8) months with body weight between (1000-1400 gm), were subjected to traumatic ulcer by 'punch biopsy' on the right side of the buccal mucosa, with diameter of (8 mm) and (2mm) depth. The animals divided into two groups; control group: the ulcer treated with sterilized distal water, the experimental group: the ulcer treated with 1% curcumin oral gel. The rabbits were sacrificed at 3rd,7th and 14th days. Clinical assessment of ulcer size and histological and histomorphometric analysis of H&E slide in both control and study groups.

Results: The present study showed that the curcumin treatment reduce mucosal ulcer area from the 3rd day till 7th day and improved ulcer healing at 14th day by activation of epithelial cell, inhibit inflammatory cells, enhance endothelial cells and promote fibroblast cells. While control group showed limited healing process of mucosal ulceration from early period of 3rd day with minimal contraction at the 7th day and incomplete healing at 14th day.

Keywords: curcumin, oral mucosa, ulcer

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INTRODUCTION

Oral mucosal ulceration is a loss of mucosal integrity that covered by a fibrinous exudate, which characterized by inflammations, local defect and excavation, with connective tissue necrosis (Gnepp, 2009).

Wound healing is the interaction of a complex cascade of cellular and biochemical actions leading to the restoration of structural and functional integrity with regain of strength-injured tissues. It involves continuous cell-cell interaction and cell-matrix interactions that allow the process to proceed in different overlapping phases and processes including inflammation, angiogenesis with of granulation tissue formation, wound contraction, reepithelialization and tissue remodeling (Eming et al. 2014).

The wound healing process can be divided into four overlapping phases:

1. Hemostasis begins immediately after the injury. Bleeding from the wound is controlled with vascular constriction, formation of a platelet thrombus, propagation of the coagulation cascade and termination with clotting (Janis and Harrison, 2016).

Inflammation

Inflammatory cells migrate to the wound site after platelet activation during the first several hours following injury. Mast cells release vasoactive cytokines such as prostaglandins (PG) and histamine, which increase capillary permeability and promote local dilation to aid the migratory process (Alam, 2014).

Within few hours of injury, inflammatory cells (neutrophil, lymphocyte and monocyte) infiltrate the clots to initiate acute inflammatory response. These cells clear the wound from bacteria and necrotic tissue through engulfment, oxygen species and enzyme release (Velnar et al. 2009).

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The proliferation phase

Re-epithelialization

Re-epithelialization from the wound edge starts within 24 hours to 48 hours after wound occurred. Keratinocytes start to migrated from edges of the wounds, where the hemidesmosomal connection was lost between epithelial cells and move through the fibrin–fibronectin matrix laterally beneath the clot through the wound until they meet their same cells (Broughton et al. 2006).

Angiogenesis

New blood vessels formation was the main source of nutrients and oxygen for wound healing that needed by rapidly proliferating reparative cells. The uninjured endothelial cells proliferate in the extracellular matrix and migrate into the provisional wound matrix to form vascular tubes and loops and form new capillaries. The angiogenic process was mediated by the secretion of angiogenic growth factors such as VEGF from macrophages, fibroblast growth factor and platelet-derived growth factor and cytokines from inflammatory cells that are secreted within the hypoxic wound (Yoo and Kwon, 2013).

The wound contraction and scarring

Contraction phase was the final stage of the wound healing process. The wound edges are union, the wound surface is reducing and the wound closure is complete. Fibroblasts differentiation into myofibroblasts represents the main step in the contraction phase (Ozturk and Ermertcan, 2011).

Curcumin (diferuloylmethane or 1, 7-bis (4-hydroxy-3-methoxyphenyl)-1, 6- heptadiene-3, 5-Dione) is a naturally polyphenolic phytoconstituents with a yellow pigment obtained from the rhizomes of *Curcuma longa* Linn (Zingiberaceae) (Shen et al. 2013).

