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RESEARCH ARTICLE

The Effect of EGF-PEG Bead Implantation in Oral Mucosal Wound on the Rabbit Salivary Gland Trace Element Levels and EGF Receptor Immunoreactivity

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Summary

Purpose : *Epidermal growth factor (EGF) is found in high concentrations in the submandibular gland (SMG) and saliva. EGF stimulates wound healing. Zinc (Zn) also accelerates wound healing and is found in the salivary gland. There are a limited number of studies on salivary glands of orally wounded animals. We thus planned to study the effect of EGF implantation in an oral incision wound on the Zn and copper (Cu) levels in salivary glands and EGF receptor (EGF-R) immunoreactivity.* ***Methods:*** *In this study, New Zealand male rabbits (n=16) weighing 2.5 ± 0.4 kg were used. After submucosal incisions were made, the rabbits were divided into two groups. The rabbits of group 1 had untreated wounds and in group 2 wounds were implanted with EGF-PEG (polyethylene glycol) beads. On the 5th day after the operation, the rabbits were killed by an excess of sodium pentobarbital anesthesia. The salivary glands were excised immediately. Zn and Cu levels were measured by atomic absorption spectrophotometer. The EGF-R immunoreactivity of the submandibular glands was determined by histological examination using a special kit. Results were compared by ANOVA and Mann-Whitney U test.* ***Results:*** *Whereas EGF implantation in oral incision wounds decreased the EGF-R immunoreactivity of the submandibular glands and Zn levels, it did not change Cu levels.* ***Conclusions:*** *There is a relationship between submandibular gland EGF-R immunoreactivity and Zn levels.*

Key Words *: Salivary gland, EGF implantation, oral incision, zinc, EGF immunoreactivity.*
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Ağız Mukoza Yarası İçine EGF-PEG Boncuk İmplantasyonunun Tavşan Tükürük Bezi Eser Element Düzeyi ve EGF Reseptör İmmünreaktivitesine Etkisi
Özet

Amaç : *Epidermal büyüme faktörü (EGF) submandibular bez (SMG) ve tükürükte yüksek konsantrasyonda bulunur. EGF yara iyileşmesini uyarır, çinko (Zn) da yara iyileşmesini hızlandırır ve tükürük bezlerinde bulunur. Ağız yaralarında tükürük bezi dokusunda yapılmış çalışmalar çok azdır. Bu sebeble oral mukozada cerrahi kesi yarasına EGF uygulamasının, tükürük bezi Zn ve bakır (Cu) düzeylerine ve EGF reseptör tutulumuna etkisini araştırmayı planladık.* ***Metod:*** *Deneylerde Yeni Zelanda albino tavşanlar (n=16, 2,5±0,4 kg) kullanıldı. Ağızda submukozal insizyonlar yapıldıktan sonra tavşanlar iki eşit gruba ayrıldılar: Grup 1 tedavi edilmeyen ağız yaraları olan tavşanlar, ve grup 2 PEG (polietilen glikol)+EGF boncuk implante edilmiş ağız yaraları olan tavşanlar. Operasyonun 5.günü tavşanlar aşırı dozda sodyum pentobarbital anestezisi ile feda edildiler. Tükürük bezleri hemen çıkarıldı. Zn ve Cu düzeyleri atomik absorbsiyon spektrofotometrisi (AAS) ile ölçüldü. Submandibular tükürük bezi EGF reseptör tutulumunu belirlemek için özel kit uygulanarak takibe alındı. Sonuçlar Anova Varyans Analizi ve Mann Whitney U testi ile karşılaştırıldı.* ***Bulgular:*** *Sonuç olarak, ağız mukozal yaralarına yerleştirilen EGF, submandibular tükürük bezi Cu düzeyine etki yapmazken, EGF reseptör tutulumu ve Zn düzeyini azaltmaktadır.* ***Sonuç:*** *Tükürük bezi EGF reseptörü tutulumu ve Zn düzeyleri arasında bağlantı vardır.*

Anahtar Kelimeler : *Tükürük bezi, EGF implantasyonu, ağız cerrahi yarası, çinko, EGF immünoreaktivitesi*

INTRODUCTION

The salivary glands are a major source of several factors which play important roles in both oral and systemic organ homeostasis and wound healing[1]. Salivary glands synthesize numerous growth factors such as insulin-like growth factor I and II (IGF-I, IGF-II), nerve growth factor (NGF), transforming growth

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factor alpha and beta (TGFα and β), and epidermal growth factor (EGF), which is effective in oral wound healing[2,3]. A biologically active growth factor, EGF is synthesized and secreted by the granular convoluted tubule cells of the salivary glands[4,5].

