Histological Evaluation of Wound Healing Effect of Topical Phenytoin on Rat Hard Palate Mucosa

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ABSTRACT

Extension and duration of wound healing following periodontal surgery are very important. The aim of present study was histological evaluation of wound healing of topical phenytoin on rat hard palate mucosa. A total of 60 rats were randomly divided into four groups of 15(n=15). A standard 4×6 mm diameter wound was created on the hard oral palate of each rat. The control group were given an equal volume of normal saline. The group of phenytoin and chitosan gel received the topical gel of phenytoin and chitosan, respectively. The fourth group were received a dosage of 10mg phenytoin daily. Five rats each were sacrificed and all sections were examined for histologic changes by light microscopy. The mean number of neutrophils, fibroblasts, macrophages, epithelialization, and the density of collagen fibers were evaluated in each group. Data were analyzed using ANOVA and Kruskal-Wallis tests. The number of fibroblasts and the rate of epithelialization in the group of phenytoin gel were significantly higher on the 7th day than the control group (P <0.05). The density of collagen fibers on the 14th day was significantly higher in the group of phenytoin gel than the control group (P <0.05). It can be concluded that topical phenytoin to promote wound healing of rat hard palate.

Key words: Wound healing, Phenytoin, Rat, Hard palate

INTRODUCTION

Localized gingival recession usually occurs due to traumatic brushing habits, incorrect occlusal relationships, periodontal inflammation, frenum (muscular attachment between two tissues), and orthodontic tooth movement [1]. Mucogingival is a wide-ranging term that comprise the modification of relationships between the gingiva and the oral mucous membrane concerning three specific problem areas: attached gingiva, shallow vestibules, and a frenum interfering with the marginal gingiva. Furthermore, mucogingival surgeries intended to preserve attached gingiva, remove frena or prevent continuous loss of attachment, and to increase the depth of the vestibule [2]. There are various techniques for increasing the zone of attached gingiva, deepening of shallow vestibules, and covering of the denuded

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root surface including free gingival autograft and free connective tissue autograft [2].

The free gingival autograft is a method for increasing the width of attached gingiva. These grafts are frequently harvested from the palate. Free gingival autograft from palatal tissue leaves an open wound in the donor site and causes a postoperative pain and morbidity (bleeding, abscess, and necrosis) and result in secondary healing. Although there is no quick fix for post-surgical healing, there are some ways to make the process go as quickly as possible. 2Various pharmacological agents such as antibiotics, vitamins, minerals, and growth factors accelerate the healing process [3-6]. The apparent stimulatory effect of topical phenytoin has prompted its assessment in wound healing [5].

Phenytoin is widely used for healing of decubitus ulcers, venous stasis ulcers, diabetic ulcers, traumatic wounds, burns, and leprosy trophic ulcers. Although the mechanism of wound-healing properties of phenytoin remains unknown, in vitro studies suggest that phenytoin may promote wound healing through multiple mechanisms, including stimulation of fibroblast proliferation, facilitation of collagen deposition, glucocorticoid antagonism, antibacterial activity, and decreasing the formation of wound exudate [7]. As well as, phenytoin can help neuropathic pain by affecting the stability of the cell membrane and nerve fiber and modulating the inflammatory response [8, 9]. The aim of present study was histological evaluation of wound healing of topical phenytoin on rat hard palate mucosa.

MATERIALS AND METHODS

The present study was a quasi-experimental study conducted in eight following stages.

Stage 1: drug preparation
Preparation of chitosan-based phenytoin was conducted at the faculty of pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. To prepare 50 g of phenytoin gel, at first, 15 cc of chitosan solution 60% (Sigma, Germany) was prepared and stored overnight. Then, 25 cc of polymeric solution containing 1g polyvinylpyrrolidone (sigma, Germany) was added to the chitosan. 0.5 g of drug was dissolved in 10 ml ethanol. The solution was added gradually with constant stirring until a viscous gel was formed. The stability of the active substance

after one and three months was investigated under controlled laboratory conditions.

Stage 2: preparation of rats
A total of 60 adult male Wistar rats weighing 200±10 g were provided from the center of laboratory animal husbandry of Ahvaz Jundishapur University of Medical Sciences (Ahvaz, Iran). Five rats per cage were housed in a temperature-controlled (20-22 °C) animal room with a 12-hour light-dark cycle.

