

Effect of Topical Erythropoietin (EPO) on palatal wound healing subsequent to Free Gingival Grafting (FGG)

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Abstract: Free gingival grafting, the most predictable technique to increase the keratinized gingiva, leaves an open wound on the palate and the resulting discomfort during the healing phase is a significant concern. This study was intended to evaluate the effect of topical erythropoietin on healing of the donor site. Twelve patients lacking an attached gingiva at two sites in the mandible were included. In the test group, 1 mL of gel containing erythropoietin at a concentration of 4,000 IU mL⁻¹ was applied to the donor site, whereas the control group was treated with 2 mL of the gel alone. On the second day after surgery, the same procedure was repeated. H₂O₂ was used to evaluate the amount of epithelialization. Clinical healing was compared using photographs and direct examination. The EPO group showed significantly better keratinization only on day 21. Comparison of clinical healing based on direct examination revealed significantly better healing in the test group on day 28. Furthermore, inflammation in the test group was lower than in the control group on the same day. Topical application of EPO improves palatal wound healing during the third and fourth weeks after free gingival graft procedures.

Keywords: Erythropoietin; Controlled Clinical Trial; Wound Healing; Palate.

Declaration of Interest: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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<https://doi.org/10.1590/1807-3107bor-2018.vol32.0055>

Introduction

A free gingival graft is considered to be the most reliable method of increasing the attached gingiva, and is one of the most frequently-used technique.^{1,2} This treatment modality is used to increase the keratinized gingiva, prevent and treat gingival recession, improve esthetics, reduce or eliminate root hypersensitivity, increase the vestibular depth and ameliorate pigmented and pathologic oral mucosa.^{3,4,5,6} Harvesting a free palatal graft leaves an open wound at the donor site, which takes two to four weeks to heal.⁷ Patients' post-operative pain and discomfort and the delayed wound healing in the palate is a great concern.^{1,8}

There have been several animal studies as well as human Randomized Clinical Trials (RCTs) which aimed to alleviate patient morbidity. Low-powered laser treatment,⁹ topical ozonated oil,¹⁰ platelet-rich plasma¹¹ and Platelet- rich Fibrin (PRF)¹² have resulted in faster healing and less pain in patients. Meanwhile, anti-advanced glycation end-product (AGE)

Submitted: October 08, 2017
Accepted for publication: April 10, 2018
Last revision: May 09, 2018



agents have been suggested to facilitate palatal wound healing by reducing AGE-associated inflammation and enhancing recovery in rats.¹³

Wound healing is a complex phenomenon involving several distinct but overlapping stages.^{8,9,14} According to some experimental studies, erythropoietin (EPO) plays an essential role in promoting skin wound healing.^{15,16,17} EPO is a 34 kDa glycoprotein hormone that belongs to the hematopoietic class I cytokine superfamily. It is responsible for controlling red blood cell mass by provoking the proliferation and differentiation of precursor cells, and hindering the programmed cell death (apoptosis) of erythroid cells in bone marrow.¹⁸ It has been demonstrated that the receptor of EPO is also expressed on cells other than hematopoietic cells. The cardiovascular system, via its endothelial cells, cardiomyocytes, and circulating endothelial progenitor cells,^{16,17,19} as well as the nervous system, via its neurons, astrocytes, and vascular endothelial brain cells, are the main nonhematopoietic targets of EPO.^{16,17,19,20} Moreover the skin is another major target for EPO and as the EPO receptor (EPOR) is expressed on endothelial cells,^{21,22} macrophages, fibroblasts, mast cells, melanocytes, and hair follicles, it has been suggested that EPO is capable of accelerating the skin wound healing process through stimulation of angiogenesis, formation of extracellular matrix and collagen, restriction of inflammatory mediators, and apoptosis in the wound healing process.^{18,23,24} Several investigations concluded that EPO plays a pivotal role in skin wound healing. It has been demonstrated that systemically-administered EPO improves skin wound healing in diabetic mice.^{15,16,17} Hamed *et al.* were the first to investigate the topical application of EPO in accelerating wound healing and showed that it can significantly promote skin wound healing in diabetic rats.^{25,26} Subsequently, Bader *et al.* were the first to demonstrate the acceleration and improved healing of acute and chronic wounds using topical EPO in human skin.²⁷ Furthermore the expression of EPORs has been observed in the basal cell layer of normal oral mucosa.²⁸ Accordingly it was hypothesized that topical application of EPO might improve wound healing in the oral mucosa.

