Journal of Oral Hygiene and Health

Papamanoli, J Oral Hyg Health 2021, 9:5

Research Article Open Access

The Role of Epidermal Growth Factor in Early Tissue Healing After Surgical Periodontal Therapy

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Abstract

The aim of the current study was to investigate the potential association of the Epidermal Growth (EGF) Factor levels of mRNA expression in oral epithelium with early healing response after surgical periodontal therapy. The study included forty-three patients undergoing surgical periodontal treatment by means of access flap in maxilla. A gingival tissue fragment was collected from the hard palate adjacent to the surgical field and was further processed for the evaluation of EGF expression in oral epithelial cells using real-time PCR. The trauma left, was followed up for the evaluation of the healing response at 1week (T1), 2 weeks (T2) and 2 months (T3). The modified Early Healing [M-EHI] and the Master Scar Proforma (M-MSPI) Indexes were used for the visual assessment of healing after intraoral photographs were taken. The effect of smoking in the healing period was also assessed. The results showed that EGF expression did not affect the healing response, as the association among the studied variables did not reach statistical significance at any time point. The results were similar even when smoking was considered. In conclusion, our study did not show any statistically significant association between the mRNA expression levels of EGF and the healing response.

Keywords: Periodontics; Epidermal growth factor; Wounds and injuries; Messenger RNA

Introduction

Growth factors are a large family of polypeptidic molecules that regulate key cellular events, including cell proliferation, chemotaxis and differentiation after binding to specific receptors [1]. Research on growth factors has undergone substantial steps during the last decades due to the pivotal role of those molecules in tissue healing, repair and regeneration [1]. Wound healing involves several stages that have been recognized as the inflammatory reaction, the granulation tissue stage and the tissue-remodeling stage [1]. During these events, cell function is regulated by a few elements, counting cell–matrix interactions and soluble molecules such as, cytokines and growth factors [2]. The sequence of cell activation by growth factors includes events that have not yet been elucidated. Platelet-derived growth factors (PDGFs), vascular endothelial growth factor (VEGF), insulin-like growth factors (IGF), fibroblast growth factors (FGFs), epidermal growth factor (EGF), transforming growth factor beta (TGF-β) and bone morphogenetic proteins (BMPs) have been extensively studied in order to understand their roles in wound healing [3]. Current evidence indicates the presence of growth factors in several stages of the healing cascade after trauma. Their release starts immediately after the disruption of blood vessels from local platelets during blood clot formation, they regulate inflammatory cell chemotaxis and proliferation of fibroblasts and monocytes and enhance vascularization [4–6].

Healing of oral mucosal trauma is a particularly interesting phenomenon because gingival tissues heal surprisingly fast when compared with other tissues [7]. Possible explanations of this phenomenon are the presence of saliva in the oral cavity and the high vascularization of the oral mucosa [8,9]. Healing after non-surgical and surgical periodontal therapy follows similar pathways including blood clot formation and tissue remodeling. Research on growth factors on periodontal wound healing and regeneration has shown the potential ability of growth factors, combined or not with bone grafts, to regenerate the periodontal attachment apparatus [1]. Interestingly PDGF-BB combined with β-tricalcium phosphate (β-TCP) has been approved by Federal Drug Administration (FDA) for use in periodontal regenerative surgeries [10].

However, when periodontal wound healing is considered, smoking is a critical constraint. Smoking disturbs healing with the effects of nicotine on cell function and hypoxic effects of carbon monoxide [11–13]. In addition it provokes vasoconstriction and reduces the rate of neoangiogenesis [12]. In periodontology smoking has been recognized as one of the major risk factors of periodontitis [14] and of course attenuates the healing response after surgical and no surgical periodontal treatment [15–17].