Stage 3: classification of rats
The rats were divided into 12 groups of 5. These groups included:
1- normal saline group (for three days), 2- normal saline group (for seven days), 3- normal saline group (for 14 days), 4- topical phenytoin gel group (for three days), 5- topical phenytoin gel group (for seven days), 6- topical phenytoin gel group (for 14 days), 7- chitosan group (for three days), 8- chitosan group (for seven days), 9- chitosan group (for 14 days), 10- oral phenytoin group (for three days),11- oral phenytoin group (for seven days),12- oral phenytoin group (for 14 days). Since chitosan polymer was used as the basis for the preparation of the phenytoin gel, in order to eliminate its interfering effect, the groups 7, 8, and 9 were included in the present study.

Stage 4: survival Surgery Procedures
General anesthesia was administered by intramuscular injection of xylazine (5 mg/kg) and ketamine (75 mg/kg). The animals were then placed on a surgical table. The mouths of the rats were completely opened by the insertion of two looped nylon yarns in the apical crown of the maxillary and mandibular central incisors. Immediately, two grooves were created in the incisive papilla area in the form of full thickness with dimensions of 6 × 4 mm and a thickness of approximately 1-2 mm. The bleeding was controlled by placing sterile cotton swabs and putting pressure on the position, and each animal was placed in its own cage. After 24 hours the wounds were cleansed with sterile physiological saline.

Stage 5:
Postoperative medication management
Postoperative medication management was started a day after surgery. The first three groups received sterile physiological saline for treatment. The second three groups received topical phenytoin gel. The third three groups received

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chitosan and the fourth three groups received 10 mg daily oral phenytoin through gastric feeding tube for wound healing. All treatments were performed for each group three times a day. For medication administration, the rat was kept in a state of consciousness by an experienced person and topical medications were administered by another person using swabs in the area. Meanwhile, the rat was prevented from eating for two hours.

Stage 6: Sacrificing the rats
The 3, 7, and 14 day groups were sacrificed on 3rd, 7th, and 4th days, respectively. The rats were sacrificed by cervical dislocation under deep anesthesia using 100 mg/kg of ketamine hydrochloride (Pfizer, Kent, UK).

Stage 7:
Preparation of the sample for histological examination
One piece of foil with dimensions of 6 × 4 mm was placed on the surgical site and the sample was cut and lifted up with a forceps from the tissue under repair. The tissues samples were fixed in 10% (v/v) neutral buffered formalin. The specimens that have been formalin-fixed embedded in paraffin and later sectioned and stained with hematoxylin and eosin (H&E).

Stage 8:
Histopathological examination of samples
Histologic examination was performed by an experienced pathologist followed by blinded review of target tissues to determine no-effect levels. The process of wound healing was evaluated by comparing histological criteria including the presence of inflammatory cells, capillary lumen, fibroblast cells, collagen fibers, and the rate of re-formation of the epithelium of the oral mucosa.

Four randomly selected sites were analyzed under the light-optical microscope at a magnification of 400x. Inflammatory cells (neutrophils and macrophages), fibroblast cells, and capillary vessel lumens were counted and their mean values were separately recorded. As well as, the percentage of epithelialization compared to the total volume of the cells of the epithelium cells lost in wound surface was recorded. In addition, to measure the amount of collagen fiber accumulated in the wound area, the density of strands was recorded in three degrees: (0= no collagen fibers),

one (1= low density), (2= medium density), and (3= high density).