It has been known that members of this peptide growth factor family have mitogenic activity, in vivo and in vitro, for many different cell types including those of the oral cavity[6,7]. It has been reported that salivary gland-derived EGF enhances the healing of gastric ulcers[8,9] and tongue lesions and modulates taste bud morphology and its maintenance[10]. Treatment with TGF-α, which is the member of the EGF superfamily, enhances the healing of experimental gastric ulcers[11-13]

The effect of EGF is triggered through its binding to a membrane receptor, EGF-R, which activates an intrinsic tyrosine kinase in the cytoplasmic domain of the receptor[14]. The protype EGF-R is a 170-kDa glycoprotein that is widely expressed in mammalian cells. It consists of ligand binding, transmembrane, and tyrosine kinase domains, in addition to a carboxy terminal span that contains tyrosine residues that undergo auto- and transphosphorylation during receptor activation[14].

Zinc (Zn), a Group IIb metal, is an essential micronutrient involved in structural and regulatory cellular functions[16] and is found in secretory granules in the salivary gland[15,17]. Zn is known to enhance wound healing by promoting the proliferation and migration of both fibroblasts and keratinocytes[18] and acts as a signaling molecule triggering Ca_i^{+2} rise[19]. Copper (Cu) is also a trace element and acts as a catalytic component for many enzymes such as Cu, Zn-superoxide dismutase (Cu, Zn-SOD), ceruloplasmin, dopamine β-hydroxylase, peptidyl alpha amidating monoxygenase, lysyl oxidase, and cytochrome c oxidase[20]. Both Zn and Cu play a role in collagen cross-linking and, therefore, help in maintaining of the tissue[21].

EGF and Zn act synergistically in maintaining oral mucosa[22-24], promoting oral wound healing[1], secreting from salivary gland[25] and acting as signaling molecules[19,26,27]. It has been reported that Zn and EGF are important molecules in saliva for oral health maintenance[28,29]. However, there is limited research about salivary gland tissue in the changing conditions in the mouth.

In this study, we described the use of a new wound model in the rabbit mouth. A 10 mm mucosal defect was surgically made to the depth of the periosteum using a round stainless steel blade in the rabbit diastema (between the incisor and molar teeth of all rabbits) on both sides of the mandibula. In this research, we planned to investigate the effect of EGF implantation after oral submucosal incision on Zn and Cu levels and EGF-R immunoreactivity of the submandibular gland, a secondary organ neighboring the mouth.

MATERIALS AND METHODS

Materials

EGF (E-4151) and polyethylene glycol (PEG) 4000 were obtained from Sigma, USA. Goat serum, EGF-R rabbit polyclonal antibody Ab-4, anti rabbit total Ig, normal rabbit IgG, avidin-biotin-complex-peroxidase and diaminobenzidine (DAB) were obtained from Oncogene Science, Manhasset, New York, USA.

Animals

New Zealand rabbits, male (n=16), five months old, weighing 2.5-3.0 kg were housed in clear plastic cages and fed with vegetables and tap water. Animals were allowed to acclimatize for at least seven days prior to surgery. Prior approval for this experiment was obtained from the Animal Experimentation Ethics Committee at Gazi University.

Preparation of EGF Beads

The beads containing EGF (Sigma E-4151) were prepared under aseptic conditions. All glass materials were sterilized by dry heat. For the preparation of beads, PEG 4000 was first melted and mixed with EGF solution. This mixture was delivered to molds with an automatic pipette. After solidification of the melted mass, the beads were used. EGF content of each bead was 40 ng. Physiologically effective dose for wound healing is daily 10 ng for 1 cm full thickness

incision[30]. The solution was sterilized by filtration. Beads were filled with EGF + PEG 4000 mixture to obtain bead-shaped forms and kept in closed ampoules.

Wound Model

A 10 mm mucosal defect was surgically made to the depth of the periosteum using a round stainless steel blade in the rabbit diastema (between the incisor and molar teeth of all rabbits) on both sides of the mandibula under xylazine (5 mg/kg) + ketamine (75 mg/kg) anesthesia. After submucosal incisions, rabbits were divided into two groups as follows: Group 1: untreated wounds and Group 2: wounds treated with EGF-PEG bead implantation.

Wounds were closed with three silk sutures (3/0). Until the end of the experiments, all rabbits were fed with vegetables and tap water ad libitum. On day 5, rabbits were weighed and sacrificed by an overdose of Na pentobarbital. Submandibular salivary glands were removed from both sides and one gland of each rabbit was transported immediately to 10% formalin for EGF-R immunoreactivity; the other submandibular gland was frozen in liquid nitrogen, and the samples were kept at -30°C until assayed.