Considering that EPOR expression has been observed in different cell types, including the basal

cell layer of normal oral mucosa,^{18,21,24,28} and that it can improve skin wound healing through multiple mechanisms,^{3,16,17,25,27} we hypothesized that topical application of EPO might improve the wound healing process in the oral mucosa. The aim of this study was to evaluate the effect of EPO treatment on palatal wound healing subsequent to free gingival grafting.

Methodology

Participants and study design

This was a randomized triple-blind placebo-controlled clinical trial using split-mouth design. The study was conducted in the periodontics department of Tehran University of Medical Sciences (Tehran, Iran; October 2016–June 2017).

Twelve periodontally-healthy patients, aged over 18 years with lack of adequate attached gingiva on at least two sites in the mandible were included in the study. Patients with any systemic disease or condition, inadequate oral hygiene, poor compliance and also smokers were excluded from the study.

Informed written consent was obtained from each patient after explanation of the study goals, design, potential risks, and benefits. The study was conducted in accordance with the Helsinki Declaration. The medical ethics committee of Tehran University of Medical Sciences approved the study protocol (approval number: 119225).

Assuming a difference rate of 40% for placebo control, a clinically-relevant treatment difference could be detected at a significance level of 0.05, considering the sample size of at least 12 subjects in a split-mouth phase 2 trial. The α and β error were estimated as 0.05 and 0.2, respectively.

Following enrollment, participants were assigned to treatment groups using balanced block randomization. The surgeon, the observers and the patients were blinded. To meet the assumptions of randomization, a number of sealed non-transparent coded envelopes were prepared with letters A, B, C, or D printed on them. Each patient was then required to choose one. If A was chosen, the patients were assigned to undergo the first surgery on the right side, followed by treatment with EPO-containing gel. A patient choosing B was assigned to the group undergoing the

first surgery on the right side, but with the application of the vehicle gel. For Patients choosing C, the first procedure was performed on the left side, followed by EPO gel application, while group D received the same treatment, but with the vehicle gel. A third individual opened each envelope and explained the sequence of surgery and gel application to the surgeon. The gels for both groups had the same appearance and there were no marks, colors or numbers on the containers, and only the third individual was aware of the gel types. Observers were not informed about the grouping of patients. Patients did not receive information regarding the type of treatment that was performed. The statistician was also blinded to the groups.

The pre-surgical and surgical phase

At the first appointment, after oral hygiene instruction, scaling and root planing was performed for each patient. Thereafter, a plaque control assessment was carried out every two weeks for one month. Once a plaque index below 15% was achieved, the patient entered the study.

The surgery was carried out according to the method of Sullivan and Atkins.²⁹ After recipient site preparation, a standardized 10 × 15 mm template was used to obtain an identical palatal wound area. A graft was harvested from the premolar and first molar area of the hard palate, and a 1.5 mm deep wound area was created in the donor site, measuring 10 × 15 mm. Harvesting grafts from the same site in all patients resulted in a uniform technique to compensate for the differences in healing potentials of the premolar and molar areas. In addition, to avoid the rugae region, the harvesting site was moved in a posterior direction to the molar area. Moreover, other well-designed studies with the same procedure, utilized the exact same technique.^{22,23} The soft tissue graft was fixed in the recipient site.

After control of bleeding at the donor site, 1 mL of gel containing 4,000 IU mL⁻¹ EPO was applied to the test site, while the control site was treated with 2 mL of gel that did not contain EPO. The wound sites were then covered with a foil and a coe-pak was placed over them.