It accelerates wound healing by exerting its action on various stages of wound repair, which are anti-inflammatory (Sugawara et al.2013),antioxidant activities (Dai et al. 2009), positive effect on granulation tissue formation (Sharma et al. 2012), collagen deposition (Dai et al. 2009), wound contraction (Jagetia and Rajanikant, 2012) and induce reepithelization (Panchatcharam et al., 2006).

MATERIALS AND METHODS

Thirty-six male white New Zealand rabbits weighting (1000-1400 gm), aged (2-8) months were used in this study, which divided into 2 groups, control group of 18 rabbits and study group of 18 rabbits. They maintained under control conditions of temperature, drinking and food consumption.All experimental procedures were carry out in accordance with the animal experimentation ethical principles that approved by the scientific committee of College of Dentistry University of Baghdad.

Induction of oral ulcer

Induction of oral mucosa ulceration of each rabbits as follows; first, the animal anesthetize via intrapretoneal injection of ketamine (50 mg/kg) (1ml/kg of body weight) and xylazine 2% (0.2 ml/kg) (Donald et al. 2008).The mucosal ulceration with 8 mm diameter and (2mm) in depth was made on the right cheek mucosa for control group and left cheek for study group by using punch biopsy (Schierle et al. 2009). In control group (18 rabbits): the ulcers treated with 10µl of sterilized distal water. While the experimental group (18 rabbits): the ulcers treated with 1% curcumin oral gel (Curenext oral gel® 10 mg/g from Abbott Healthcare Pvt., Ltd., Mumbai, India). Applied topically to ulcer area by mucoperiosteal elevators as three layer, first layer applied to ulcer and then doing rubbing action by mucoperiosteal elevator for one minute until material completely absorbed, then second layer applied to ulcer and do same rubbing action by mucoperiosteal elevators for one minute until material completely absorbed, and the third layer as dressing that completely covered the ulcer (Mayo clinic, 2020). Then the animals were sacrificed according to 3 healing intervals into 3,7, and 14 days (12 rabbits from both groups in each periods).

Ulcer size assessment

Ulcer size has been measured in the day of ulceration and in the day of sacrifiction, through the following formula: $A = \pi \cdot R \cdot r$, where "A" represents the area (mm^2), "R" larger radius, and "r" smaller radius (each diameter calculated by digital paquimeter) (Franco et al. 2012). The percentage of the ulcer size reduction (percentage of ulcer healing) calculated by using simple equation:

"[(Wound area day 0 - Wound area relative day)/Wound area day 0] × 100" (Chen *et al*., 2019).

Intensity of inflammatory reaction

The inflammatory cells in histological section was counted for each specimen, in five microscopic fields at 40x magnification. The intensity of inflammation was calculated according to the amount of inflammatory cells as follows (Accorinte et al. 2008):

1. Absent or few inflammatory cells 1-4.
2. Mild: average number less than 10 inflammatory cells.
3. Moderate: average number 10-25 inflammatory cells.
4. Sever: average number greater than 25 inflammatory cells.
5. Histological and histomrphometrical evaluation

The histomrphometric assessments of the blood vessels account and the epithelium thickness at 3rd, 7th and 14th days were performed by using "Image-J" image analysis software.

The images of histological samples were captured using digital camera and light microscope, and saved to the computer in a JPEG format and examined using high