Immunohistochemical Procedures

Preparation of tissue samples

Submandibular glands were fixed in neutral formalin for 72 h. They were later examined by usual light microscope follow-up and embedded in paraffin, and cross-sections (4-5 μm) were placed on polylysine-covered slides. Each section of tissue blocks was deparaffinized with xylene and rehydrated.

Antibodies and staining procedure

Firstly, slides were incubated for 10 min in 3% hydrogen peroxide to eliminate endogenous peroxidase activity in tissues and were incubated with saponin to facilitate the binding of primary antibodies to antigenic areas. Epitopes wer e stabilized by application of serum blocking solution for 20 min. Sections were incubated with EGF-R rabbit polyclonal antibody Ab-4 (100 μg/ml) diluted 1:20 in PBS overnight at +4°C. The secondary antibody, a 1% diluted biotin labeled anti rabbit total Ig, was applied for 30 min at room temperature. A negative control was done using normal rabbit IgG instead of the primary antibody. After washing with PBS, avidin-biotin-complex-peroxidase was applied to the slides. Diaminobenzidine (DAB) was used as a chromogen. Afterwards, the slides were counterstained with hematoxylin for 1 min, dehydrated in graded ethanol and mounted in a conventional medium.

In this study, the aim of histological examination was to evaluate EGF-R immunoreactivity in the submandibular salivary gland by a qualitative system using immunohistochemical method. All slides were stained on the same day under identical conditions to minimize variability of the staining.

Scoring Criteria

Membrane or both membrane and cytoplasm immunoreactivity were evaluated. Immunohistochemical immunoreactivity was assessed by combining the proportion of stained cells and the intensity of the staining. The intensity of the staining was evaluated on a semiquantitative 4-point scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining[31]. The final EGF-R staining score was calculated by multiplying the percentage of positively stained cells by the intensity of the staining.

Zn and Cu Determination

Zn concentrations were measured by atomic absorption spectrophotometer (AAS) (Philips Model PU-9200). Tissue specimens were placed in a 125 ml Erlenmeyer flask containing an equal weight of acid washed glass beads and 25 ml of water. Ten milliliters of concentrated HNO_3 concentrated $HClO_4$ (1:1) were added and the solution boiled until it was clear. The solution was then transferred to a 100 ml volumetric flask and brought to volume with water[32]. The assay was performed by AAS. Results are expressed in microgram per gram wet weight for tissues.

Statistical Analysis

Data are presented as mean ± SD. The results were compared statistically by ANOVA and Mann-Whitney U test using the Statview program. Significance was set at $P< 0.05$.

RESULTS

Salivary gland Zn and Cu levels

The submandibular salivary gland Zn level of the EGF-implanted experimental group was significantly decreased when compared to the control group. Submandibular gland Zn levels in EGF-implanted group were 114 ± 49 µg/g wet weights versus 151 ± 61 µg/g wet weight in the control group (Table 1).

Table 1. The effect of EGF implantation after incisional surgery in oral submucosa on zinc and copper levels of submandibular gland

Treatment (n=16)	Zn levels (µg/g wet weight)	Cu levels (µg/g wet weight)
1. Untreated control (n=8)	151 ± 61*	6.13 ± 2.08
2. EGF-implanted group (n=8)	114 ± 49**	5.07 ± 2.70

Values are expressed as mean ± SD. Difference statistically significant *-**P < 0.05 by Mann-Whitney U test.

Although salivary gland Zn levels were changed in the experimental group, submandibular gland Cu levels were not significantly changed in either group. There was decrease in Cu levels of the submandibular glands, but the decrease was not significant in the experimental group when compared to the control.

Immunohistochemical results of submandibular salivary glands:

Control group: Weak apical cytoplasmic and moderate basal cytoplasmic reactivity was observed in the striated duct cells of the submandibular glands (Fig. 1a). Strong membranous and moderate cytoplasmic EGF-R immunoreactivity was seen in interlobular duct cells (Fig. 1b).