The role of EGF in periodontal wound healing has not been clearly described yet. EGF is a small polypeptide growth factor found to be involved in control of epithelial growth and differentiation of periodontal tissues [18]. EGF has been found in milk, urine, amniotic fluid and saliva [19–21] and is involved in several events including embryonic development [22] tissue regeneration [23–25] and healing response [26,27]. Both animal and human trials indicated its role in healing after trauma and ulceration [28–30]. Clinical studies have shown that EGF enhances the proliferation of epithelial cells and fibroblasts [31]. In addition, it has been found in elevated levels in saliva after surgical periodontal therapy [32] and topical application of the growth factor has shown signs of rapid healing [33], indicating its potential role in the repair of oral tissues.

The scope of the current study was to investigate the potential association among the pre-surgical levels of EGF-mRNA in gingival tissues with the early healing rate. In addition, the role of smoking in the previous association was examined.

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Received: April 05, 2021; **Accepted:** April 19, 2021; **Published:** April 26, 2021

Citation: Papamanoli S, Fragkioudakis I, Kalamaki MS, Makris G, Doufexi AE (2021) The Role of Epidermal Growth Factor in Early Tissue Healing After Surgical Periodontal Therapy. J Oral Hyg Health 9: 278.

Materials and Methods

Study design and sample size calculation

The study was designed as a cross-sectional study including patients attending the Postgraduate Clinic of the department of Periodontology and Implant biology of Dental School, Aristotle University of Thessaloniki, for periodontal therapy. All participant's undergone periodontal surgery between September 2018 and May 2019.

The study protocol was approved by the ethics committee of The Faculty of Dentistry of the Aristotle University of Thessaloniki (protocol number, 4/13.02.2019). All participants received written information regarding the aim and the procedures of the study, and they were asked to sign a consent form.

The association among the pre-surgical EGF-mRNA levels and the rate of healing was examined with the Spearman's rank correlation coefficient. The statistical significance level was set at p-value ≤ 0.05, thus the sample size calculation resulted in 38 patients.

Inclusion exclusion criteria

Patients included in the study were referred to the postgraduate periodontology clinic for periodontal treatment. The participants were planned to receive surgical periodontal therapy (access flap). Only systemically healthy adult patients were considered eligible for the study. All participants had to sign their inform consent. Participants were divided in 2 separate groups concerning smoking. Exclusion criteria were set as systemic disease which would not allow surgical periodontal therapy, systemic medication or drugs affecting gingival tissue healing (insulin, calcium channel blockers, bisphosphonates, immune suppressants and anticonvulsants), use of antimicrobials during the last 6 months, pregnancy and lactation and acute inflammation in the oral cavity the previous 3 months.

Surgical process

All surgical procedures were carried out by postgraduate students of the department of Periodontology and implant biology. The main surgical scheme was an access flap for periodontal debridement in the maxilla, with osteotomy or osteoplasty was indicated. The surgical process included local application of anaesthesia, elevation of full-thickness flaps, scaling and root-planing with removal of the granulation tissue, osteoplasty, when needed, and suturing.

Collection of gingival tissue

In each surgical procedure an incision 2 mm in length was made 2mm palataly of the gingival margin and 3mm in depth. From each incision a small fragment of epithelial and connective tissue (0,5 mm) was collected and stored in RNA later (Sigma) at -80° C until RNA isolation.