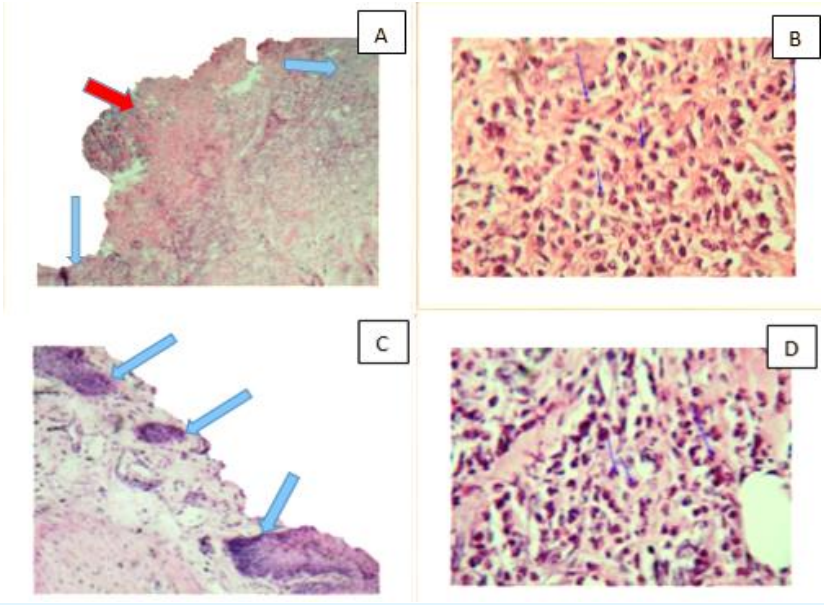

Fig. 1. Photomicrograph shows ulcer at 3rd day in the control group, (A) Limited epithelium proliferation to word the ulcer center (blue arrows), and exudate fill the surface (red arrow), H& E stained slide (x4), (B) Magnifying view, shows granulation tissue with heavy inflammatory cells infiltration (blue arrows) (x40). Photomicrograph shows ulcer at 3rd day in study group: (C) Epithelium migration front from the margin toward the center area (blue arrows), H& E stained slide (x4), (D) Magnifying view, shows granulation tissue with the blue arrow points to an inflammatory cell (x40)

resolution display monitor and the Image-J (Java based image processing program developed at the National Institutes of Health, USA).

Assessment of blood vessels account

Assessment of the blood vessels was performed at all healing period in both groups. Three fields were selected for each slide, where light microscope at x40 with slide moving in clockwise direction, three digital images were imported to the image analysis software Image-J, and the area was counted by multi- doted or point command, then the mean of the three areas was calculated (Abed and Al-Ghaban, 2018).

Assessment of epithelial thickness

Assessment of the epithelial thickness was performed in all healing period in both groups. The epithelial thickness was measured in a three different areas captured at 10x magnification from the surface of the epithelium to the basement membrane of a deepest area of rete redge in a chosen field (µm),three fields were chosen and the mean obtained was considered as the epithelial thickness per slide (Paul et al. 2017).

RESULTS

Ulcer size

The control group

The ulcer area at 3rd day was crater form, bleeding with exudate formation. The ulcer diameter with minimal reduction in size. At 7th day, there was obviously reduction in the ulcer size, also presence redness at the injured area surrounded by a white halo. At the 14th day, there was a small red area in the center of the ulcer.

The study group

In the study group, ulcer area after 3rd day of ulceration, showed observably reduction in the diameter of the ulcer, no exudate formation, with irregular borders and shallow depth that covered by whitish or yellow pseudomembrane. At the 7th day, the ulcer surrounded by white halo and reduced in size, with mild redness. At 14th day, virtually they were completely cicatrized, uniform and presented with normal mucosa.

The histological and histomorphometrical results At the 3rd day:

The control group

Under the light microscope examination the control group at 3rd day showed limited regeneration of the epithelium from the ulcer margin toward the central defect (Fig.1-A).The microscope examination of lamina properia showed heavy infiltration of acute and chronic inflammatory cells to the central area of the ulcer associated with necrotic tissue, few fibroblast cells, with sparse and thin blood vessels were seen below the necrotic area (Fig.1-B).

The study group

In the study group, the keratinocytes showed active proliferation of epithelium from the ulcer margin toward the central defect with obvious reduction in ulcer area through approximation of two ulcer edges (**Fig.1C**).The lamina properia of ulcer area showed early immature granulation tissue formation with moderate inflammatory cells infiltration, presence of moderate count blood vessels, plenty fibroblasts cells, and presence of new collagen fibrils distributed randomly (**Fig.1D**).