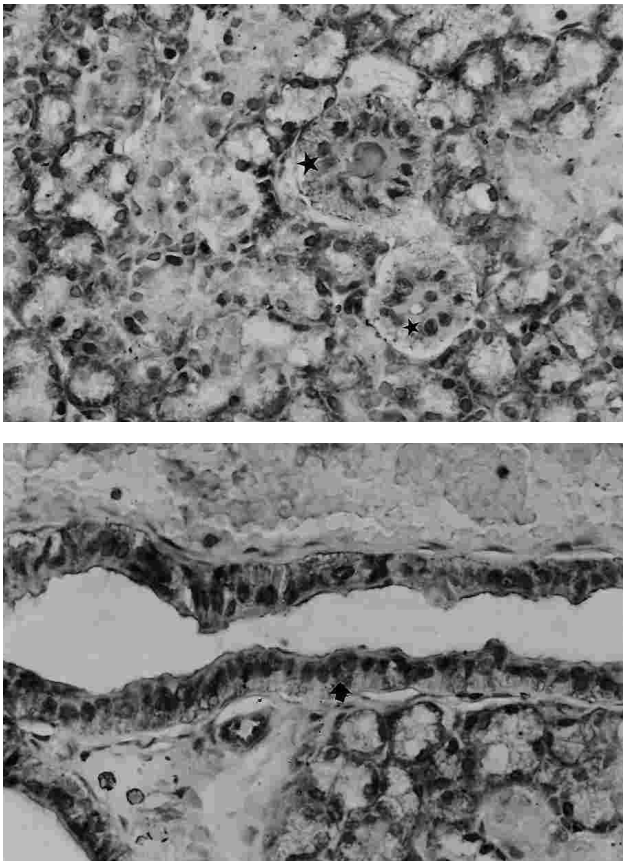

Figure 1. a, b EGF-R immunoreactivity of rabbit submandibular gland tissues in the control group. a. Weak apical, moderate basal (★) cytoplasmic reactivity was observed in the striated duct. (Immunoperoxidase – hematoxylin X 400).
b. Strong membranous (➔), moderate cytoplasmic reactivity was seen in the interlobular duct (Immunoperoxidase – hematoxylin X 400).

Incision group: Weak cytoplasmic immunoreactivity was determined in striated duct cells (Fig. 2a). Strong membranous and apical cytoplasmic reactivity was observed in cells of the interlobular duct, but moderate cytoplasmic staining was seen in basal parts of these cells (Fig. 2b).

Incision + EGF-applied group: In general, weaker reactivity was seen in this group than in the incision group. Weak cytoplasmic reactivity was observed in striated duct cells (Fig. 3a). In cells of the interlobular duct, moderate membranous and weak cytoplasmic immunoreactivity was seen (Fig. 3b).

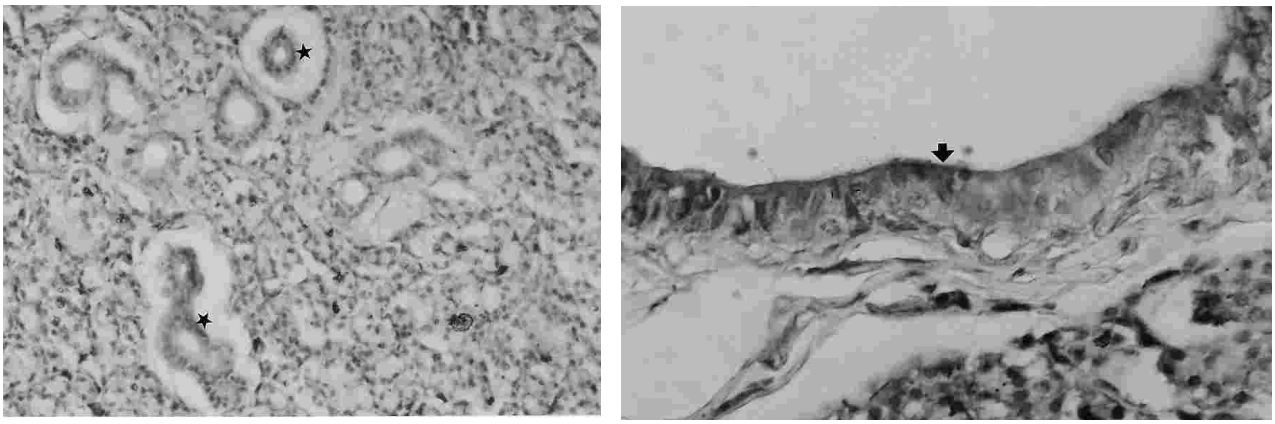

Figure 2. a, b EGF-R immunoreactivity of rabbit submandibular gland tissues in the incision group. a. In the striated ducts, weak EGF-R immunoreactivity was seen (★). (Immunoperoxidase – hematoxylin X 400). b. In the interlobular duct, strong apical (➔) staining was observed (Immunoperoxidase – hematoxylin X 400).