EPO-containing gels were prepared by mixing 4,000 IU EPO with 1 mL of 3% hydroxy ethyl cellulose

the day of surgery, while the same gel with 1.5% concentration was applied at the control site.

Because of the favorable consistency of the gel applied, there was no concern about its displacement during or after surgery. Postoperative instructions were described for each patient after surgery. Another gel application was performed 2 days after surgery and the periodontal pack was placed on the wound area.

Clinical examinations

Epithelialization

A 3% H₂O₂ solution was utilized to evaluate the wound epithelialization rate. When no epithelium is formed in the wound, H₂O₂ interacts with the catalase in the connective tissue and bubble formation occurs. Conversely, no bubbles develop if the epithelium covers the wound surface³⁰. The amount of epithelialization was assessed on days 7, 14, 21, and 28 after surgery. The results were reported as 0, $\frac{1}{3}$, $\frac{2}{3}$, $\frac{3}{3}$, for each wound site.

Healing and inflammation

Evaluation of the healing rate and the amount of inflammation was performed in two ways:

Direct observation of the region by a blinded periodontist on days 2, 7, 14, 21, and 28 after surgery.

Observation of photographs by three periodontists; photographs taken from test and control areas on days 2, 7, 14, 21, and 28 were prepared in one slide using a graphic software program (Power Point, using the ACDC program).

Three periodontists observed the slides and determined which site had better healing after considering the color match, the tissue texture and contour. The inflammatory halo around the wound was used to characterize the site with more inflammation (Figure 1).

Statistical analysis

The Wilcoxon sign test was performed to identify significant differences in epithelialization between the test and control groups. The degrees of inflammation and repair were compared between groups using the sign test. The level of significance for rejection of the null hypothesis was set at 0.05. All statistical analysis were performed with SPSS, version 22 (SPSS Inc., Chicago, IL, USA).