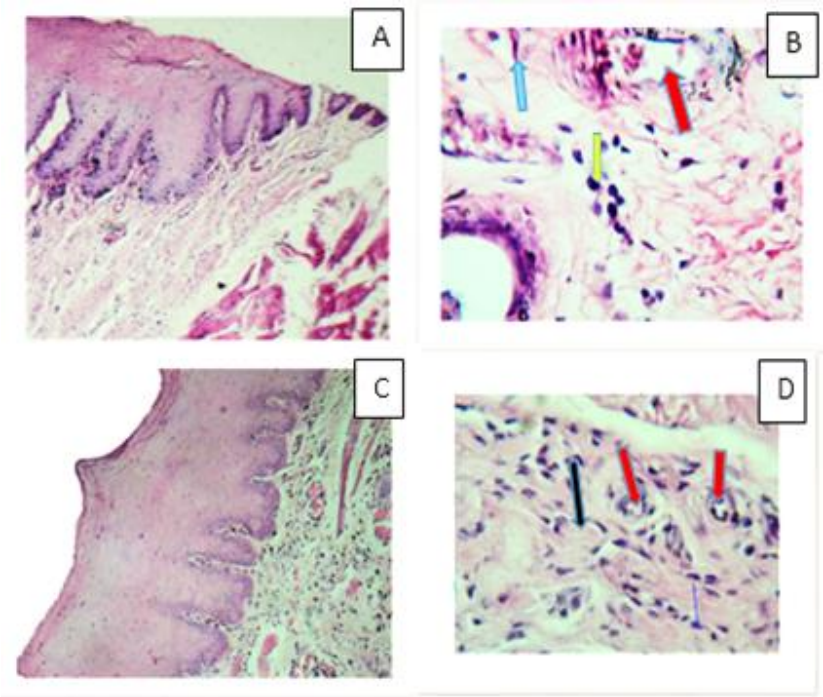

Fig. 2. Photomicrograph shows ulcer at 7th day in control group, (A) Incomplete epithelium with thin new layer epithelium (x10), (B) Magnifying view, shows granulation tissue with presence of inflammatory cells (yellow arrow), fibroblast cells (blue arrow), and dilated blood vessel (red arrow) (x40). Photomicrograph shows ulcer at 7th day in study group (C) Mature keratinized stratified squamous epithelium, H& E stained slide (x10), (D) Magnifying view, shows mature granulation tissue with fibroblast (blue arrow), blood vessel (red arrow), and scattered collagen fibers (black arrow) (x40)

At the 7th day

The control group

The histological picture of control group at the 7th day after ulceration, showed newly formed thin epithelium in ulcer area (**Fig.2A**).The lamina properia showed early granulation tissue formation with moderate to severe number of inflammatory cells infiltration, scanty collagen fibers and few blood vessels (**Fig.2B**).

The study group

The histological picture of the ulcer showed new well-defined keratinized squamous epithelium with prominent rete ridge. Closed approximation at wound edges with strong epithelial activity and high maturity of epithelial cell layers (**Fig.2C**). Lamina properia showed mature granulation tissue that characterized by, signs of collagen fibers bundle, copious amount of numerous blood vessels, reduction in the inflammatory cells infiltration (mild to moderate inflammatory cells) and increase fibroblast cells number (**Fig.2D**).

At the 14th day

The control group

The control group showed incomplete re-epithelization with well-defined cell layers of keratinized stratified squamous epithelium (**Fig.3A**). Lamina properia showed granulation tissue with numerous blood vessels, fibroblast cells, moderate number of inflammatory cells, and thin collagen fibers (**Fig.3B**).

The study group

The study group showed complete keratinized stratified squamous epithelium with well-defined cell layers associated with rete ridges (**Fig.3C**). In the Lamina properia, showed fibrotic tissue characterized by remodeling of collagen fibers (appeared as highly organized bundles), sufficient amount of myofibroblast, absent of inflammatory cells, and reduce number of blood vessels (**Fig.3D**).