RESULTS
The number of neutrophil cells was observed best on the 3rd day and then gradually decreased on the 7th and 14th days, respectively ($p < 0.05$). There was no significant difference between the groups in the 3rd day (P = 0.22), 7th day (P = 0.20) and 14th day (P = 0.35). On the 3rd day, macrophage cells had the highest number in all groups and were decreased on the 7th and 14th days. The rate of macrophage cell reduction on 14th day compared to the 7th and 3rd days was not statistically significant in any of the groups (P> 0.05). No significant difference was observed among all groups on the 3rd day (P = 0.22), 7th (P = 0.26) and 14th days (P = 0.90). The number of fibroblastic cells increased from day 3 to 7, respectively and this increase was significant in all groups, control group (P = 0.004), phenytoin gel group (P = 0.002), oral phenytoin group (P = 0.04). The number of fibroblastic cells on the day 14 compared to the 7th day decreased significantly in all groups, control (P = 0.005), phenytoin gel (P = 0.006), oral phenytoin (P = 0.026), chitosan base (P = 0.04). On the 7th day, the number of fibroblastic cells in the phenytoin gel group was higher than the other groups. There was a significant difference between the phenytoin gel group and control group (P = 0.015). The rate of blood vessels was increased on the 7th day compared to the 3rd day in all groups and this increase was significant only in the phenytoin gel group (P = 0.04). After a while, the rate of blood vessels was gradually decreased in all four groups up to the 14th day, but this decline was not significant in any of the groups (P> 0.05). There was no significant difference among the 3rd (P = 0.75), 7th (P = 0.15) and 14th days groups (P = 0.22). The rate of epithelialization was gradually increased from day three to 14th day in all groups. This increase was not significant in any of the groups from day three to seventh (P> 0.05), but from the 7th day to the 14th day, this increase was significant in all groups, control group (P = 0.004), phenytoin gel group (P = 0.003), oral phenytoin group (P = 0.086), and control group (P =0.026). On the 3rd day, there was no significant difference between the groups (P> 0.05). On the 7th day, the rate of epithelialization in the phenytoin gel group was higher than the other groups, oral phenytoin group (P = 0.02), chitosan base group (P = 0.004), and control group (P = 0.003). The collagen fibers were increased in all groups from 3rd to 14th day.

The increase of collagen fibers from the 3rd to the 7th day was not statistically significant (P> 0.05). The increase of collagen fibers was significant on the day 14 compared to the 7th day in the chitosan base group (P = 0.02) and phenytoin gel group (P = 0.004).

There was no significant difference among all groups in the 3rd and 7th days (P> 0.05). On the 14th day, the highest density was observed in the phenytoin gel group, which showed a significant difference compared to the control group (P = 0.001) and oral phenytoin group (P = 0.002).

DISCUSSION

Phenytoin is an anti-epileptic drug, also known as an anticonvulsant. It works by slowing down impulses in the brain that cause seizures, but various side effects have been reported. The common side effect of phenytoin is the development of fibrous overgrowth of gingiva and the same effect has led to the use of this drug in accelerating wound healing [5]. In acute wounds, the provisional wound matrix, containing fibrin and fibronectin, provides scaffolding to direct cells into the injury, as well as stimulating them to monocytes/macrophages. Neutrophils are usually depleted in the wound and replaced by macrophages, lymphocytes and mast cells. These inflammatory cells secrete many factors that can recall the resident fibroblasts and mesenchymal progenitor cells into a temporary matrix [10]. Experimental studies suggest that using phenytoin cause stimulation of fibroblastic proliferation, enhancing the formation of granulation tissue, decreasing collagenase activity (by reducing its production or secretion or both), promoting deposition of collagen and other connective tissue components, decreasing bacterial contamination, and reducing exudates [11].

In the present study, the highest expression quantities in neutrophil cells were observed on the 3rd day and gradually decreased on the 7th and 14th days. But, on the 3rd, 7th, and 14th days, there was no significant difference among the groups. Based on the obtained results, the chitosan base group reduced the neutrophil count more on the 3rd day, and this decrease was not statistically significant. Reduction of neutrophil counts on the 3rd day in the chitosan group can be attributed to its antimicrobial and bacteriostatic activity [12]. Chitosan exhibits potent

efficacy against various bacteria such as *E.coli, S.aureus, P.aeruginosa, B.subtilis, and A.niger* [13]. Histological analysis showed that neutrophils decreased more on the 7th and 14th day in the group of phenytoin gel, but there was no statistically significant difference among the groups. Elzayat *et al*., 1989 in a study concluded that phenytoin decreases the bacterial load of wounds and reduces the *S. aureus, E. coli, and Klebsiella* bacteria in the initial 7-10 days of the wound creation [14].

M.Ali *et al*., 2012 in a study suggested that phenytoin has antimicrobial properties against *E. coli, B. subtilis, S. aureus, A. niger,* and *Candida albicans* [15]. In the present study, macrophage cells had the highest rates on the 3rd day and then gradually decreased, but there was no significant difference on 3rd, 7th, and 14th days among the groups. The macrophage level has remained highest in the phenytoin gel group.

Macrophage is the largest cell mediator in the turnover connective tissue, and it is considered to be the most important coordinator of wound healing. This cell is involved in the processes of receiving, augmenting, and transmitting signals to fibroblasts, endothelial, and smooth muscle cells by producing catabolic proinflammatory cytokines. As well as, macrophages influence wound healing through the secretion of growth factors that promote cell proliferation and protein synthesis.16 Macrophage serves as the coordinator of wound healing process, by producing growth factors such as TGFB1, FGF, and vascular EGF (VEGF) [10].