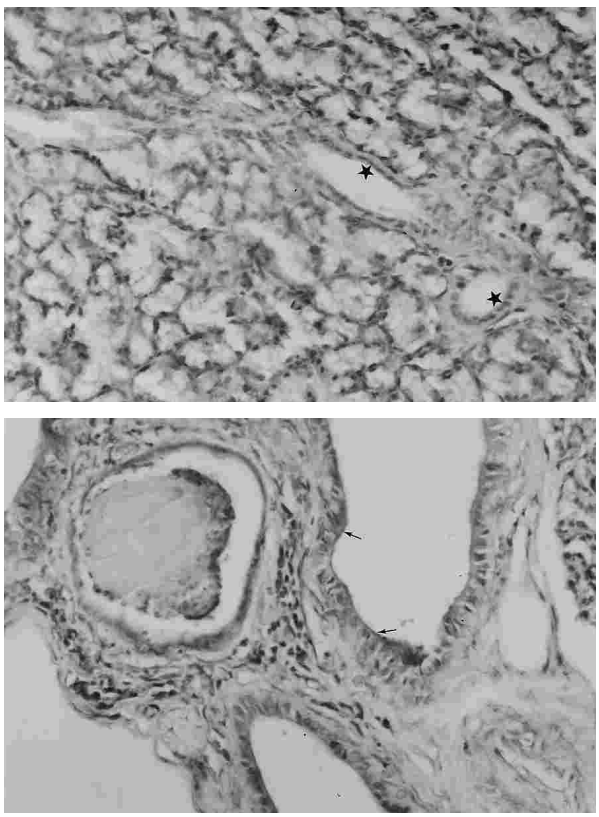

Figure 3 . a, b EGF-R immunoreactivity of rabbit submandibular gland tissues in incision + EGF-R applied group. a. Weak cytoplasmic reactivity was observed in the striated duct (★) (Immunoperoxidase – hematoxylin X 400). b. Weak cytoplasmic (➔) reactivity was detected in the interlobular duct (Immunoperoxidase – hematoxylin X 400).

DISCUSSION

Growth factors are mediators with essential importance in the normal repair process after wounding. It has been suggested that oral or juxtaoral surgery stimulates increased synthesis and secretion of growth factors in the saliva as well[33,34]. It has been suggested that EGF is effective on experimentally induced incision wounds and gastric ulcers[11,35,36]. EGF-R has also been detected in the salivary glands[37,38] and is an important mediator of cell growth, differentiation, and survival. Its effect at the nuclear level is mediated by tyrosine kinase activity[39].

Enhanced activity or over expression of EGF-R has been associated with tumor progression in many epithelial malignant tumors from different locations (e.g., head and neck, gastrointestinal tract, lung, breast, and brain)[40,41]. However, the alteration in submandibular gland EGF-R immunohistochemistry following EGF implantation in oral submucosa after incision has not been reported. The present study is the first to specifically examine the salivary gland EGF-R immunohistochemistry after incision with EGF implantation in the oral mucosa wound model. In this study, we determined that submandibular gland EGF-R immunohistochemistry decreased after EGF with PEG implantation in the wound. With exogenous EGF administration in the oral mucosal incision, the EGF-R immunoreactivity (complex) of the submandibular salivary gland decreased by the down regulation mechanism. In our previous study,

we determined tissue response for PEG without EGF[30]. We thus decided to use inert PEG as an excellent carrier for EGF.

Organic and inorganic salivary secretion from the submandibular salivary gland may be responsible for the increased susceptibility to oral infections and impaired wound healing. Zn and Cu play an important role in catalytic activity of hundreds of enzymes of every type[16] and Zn also has a regulatory role in cell signaling[42]. Zn is related not only to EGF but also to EGF-R by functional mechanisms. Numerous studies have shown that Zn ion causes EGF-R phosphorylation and induces EGF-R-dependent signaling, including Ras and MAPK activation[43,44]. From these findings, it is reasonable to assume that there is a relationship between EGF-R and Zn^{+2} ions because of their interaction with each other.

In our study, we found a relationship between EGF-R immunoreactivity and Zn levels in the submandibular gland after EGF implantation in an oral wound. We demonstrated that submandibular salivary gland EGF-R immunoreactivity and Zn level decreased after EGF implantation in an oral wound, but Cu levels in the salivary gland did not change. Although Cu is required for many biochemical processes as a cofactor, in this study with EGF administration, Cu levels in the submandibular gland were unchanged in the experimental group.

It has been shown that infrared laser irradiation of the biological system decreased salivary gland Zn and Cu levels[45]. In this research, we found that Zn levels decreased in the submandibular glands of the EGF-treated group. However, submandibular salivary gland Cu levels were not affected by this process.

Based on the present study, as a result of exogenous EGF implantation in the incisional wound, not only oral tissue but also the submandibular gland as a secondary organ may undergo alterations related to EGF-R immunoreactivity and Zn levels.

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