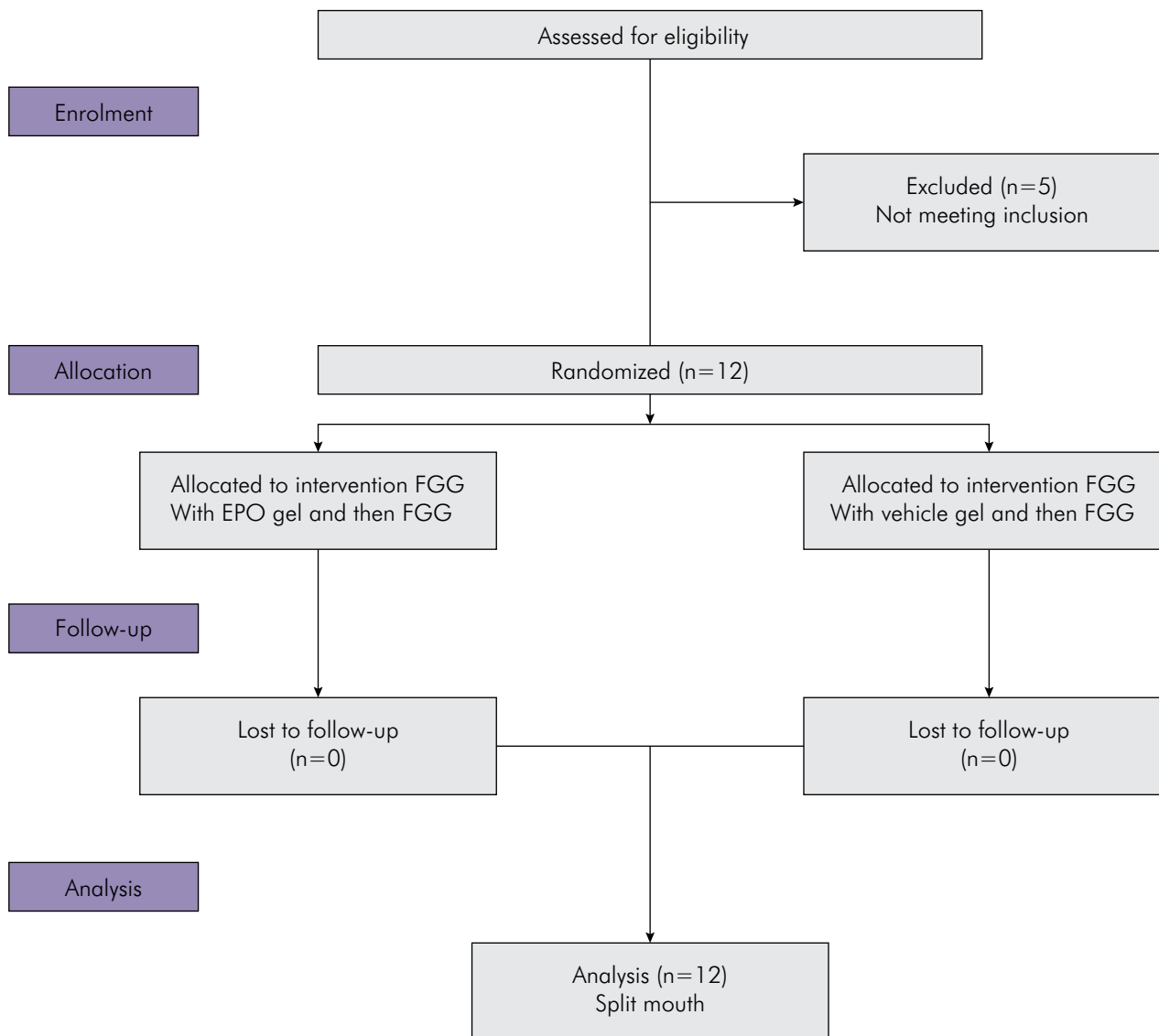


Figure 1. Clinical views of wound area, in the EPO and Control group: (a, b) harvesting free gingival graft; (c, d) 2 days after surgery; (e, f) healing on day 7; (g, h) healing on day 14; (i, j) healing on day 21; (k, l) healing on day 28; and (m, n) healing on day 60.

RESULTS

Participants' demographics

Twelve patients (three males and nine females), aged between 30 and 53 years, with a mean age of 44.58 ± 7.5 were evaluated for 28 days postoperatively (Figure 2).

Clinical parameters

None of the patients complained of loss of sensation or bleeding, and healing was uneventful.

No unwanted side-effects were observed after application of the gel in either group. By the end of the second week one patient in each group achieved complete epithelialization, with no significant difference between groups (Table 1). At the end of the third week, complete epithelialization was observed in ten subjects from test groups and six patients from control groups, with a statistically-significant difference in favor of the test group ($p < 0.05$). By the 30th day after surgery almost all patients exhibited complete epithelialization.