Clinical results

The ulcer size analysis

Both control and study groups showed decrease in the size of ulcer (mm^2) beginnings from the 3rd day to the 7th day and the lowest area value (mm^2) for final ulcer size was seen at the 14th.

The result showed highly significant difference ($p<0.001$), in the mean of the ulcer size between control and study group at all healing periods by using T- test (**Table 1**).

Percentage of ulcer healing

The results of present study showed there were high percentage of ulcer healing in study group from early period of treatment (3rd day) and increase prominently at mid healing period (7th day) till maximum healing percentage at 14th day in comparison with minimal percentage of healing in control group. The T- test showed highly significant difference ($p<0.001$) of ulcer

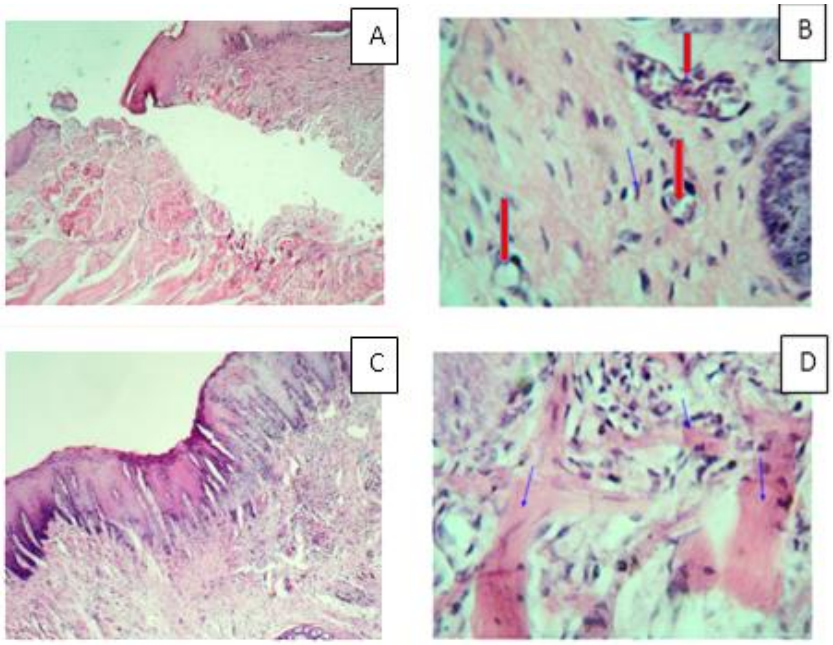

Fig. 3. Photomicrograph shows ulcer at 14th day in control group (A) Incomplete epithelium. H&E stained slide (x10), (B) Magnifying view, shows granulation tissue fibroblast (blue arrow), and blood vessel (red arrow) (x40). Photomicrograph shows ulcer at 14th day in study group (C) Well defined keratinized stratified squamous, H&E stained slide (x10), (D) Magnifying view, shows remodeling collagen fibers (blue arrow) (x40)

Table 1. The T-Test between control and study groups of final ulcer sizes (mm^2) for each duration

DAY	Control Group Mean±S.D	Study Group Mean±S.D	T.TEST	P-VALUE
3rd	150.73± 3.52	95.99 ± 0.57	37.52	0.00 HS
7th	127.68 ± 1.75	44.67 ± 3.94	47.03	0.00 HS
14th	2.14 ± 0.12	0.04 ± 0.01	39.69	0.00 HS

Table 2. The T-Test between control and study groups of percentage of ulcer healing for each duration

DAY	Control Group Mean±S.D	Study Group Mean±S.D	T.TEST	P-VALUE
3rd	11.08 ± 4.68	43.2 ± 1.65	-15.81	0.00 HS
7th	26.98 ± 1.81	74.47 ± 2.16	- 41.2	0.00 HS
14th	98.71 ± 0.08	99.96 ± 0.07	- 35.53	0.00 HS

Table 3. Comparison of inflammatory scores between control and study groups for each duration

Day	Control group Mean±S.D	Study group Mean±S.D	(K.W) test
3rd	4 ± 0.00	2.66 ± 0.51	Chi-Square= 9.9 d.f= 1 P-value= 0.00
7th	3.66 ± 0.51	2.5 ± 0.54	Chi-Square= 6.54 d.f= 1 P-value= 0.01
14th	2.5 ± 0.54	1.33 ± 0.51	Chi-Square= 6.54 d.f= 3 P-value= 0.01

healing percentage between control and study groups at all healing periods (**Table 2**).