RNA isolation and reverse transcription

Total RNA was isolated from the tissue fragment using the Nucleospin RNA Kit (Macherey Nagel) with modifications. Briefly, the tissue fragment was removed from the RNA later solution and was ground in lysis buffer in a 2 mL tube containing a mix of 1.4 mm and 2.8 mm zirconium oxide beads (CK mix, Bertin Instruments). Three cycles of homogenization at full speed for 30 s were performed in a Minilys homogenizer (Bertin Instruments) with a 60 s rest on ice. After homogenization, the samples were treated as described in the Nucleospin RNA kit protocol. RNA concentration in each sample was measured using a Genova Plus spectrophotometer (Jenway). 200 μg RNA from each sample were reversibly transcripted to cDNA using the PrimerScript II 1st strand cDNA synthesis kit [Takara] following manufacturer's protocol, using random hexamer primers. The cDNA samples were stored at -20°C until further processing. Quantitative real-time PCR [RT-PCR] was performed in 96-well PCR plates in triplicate. Each 20 μl reaction contained 2μL cDNA, 1μL of each primer [10 μM], 10 μL 2x Luna ® qPCR Master Mix (New England Biolabs) and 6μl RNAse free water. The primers used for EGF expression were EGF-Forward: 5′-CAACCAGTGGCTGGTGAGGA-3′, and EGF-Reverse: 5′-GAGCCCTTATCACTGGATACTGGAA-3′ [34]. The gene encoding for the human β-actin protein was used as a reference gene to normalize target gene expression. Actin primers were Forward: 5' CATGGATGATGATATCGCCGCG 3' and Reverse: 5'ACATGATCTGGGTCATCTTCTCG 3' [35]. The thermal cycler protocol was comprised of an initial denaturation step at 95° C for 3 min, followed by 40 cycles of heating at 95° C for 15 s, then 60° C for 30s followed by a plate read. A melting curve was constructed between 95° C and 60° C with plate reads every 0.5° C to verify the amplified products. A threshold cycle (Ct) was set according to the standard curves constructed for each gene. Relative gene expression analysis was achieved according to the double delta Ct analysis (ΔΔCt) method [36].

Evaluation of the healing response

Post-surgical gingival tissue healing evaluation included the examination of intraoral photographs at 3 different time points after the surgical procedure [1week (1), 2 weeks (T2), 2 months (T3)]. All photos were assessed independently by two different examiners (S.P., E.D.). Two different indexes were used for the assessment of early healing. The early wound healing-index (EHI) proposed by Wachtel and co-workers [37] was modified and used for the evaluation of the healing response during the 1st (T1) and 2nd (T2) week of healing (Figure 1). More specifically the modified Wachtel index (M-EHI) includes.

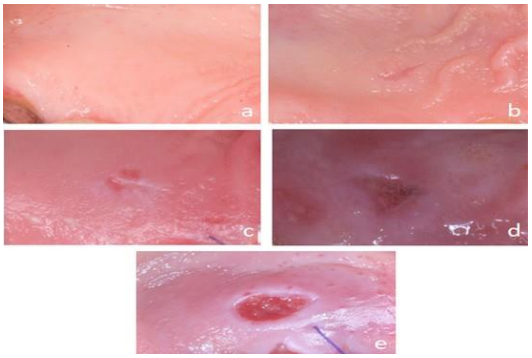


Figure 1: The Modified Early Wound Healing Index (M-EHI).

1: Complete trauma closure – no fibrin line present between the wound edges (Figure 1a).

2: Complete trauma closure – fine fibrin line present wound edges (Figure 1b).

3: Complete trauma closure – fibrin clot in the area wound edges (Figure 1c)

4: Incomplete trauma closure – partial necrosis of the tissue wound edges (Figure 1d).

5: Incomplete trauma closure – complete necrosis of the tissue wound edges (Figure 1e).

The "Manchester scar Proforma" index [38] was modified (M-MSPI)

and used for the evaluation of the scar formation in the 1st (T1), 2nd (T2) and 3rd (T3) week of healing. The modified index included a 0-6 scale depending on the color, distortion and contour of the tissues (Table 1). The lower the final score, the better healing capacity.

(A) Color (Compared With Surrounding Skin)	(B) Contour	(C) Distortion
0 Perfect	0 Flush with surrounding skin	0 None
1 Slight mismatch	1 Slightly proud or indented	1 Mild or moderate
2 Obvious or gross mismatch	2 Hypertrophic	2 Severe

Table 1: Modified Manchester Scar Proforma index (M-MSPI) for clinical scar assessment (score range: 0–6; lower values represent improved healing quality, i.e., less scar), Adapted from Wong et al, 2009.

Statistical analysis

The association among the pre-surgical EGF-mRNA levels and the rate of response was examined with the Spearman's rank correlation coefficient. Linear regression model was used for the investigation of the potential association among EGF levels and the expression of healing with the Wachtel and Master Scar Proforma Indexes in each time point. Multiple linear regression models were used for the examination of the effects of smoking on the previous association. For the statistical analysis IBMM SPSS Statistics 20 was used. The statistical significance level was set at p-value ≤ 0.05.