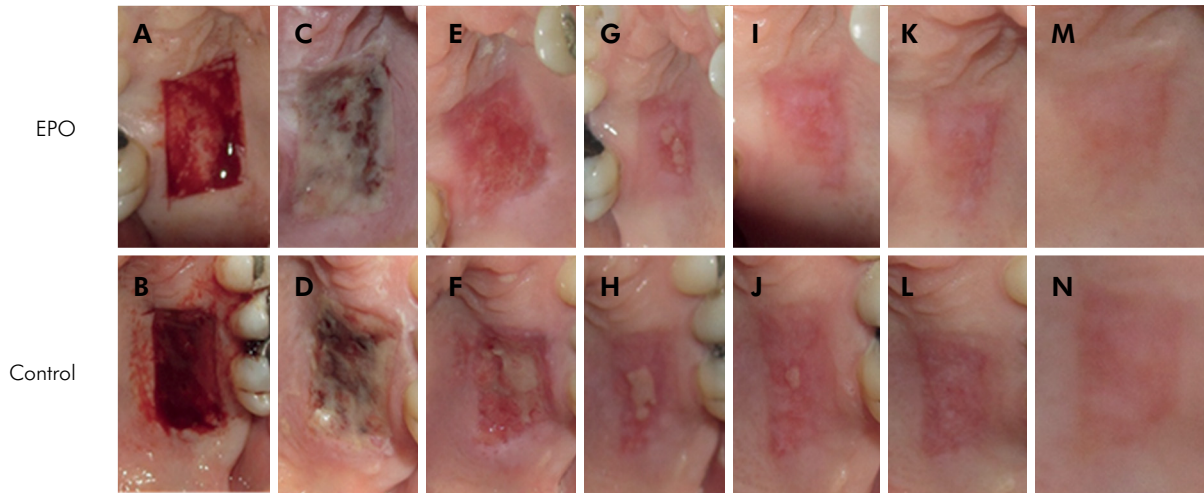


Figure 2. CONSORT statement flow diagram.

Table 1. Comparison of epithelialization rate.

Epithelialization	0	1/3	2/3	3/3	p-value
Day 7					
EPO	9	2	1	0	
control	11	1	0	0	0.08
Day 14					
EPO	0	3	8	1	
control	1	4	6	1	0.2
Day 21					
EPO	0	0	2	10	
control	0	1	5	6	0.02*
Day 28					
EPO	0	0	0	12	
control	0	0	1	11	0.1

EPO; Erythropoietin. *significantly different between two groups; No difference was found by intergroup comparison ($p > 0.05$).

Regarding clinical healing based on direct examination of the area, during the first three postoperative weeks, no statistically-significant difference was observed. Only on the 28th day, the test group showed significantly better healing compared to the control group (Table 2). Moreover the test group displayed significantly less inflammation on the same day (Table 3).

According to photographs observed by three periodontists, no difference was demonstrated between the groups regarding the clinical healing rate or the amount of inflammation (Tables 2 and 3).

Table 2. Comparison of healing rate based on photography and direct examination.

Healing		EPO > control*	EPO < control	EPO = control	p-value
Observer 1	Day 2	5	3	4	0.3
	Day 7	4	3	5	0.4
	Day 14	4	4	4	0.5
	Day 21	5	4	3	0.4
Observer 2	Day 28	2	1	9	0.2
	Day 2	4	2	6	0.2
	Day 7	4	4	4	1.2
	Day 14	3	4	5	0.5
Observer 3	Day 21	4	1	7	0.1
	Day 28	1	0	11	0.1
	Day 2	4	4	4	0.5
	Day 7	5	4	3	0.4
Direct examiner	Day 14	5	4	3	0.4
	Day 21	5	3	4	0.3
	Day 28	5	3	4	0.3
	Day 2	5	2	5	0.2
Direct examiner	Day 7	5	2	5	0.2
	Day 14	5	3	4	0.3
	Day 21	5	2	5	0.2
	Day 28	4	0	8	0.02*

EPO: Erythropoietin. No difference was found by intergroup comparison ($p > 0.05$).

Table 3. The amount of inflammation based on the photography and direct examination.

Inflammation	EPO < control	EPO > control	EPO = control	p-value	
Observer 1	Day 2	5	3	4	0.3
	Day 7	5	3	4	0.3
	Day 14	6	2	4	0.1
	Day 21	5	3	4	0.3
	Day 28	2	1	9	0.2
Observer 2	Day 2	4	1	7	0.1
	Day 7	4	4	4	0.5
	Day 14	3	2	6	2.9
	Day 21	3	2	7	0.3
	Day 28	1	0	11	0.1
Observer 3	Day 2	5	3	4	0.3
	Day 7	6	3	3	0.3
	Day 14	4	5	3	0.6
	Day 21	5	3	4	0.3
	Day 28	5	3	4	0.3
Direct examiner	Day 2	5	1	6	0.08
	Day 7	5	3	4	0.3
	Day 14	5	3	4	0.3
	Day 21	5	1	6	0.08
	Day 28	4	0	8	0.02*

EPO: Erythropoietin. No difference was found for intergroup comparison ($p > 0.05$).