Histological findings

The results of this study showed high mean of inflammatory score in the control group than mean of inflammatory score of study group.

Table 4. The T-test between study and control groups of the epithelium thickness measurement (µm) for each duration

DAY	Control Group Mean±S.D	Study Group Mean±S.D	T.TEST	P-VALUE
3rd	313.15 ± 95.62	427.24±127.75	-3.91	0.00 HS
7th	413.24 ± 97.00	492.36 ± 134.2	-2.61	0.01 S
14th	395.33± 154.52	468.82±123.66	-2.03	0.04 S

In comparison between control and study groups, using the Kruskal-Wallis (K.W) test showed highly significant difference ($p<0.001$) at the 3rd day and significant ($p<0.05$) at the 7th and 14th days (**Table 3**).

Histomorphometrical analysis

The epithelium thickness

The results revealed that mean values of epithelium thickness (µm) were higher in study group than in control group at all healing periods. They found highly significant difference ($p<0.001$), in the mean thickness of the epithelium between control and study groups at 3rd day and significant difference ($p<0.05$) at 7th and 14th days (**Table 4**).

The blood vessels count

The mean values for blood vessels number in both control and study groups increased with time, the results showed increased the mean values of study group than control group at all healing periods.

The comparison between control and study groups by using t-test revealed that they were highly significant difference ($p<0.001$) at the 3rd, 7th days and 14th day (**Table 5**).

Table 5. The T-test between control and study groups of the blood vessels counts for each duration

DAY	Control Group Mean±S.D	Study Group Mean±S.D	T.TEST	P- VALUE
3rd	3.6 ± 2.19	5.4 ± 0.81	4.21	0.00 HS
7th	2.4 ± 1.52	11.66 ± 6.26	7.87	0.00 HS
14th	1.46 ± 1.16	5.3 ± 4.24	4.77	0.00 HS

DISCUSSION

Any type of trauma to oral mucosa caused disrupting to its continuity and result a painful condition affects normal physiological function of the oral cavity and life quality. For that reason a safe and quick treatment with good result, was extremely needed, to resolve the problem and accelerates the healing process (Abed, 2018).

Curcumin is a nontoxic, highly promising natural antioxidant compound having a wide spectrum of biological functions. Curcumin was a novel medication in the near future to control various oral mucosal disorders. Curcumin, fulfills many roles in the putative treatment of oral mucosal disorders, as an anti-inflammatory, antioxidant, antimicrobial, and chemopreventive agent (Rai et al., 2019).

Ulcer size

The results of this study reported curcumin treated major oral ulceration with potent reduced ulcer size through all treatment period 3rd,7th and 14th days, that agree with Panchatcharam et al., 2006 and Kulac et al., 2013 reported curcumin was potent tissue repair medication that improve wound contraction at 4th, 8th and 12th days post wound respectively, which enhance centripetal movement of the edges of a full-thickness wound to facilitate closure of the defect, though curcumin enhance reduce wound size and faster healing of wound.

Also Kant et al., 2014 demonstrated gross evaluation of wound revealed that topical curcumin application decreased the wound size with significant increase percent of wound contraction, as showed faster cutaneous wounds closure in the curcumin-treated rats.

Mani et al., 2002 reported curcumin was potent enhanced reduction of wound size with appropriated percentage of wound healing in treatment of dexamethasone impaired wound and unimpaired secondary wound at 4th and 7th days in compare with normal wound healing.