Results

The study included 44 patients according to the established inclusion criteria, however, one patient had to be excluded from the final analysis due to zero amplification of EGF and β-actin, resulting in a final sample of 43 subjects. The mean age of the patients was 52.8 years (Table 2). Among the included participants 62.79% [27] were nonsmokers and 37.21% (16) smokers. The expression of EGF mRNA levels in each subject is illustrated in Graph 1 (Figure 2).

	N (%)
Sex	
Men	21 (48.84)
Women	22 (51.16)
Smoking	
Non-Smoker	27 (62.79)
Smoker	16 (37.21)
Age (Mean ± S.D.)	52.79 ± 12.07
Mean ± S.D., Mean, Standard Deviation	

Table 2: Demographic characteristics of the participants.

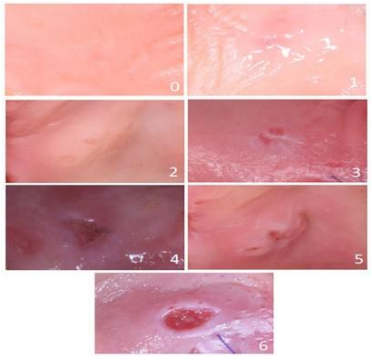
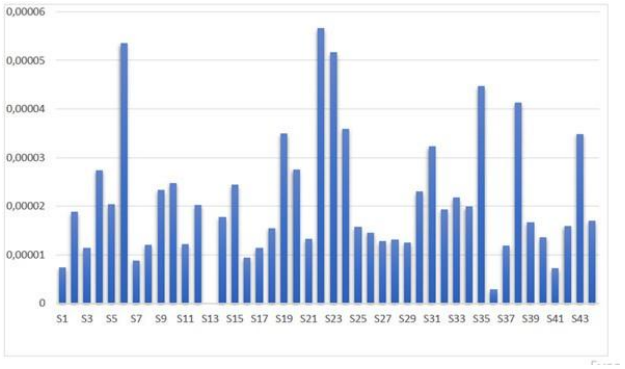


Figure 2: Modified-Master scar proforma index.



Graph 1: Expression of EGF in each subject (EGF μg/μl).

Evaluation of the healing response

The mean values of the M-EHI during the 1st week (T1) were 2.08 ± 1.05. Among the 42 patients attending the T1 evaluation, 16 presented a mean value of 1, 12 a mean M-EHI of 2, 10 a mean value of 3 while 3 patients had a mean value of 4. Only 1 patient presented a mean value of 5 accompanied with partial necrosis of the wound. In the 2nd week examination (T2), the mean values of M-EHI were 1.53 ± 0.91 with most of the participants presenting a value of 1 or 2 (76% of patients). The mean values and the standard deviation for the M-EHI in each examination (T1, T2) are depicted in Table 3.

Index	Value (Mean ± S.D)
M-EHI – T1	2.08 ± 1.05
M-EHI – T2	1.53 ± 0.91
M-MSPI – T1	2.55 ± 1.72
M-MSPI – T2	1.74 ± 1.31
M-MSPI – T3	0.89 ± 1.32
Mean ± S.D., Mean, Standard Deviation M-EHI, Modified Early Healing Index M-MSPI, Modified Master Scar ProForma Index	

Table 3: Healing response indexes mean values.

The M-MSPI, used for the evaluation of scar formation, had a mean value of 2.55 ± 1.72 at T1, 1.74 ± 1.31 at T2 and 0.89 ± 1.32 at T3 (Table 3). More specifically during the first two weeks of healing (T1, T2) most of the patients presented a value of 1 or 2 (59% of patients at T1, 86% at T2) while only 3 and 6 patients presented a value of 0 at T1 and T2 respectively. In the second month of healing (T3) most of the participants presented a value of 0 or 1 (69% of patients), while none of the patients had a value of 6.