Discussion

Free gingival grafting is one of the most common modalities for increasing the keratinized gingival.² After harvesting the graft, approximately two to four weeks is required for the open wound left at the donor site to heal, and as a result, patients experience postoperative pain, excessive bleeding and discomfort during the early healing phase. This morbidity and the delayed wound healing in the palate has been a cause of great concern.^{7,8}

Various efforts have focused on improving palatal wound healing. Keceli et al.³¹ evaluated the effectiveness of an herbal extract on early wound healing following FGG and found fewer incidences of primary and secondary bleeding as well as lower pain levels during the first postoperative week. In a human study Patel et al.¹⁰ demonstrated that ozonated oil was

able to accelerate palatal wound epithelialization in a significant manner, compared with the control group. Almedia *et al.* investigated the effect of a low-density laser. They found a slight improvement in clinical healing following laser application although this was not statistically significant.² In an animal study, Shayesteh et al.¹¹ showed that a single dose of platelet-rich plasma did not improve palatal wound healing in dogs following a connective tissue graft. This is the first study conducted to evaluate the therapeutic effects of EPO on palatal wound healing following harvesting of a free gingival graft.

Wound healing is a complex process comprising parallel overlapping events: the hemostatic phase leads to sealing of the wound by a clot formed by extravasated platelets and other blood-derived cells in a network composed of fibrin, fibronectin, and vitronectin. The inflammatory phase develops a catabolic inflammatory process mediated by neutrophils and monocytes which leads to wound cleansing. The new tissue formation phase is initiated by formation of highly-vascularized granulation tissue which is replaced by provisional connective tissue. Three major cell types are involved in this anabolic phase: (1) Endothelial cells and progenitors for the formation of capillaries. (2) Fibroblasts and myofibroblasts required for connective tissue formation and wound shrinkage. (3) Epithelial cells which contribute to re-epithelialization. The remodeling phase leaves a collagen-rich dense matrix containing a few cells which regenerates a stable tissue in the long term.

Positive effects of EPO on wound healing have been demonstrated in several animal studies. Buemi et al.¹⁶ showed that 7-day subcutaneous EPO advanced early and late stages of ischemic wound healing in healthy rats by stimulating angiogenesis and reducing inflammation. In another study they attributed these effects to increased Vascular Endothelial Growth Factor (VEGF) and collagen content.¹⁵ Galeano et al.³² found that two weeks of systemic EPO treatment accelerated wound closure in deep dermal second-stage burns in rats. This improved healing was associated with lower cellularity of the inflammatory response which led to enhanced resolution of granulation tissue and faster tissue remodeling. Tobalem et al.³³ reported a dose-dependent influence of early treatment with systemic

EPO on vascular perfusion and inflammation which could prevent deterioration of burn wounds³³. Studies on human chronic skin wounds have demonstrated the presence of EPO receptors on various cell types such as endothelial cells, macrophages and fibroblasts^{18,21,23,24} as well as its expression on the basal cell layer of the normal oral mucosa.²⁸ Consequently, it is expected to accelerate the wound epithelialization rate. These findings prompted us to investigate its influences on palatal wound healing created following free gingival grafting.

The effect of EPO on palatal wound epithelialization was one of the parameters evaluated in this study. Re-epithelialization is considered an essential part of wound healing. Following tissue injury and epithelial disruption, it is of paramount importance that epithelialization takes place as soon as possible. It takes about 24 hours for keratinocytes to start to migrate into the defect. This phenomenon is achieved through a variety of interactions between cells and the extracellular matrix, and a diverse range of growth factors and cytokines.^{10,14,34,35} The basal keratinocytes at the wound margin change from the stationary to the migratory type when exposed to connective tissue matrix, and migrate to the wound bed. This migration pattern seems to be similar in skin and mucosa, except for hair follicles and sweat glands which serve as migratory cell sources for the skin only.¹⁴

The results of this study showed a significant difference in epithelialization in favor of the test group on day 21. Although not statistically significant, the epithelialization rate was faster in the test group at other follow up visits. The acceleration of acute and chronic wound healing in human skin using topical EPO was first demonstrated by Bader et al.²⁷ They suggested that EPO could considerably improve the wound healing process. They applied a mixture of 3,000 IU EPO and 20 g of hydrogel on an 8 × 24 cm skin graft donor site, which was 0.3 mm deep. The mixture was applied again after 3 and 6 days. Their findings were in line with our study. At the test site the wound was epithelialized within 7 days, whereas the control site showed incomplete epithelialization after this time interval. The greater frequency of drug application and the lesser wound depth of 0.3 mm in their study could justify their better results compared with our findings.