The histomorphometric and histological findings

Inflammation

The results of this study showed antiinflammatory effect of curcumin during all phases of wound healing, in study group treated with curcumin with less inflammatory intensity than in control group at the 3rd, 7th and 14th days.The results agree with Margina et al., 2013; Cheppudira et al.,2013 reported curcumin was the active ingredient of curcuminoid with potent anti-inflammatory effects that reduced the inflammatory reaction.

Meng et al.,2013 reported curcumin down-regulating the activity of cyclooxygenase-2 (COX-2), that interfere with the signaling mechanisms governing the transcription of COX-2 should also inhibit the catalytic activity of the COX enzyme and decreases COX-2 expression at the transcriptional level, which inhibit inflammation through reduction PG synthesis (main product of cox-2 pathway) might influence inflammatory cell growth, metabolism, proliferation.Merrell et al. 2009 revealed the anti-inflammatory effect of curcumin through suppress NF-κB activation and proinflammatory gene expression by blocking phosphorylation of inhibitory factor I-kappa B kinase (IκB), that reduce COX-2 and iNOS expression, thus inhibiting the inflammatory process.

Reepithelization

In the present study, the histological examination demonstrated that the re-epithelization process in the study group was much faster than control group. Curcumin induce epithelial proliferation and movement toward the center area of the ulcer in study group, at the 3rd day was faster and produced obviously reduction in ulcer diameter, at the 7th day nearly complete reepithelization and at 14th day complete reepithelization, while in control group, the epithelial proliferation was limited rates, with incomplete reepithelization at 14th day.

The results of present study agree with Kant et al., 2014 who reported curcumin treatment reduced time to epithelialization, by switching of keratinocytes from sedentary phenotype to migratory and proliferative phenotype, which accelerate wound closure, by reepithelialization of the epidermis.

Also Partoazar et al. 2016 documented that curcumin induce complete and mature re-epithelization in epidermis through stimulation of keratinocyte migration and proliferation that restore epithelial barrier of burn wound faster than control group.

Also Kulac et al., 2013 reported that curcumin enhance epithelial keratinocyte to restore wound healing, which accelerated epithelialization to begin at 4th days and mediate potent epithelilazation at 8th days and complete healing at 12th days.

Curcumin induced wound healing faster, Thangapazham et al. 2007 reported curcumin act as antioxidant and reactive oxygen species scavenger, which protects keratinocytes from the oxidative damage of lipid peroxide and improved activity of keratinocyte during wound healing.

Curcumin maintained the keratinocytes activity**,** Gadekar et al. 2012 reported Curcumin possessed powerful inhibitory capacity against H2O2 that induced

damage in human keratinocytes and fibroblasts lead protection effect and contribute to wound healing.

Angiogenesis

Angiogenesis was important point for tissue engineering, to enhance cell proliferation and promote wound healing (schultze et al. 2003). The results revealed the proangiogensis effect of curcumin by inducing new blood vessel formation in wound bed especially at 7th day of healing period.

The results agree with Kant et al. 2014 reported topical application of curcumin significantly up-regulated the mRNA and protein expression of vascular endothelial growth factor (VEGF) and transforming growth factor beta 1(TGF-β1) mainly from day 7 till day 14 of wound healing. VEGF has been shown to induce angiogenesis by promoting endothelial cell proliferation and prevent their apoptosis and TGF-β1 involved in angiogenesis by several possible molecular signaling including enhancing VEGF synthesis through Akt and ERK pathways or by recruiting VEGF-expressing hematopoietic effecter cells (Mallet et al., 2006).

Curcumin acted as proangiogenic medication to induced wound healing, therefore it revealed synergistic effect of new blood vessel formation and stimulation of endothelial cell by increase expression of angiogenic growth factors such as VEGF, Angiopoietin 1(Ang-1), basic fibroblast growth factor (bFGF) (Milovanova et al. 2008; 2009).

CONCLUSION

Curcumin enhance oral mucosal ulcer healing faster than in normal healing.

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