Association of EGF expression in gingival tissue and early healing response

The association of the EGF pre-surgical mRNA levels and the M-EHI values are depicted in Table 4. During the 1st week of healing (T1) the increase of EGF expression was accompanied with a trend towards a reduction in the M-EHI values, however the later association did not reach a statistical significance (p-value, 0.813>0.05). Smokers had an increased value of M-EHI (+0.29) in comparison with non-smokers, however the difference was not statistically significant. In the second week (T2) (Table 4), the trend was reversed, as increasing EGF levels were accompanied with an increase of M-EHI values. Nor this association was significant, however (p-value, 0.541>0.05). Again, smokers and non-smokers did not differ in the M-EHI values. In addition, smoking did not affect EGF expression in any time points (p-value: 0.382 at T1, 0.174 at T2).

As for the M-MSPI statistical analysis revealed a trend for a reduction in the indexes levels as the EGF expression increased, both in T1 and T2 (Table 5). This association was not significant (p-value, 0.271 at T1, 0.417 at T2). The trend was reversed in the second month of healing (T3) but neither at this time the association did not reach a statistical significance. Smoking did not affect either the EGF expression or the association among the variables at any time point (Table 5).

		Single Variable Analysis			Multi Variable Analysis		
	Variable	**Evaluation**	**95% CI**	**p-value**	**Evaluation**	**95% CI**	**p-value**
T1	EGF	-3031.65	-28705.35, 22642.06	0.813	-4513.6	-30512.83, 21485.63	0.727
	Smoking						
	Non-Smoker	-	-		-	-	
	Smoker	0.29	-0.40, 0.97	0.404	0.3	-0.40, 1.00	0.388
T2	EGF	6735.93	-15349.68, 28821.54	0.541	6638.59	-15975.74, 29252.91	0.556
	Smoking						
	Non-Smoker		-			-	
	Smoker	0.04	-0.55, 0.63	0.883	0.02	-0.58, 0.62	0.952
CI, Confidence interval							

Table 4: Linear Regression Model of The Association of M-EHI with smoking and EGF expression in the 1st and 2nd week of healing.

		Single Variable Analysis			Multi Variable Analysis		
	Variable	**Evaluation**	**95% CI**	**p-value**	**Evaluation**	**95% CI**	**p-value**
T1	EGF	-22935.24	-64425.06, 18554.58	0.271	-23491.19	-65893.27, 18910.88	0.269
	Smoking						
	Non-Smoker		-			-	
	Smoker	0.03	-1.10, 1.16	0.958	0.11	-1.03, 1.25	0.842
T2	EGF	12884.74	-18859.55, 44629.03	0.417	13598.86	-18866.33, 46064.06	0.402
	Smoking						
	Non-Smoker		-			-	
	Smoker	-0.08	-0.93, 0.77	0.847	-0.13	-1.00, 0.73	0.756
T3	EGF	3560.47	-29411.57, 36532.51	0.828	5319.38	-28656.83, 39204.58	0.752
	Smoking						
	Non-Smoker		-			-	
	Smoker	-0.25	-1.18, 0.69	0.593	-0.28	-1.24, 0.69	0.565
CI, Confidence interval							

Table 5: Linear Regression Model of The Association of M-MSPI with smoking and EGF expression in the 1st, 2nd week and 2nd month of healing.

Discussion

EGF is a potent mitogenic factor, induces epithelial cell proliferation and division, improves collagen construction and has also chemotactic effects on vascular endothelial cells and fibroblasts [39]. The role of EGF in oral wound healing has been investigated mainly in studies using saliva [40,41]. A temporal increase in EGF protein concentration in saliva has been found after 3rd molar extraction, tumor removal from parotitis gland and after surgical periodontal therapy [32], while local application of the factor in skin grafted sites seems to promote the healing ability [33]. Furthermore, EGF has been found to be expressed in gingival tissues and periodontal ligament cells [34,42]. Application of human gingival fibroblasts in cutaneous radiation burns accelerated wound healing by modulating expression of FGF7, EGF and VEGF [43]. Moreover, mucosal transplants accelerated wound repair in rats' abdomen compared to skin transplants by upregulating EGF and VEGF expression [39]. Those findings suggest a potential role of EGF in early healing of mucosal trauma.