Our findings in this study are in accordance with those of Hamed et al.²⁶ They showed that topical application of EPO to a full-thickness skin wound in diabetic rats improved the healing process in a dose-dependent manner (600 IU mL⁻¹ EPO compared with 3,000 IU mL⁻¹ EPO). Takahashi *et al.* have shown that EPO can stimulate the production of collagen by activating fibroblasts.³⁶ Hamed et al.²⁶ suggested that the underlying mechanisms arising from topical application of EPO in accelerating the wound healing process may include: inducing capillary formation, increasing VEGF and collagen synthesis, and inhibiting apoptosis. Furthermore, angiogenesis could indirectly increase collagen synthesis in the wound area through delivery of more oxygen and other nutrients.¹⁵ However, the test group in the study by Hamed *et al.* showed a significant difference in the healing process from the sixth day. The higher frequency of drug administration in their study (every 2 days for 12 days) could have enhanced the healing rate compared with our study. Furthermore, the larger wound size (9 cm²) and the diabetic status of the rats may be responsible for obtaining more favorable results. Since the diabetic rats showed a decrease in vascularization and collagen production and also an increase in apoptosis in their wound area, the drug could have a more significant effect compared with non-diabetic rats. Moreover, the extra sources of receptors for EPO in the skin compared with the mucosa should not be disregarded.²⁶

In some experimental studies, EPO was administered by intraperitoneal injection and was reported to accelerate skin wound healing.^{37,38} Furthermore, Sorg et al.³⁸ explained the dose-related manner of the effect of EPO on skin wound healing, in which repeated low doses and a single high dose of EPO could improve cell migration and wound healing, whereas repeated high doses of the drug would impair the healing process. In addition, in some animal studies it has been shown that EPO can improve wound healing by means of its anti-inflammatory effect, which is one of the basic mechanisms of healing. Hamed *et al.* demonstrated that EPO decreases the levels of inflammatory cytokines in the skin wounds of diabetic rats.²⁵ In our study we observed a considerable decrease in clinical inflammation in the test group on day 28 compared to vehicle controls.

Comparing the clinical healing based on direct examination of the area, the test group showed significantly better healing only on day 28 ($p < 0.02$), while the healing rate was not significantly better in the test group at any other time-points. According to the photographs observed by three periodontists, however no significant difference was demonstrated between groups at any time-point. Based on these findings, although low frequency topical application of EPO improved the rate of epithelialization on day 21, it did not markedly affect either palatal tissue epithelialization at earlier time-points, or the texture, color and contour of donor tissue at any time-point except for day 28. Overall therefore, it did not show a positive clinical effect on postoperative complications during the first week. Gunter et al. (2013) conducted a prospective, double blind, randomized, multicenter study which investigates the clinical outcome, laboratory tests, gene, and protein expression following systemic administration of EPO in 150 severely burned patients. The patients in the test group are assigned to receive 150 IU/kg EPO every other day for 21 days.³⁹ Accordingly, it could be suggested that clinical outcome could be ameliorated

by increasing the dose of the drug or the rate of its application. Furthermore, we did not use a surgical stent, thus eliminating its protective effect during the healing period. On the other hand, it could lead to rapid drug expulsion and a reduced effect on wound healing.

Limitations: Small sample size, low frequency of drug administration and small wound size were the limitations of our study. In addition subjective data such as pain or number of mediations were not assessed in our study.

Conclusion

According to the results of this study, topical application of EPO could improve palatal wound healing during the third and fourth weeks subsequent to free gingival grafting procedures.

Acknowledgements

The authors acknowledge the assistance of periodontal residents of Tehran University of Medical Sciences in management of patients enrolled in this study.

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