Sayar *et al*., in 2012, showed that following the use of phenytoin TGFB and VEGF were increased [17]. Serra *et al*., 2010 examined the effect of phenytoin on cytokines and lipopolysaccharide-induced macrophage and concluded that macrophages secrete less TNFα in the presence of phenytoin, which allows inflammation and collagen accumulation [16].

In the present study fibroblasts were increased on the 7th day than the 3rd day, and then gradually decreased until the 14th day. There was no significant difference in the number of fibroblast cells in the 3rd day among all groups. The difference was significant among the 7th day groups and the number of fibroblasts in the phenytoin gel group was higher than the other groups. The only significant difference was observed between the base and control groups. It

can be suggested that the phenytoin gel proliferates fibroblasts in the first week, possibly due to the secretion of growth factors produced by macrophages. Kanaparthy *et al*., 2012 examined fibroblasts and phagocytic cells in phenytoin-induced connective tissue proliferation and concluded that phenytoin increase fibroblast, macrophage, lymphocyte, collagen and capillary vessels, which consistent with the results of the present study [18].

Sayar *et al*., 2012 in an animal study showed that phenytoin accelerates epithelialization, vascular granulation tissue, fibroblast count, and collagen synthesis [17]. MOY *et al*., 1985 concluded in their study that the effect of phenytoin on fibroblasts depends on its concentration and duration of shelf-life, and stated that the phenytoin would clearly increase proliferation of fibroblasts at low concentrations (5mg/l) and short-term incubation (3h) [19].

According the results of present study, the vascularization on the 7th day compared to that of the 3rd day increased in all groups, and then gradually decreased until the 14th day. However, there was no significant difference among the groups in the 3rd, 7th, and 14th days. Vascularization was higher in the group of phenytoin gel on all days.

As it was previously mentioned, the fibroblast cells increased in the first week and then gradually declined in the second week. The vascularization also follows the same pattern (increase in the first week and decrease in the second week), which can be attributed to growth factors secreted from macrophages. The growth factor is a naturally occurring substance capable of stimulating cellular growth (vascular endothelial growth factor and basic fibroblast growth factor levels) during the first week after wound healing. The result of present study is consistent with the results of the Sayar *et al*.'s study, but the difference was not statistically significant.

Turan *et al*., 2004 in a study concluded that topical application of phenytoin would increase fibroblast, collagen, vascular endothelial growth factor, and basic fibroblast growth factor [20]. In the present study, the collagen fibers were increased from 3rd day to 14th day, while there was a statistically significant difference in the 14th day groups. The highest level of collagen fibers was related to phenytoin gel group and the lowest was observed in the control group.

The results of present study are consistent with the Sayar, Kanaparrty, and Turan studies. Shikata *et al*., 1993 in a study showed that the increase deposition of type VI collagen in phenytoin-induced gingival enlargement was significantly higher than predicted [21].

Therefore, it can be suggested that phenytoin increases the secretion of growth factor through stimulation of macrophages release. Fibroblasts cells subsequently increase and leads to increased collagen deposition in wound tissue.

The present study showed that the rate of epithelialization has been gradually increased in 14 days in all groups. The difference between the percentage of epithelialization in the 3rd and 14th days was not statistically significant among all groups, but there was a significant difference on the 7th day group. In the phenytoin gel group, the highest epithelialization rate was seen on day 7, which is consistent with the results of other studies.

Sayar *et al.,* study in 2012 also showed that the use of topical phenytoin accelerates epithelialization [17].

Similarly, Meena *et al.,* 2011 examined the effect of topical phenytoin on burn wound healing in rats. They showed that phenytoin accelerates wound healing and reduces epithelialization and wound closure more quickly than control group [22].

Pereiva *et al.,* 2010 examined the effect of phenytoin on cutaneous healing from excision of melanocytic nevi on the face and concluded that phenytoin exhibits better therapeutic and cosmetic results, faster healing with more intense epithelialization in wounds compared to control group [23].

Jyoti *et al.,* 2001 in a study stated that stimulated epithelialization of wounds and accelerated healing using topical phenytoin are attributed to keratinocyte growth factors (KGF) [24].

CONCLUSION

According to this study, phenytoin showed significant healing improvement by stimulating fibroblast proliferation, increasing collagen deposition and accelerating epithelialization.

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