In the current study the expression of EGF mRNA in keratinized oral epithelium was examined, while the association between mRNA levels and the healing ability was also studied. It is known that early wound healing is influenced by a variety of factors such as surgical experience, tissue management, primary or secondary wound closure wound and patients' post-operative behavior, such as oral hygiene performance and smoking habits. Consequently, in order to eliminate all these critical factors for the wound healing response, we decided to examine wound closure in a small tissue fragment 2 × 2 × 3 mm was removed from the hard palate and the wounded area was closed with 2 interrupted sutures. For the clinical evaluation of the early healing response the Wachtel early healing index [37] was modified (M-EHI) to assess trauma closure and during the first two weeks of healing. Moreover the <<Manchester Scar Proforma>> index [38] was used for the evaluation of scar tissue formation at 1 and 2 weeks, and at 2 months of healing.

The results of the study unveiled a trend towards a better healing response in the first week of healing as EGF mRNA levels increased and as evaluated by the two clinical indexes. This association did not reach statistical significance.

Surprisingly in in the second week and second month of healing the later association was reversed, suggesting a compromised healing response in the cases with increased EGF levels. However, neither this association was significant. Smokers and non-smokers were evaluated in a separate analysis and smoking did not seem to affect healing at any time point. It is possible that EGF might be implicated in the quality of tissue response, but its expression increases after the initiation of trauma. Similarly with this hypothesis, a previous study examining the EGF levels after mucosa grafting in rats' abdomen showed a substantial increase in EGF mRNA and protein expression 3 days after grafting and this increase was higher in mucosal compared to dermal grafts [39]. As a result, it was impossible to detect any association at the time of tissue harvesting. If we could harvest tissue at later time points, we might have seen an association between a better healing response and increased EGF mRNA levels.

Another possible explanation is that an increase in any protein expression does not necessarily mean an increase in mRNA levels, since there are various post-transcriptional mechanisms involved before and during translation [44]. One of those common mechanisms is RNA silencing, which means RNA degradation by small double stranded RNAs [45]. Moreover, only EGF secreted in saliva and not the one present in gingival epithelial cells or fibroblasts might be implicated in tissue healing response.

In addition, EGF might play a role in wound healing only in the presence of inflammation. It was previously shown that one of the virulence factors of Porphyromonas gingivalis (The peptidylarginine deiminase PPAD), was inhibition of the ability of EGF to accelerate cell proliferation [46], affecting the function of many different signals. It is possible that our study did not find an association between EGF and wound healing, because we examined only healthy gingival tissues. Finally, we can also speculate that since early wound healing is a complicated mechanism, other growth factors are important for the quality of healing response.

Conclusion

The current study suggests a potential role of EGF in early gingival healing, although the results did not reach statistical significance. Future studies could include a larger sample and correlation of both EGF mRNA and protein levels at different time points after gingival injury.

Acknowledgements

The authors would like to thank Apostolos Angelidis, Department of Food Hygiene and Technology of Food of Animal Origin, for providing the laboratory equipment for the mRNA isolation. Also Persefoni Talimtzi for helping in the statistical analysis of the results.

Authors' Contributions

The conception of the study belongs to Makris Georgios and Aikaterini-Elisavet Doufexi, who also designed part of the acquisition of the study. Aikaterini-Elisavet Doufexi supervised the clinical work, took part in the analysis of the data and was responsible for the final approval for the work to be published. Stergoula Papamanoli worked on all the needed clinical processes for the acquisition of the sample, followed up the clinical examinations of the patients and performed the mRNA extractions. Ioannis Fragkioudakis did part of the clinical work, drafted and revised the manuscript. Mary Kalamaki designed and performed the gene expression experiments, analyzed the data and revised the text before the final submission.

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.

Funding

This study was funded by the department of Periodontology and Implant Biology, Dental School, Aristotle University of Thessaloniki.

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