The time-dependent effect of exogenous epidermal growth factor administration on oxidant events in submandibular glands of New Zeland-type male rabbits

Seda Tasir PhD1, Emre Avci PhD2, Emine Gulceri Gulec Peker PhD3, Sule Coskun Cevher PhD4

Abstract

BACKGROUND AND AIM: Epidermal growth factor (EGF) plays an important role in the wound healing process. The main purpose of this study is to investigate the effects of EGF on salivary gland oxidative events due to the incision wound created in the oral mucosa.

METHODS: In the experiments, 42 New Zealand male rabbits weighing about 2.5 kg were used. Then submucosal incisions were created and subjects were divided into groups: control group (only the incision wound was not applied), untreated incisional group, and EGF therapy group. Subjects were anesthetized on days 1, 3, and 5 after injury and the tissues were removed immediately. Thiobarbituric acid reactive substances (TBARS), the end product of lipid peroxidation, and glutathione (GSH) and ascorbic acid (AA) levels, which are important antioxidants, were measured by spectrophotometric methods in salivary glands. The results were compared with Mann-Whitney U and analysis of variance (ANOVA) tests.

RESULTS: The thiobarbituric acid (TBA) reagent levels of the EGF-treated group were found to be significantly reduced on the 1st, 3rd, and 5th days when compared with the data of other group. It was determined that there was a reduction in GSH data from day 1 to day 5 with a similar ratio in both untreated incisional group and EGF therapy group. No statistically significant difference was observed in the days examined within both untreated incisional group and EGF therapy group.

CONCLUSION: It was observed that processes including submandibular gland oxidative events and antioxidant responses during wound healing were affected by EGF.

KEYWORDS: Epidermal Growth Factor; Submandibular Gland; Thiobarbituric Acid Reactive Substances; Glutathione; Ascorbic Acid


Growth factors that are synthesized by the salivary glands may play a crucial role in healing wounds within oral mucosa rapidly.1 Many researchers have reported that the epidermal growth factor (EGF) synthesized in the salivary glands is effective in healing dermal and gastric wounds, and tissue damage leads to rises EGF synthesis in saliva.2-5 It was reported by different researchers that changes had been observed in relation to both oxidant events and involvement of EGF receptor (EGFR) in submandibular salivary glands that are source of endogenous EGF.6,7 Oxidative events develop during repairing of tissue integrity impaired by the wounding. Free radicals increase in the process of wounding and following this, its repair leads to oxidative damage.8 Lipids are the most sensitive ones among biomolecules, and free radicals, by becoming effective in lipids in organism, lead to lipid peroxidation.9

1- Assistant Professor, Biotechnology Institute, Ankara University, Ankara, Turkey
2- Associate Professor, Department of Molecular Biology and Genetics, School of Arts and Sciences, Hitit University, Corum, Turkey
3- Assistant Professor, School of Health Sciences, Giresun University, Giresun, Turkey
4- Professor, Department of Biology, School of Arts and Sciences, Gazi University, Ankara, Turkey
Address for correspondence: Emre Avci PhD; Associate Professor, Department of Molecular Biology and Genetics, School of Arts and Sciences, Hitit University, Corum, Turkey; Email: emreavci@hitit.edu.tr

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Malondialdehyde (MDA) is a lipid peroxidation product. Thiobarbituric acid reactive substances (TBARS) are being used as common indicators of oxidative damage.\textsuperscript{1,10} There are miscellaneous studies showing TBARS increase in the wounded tissues or various tissues of animals in which the wound is made.\textsuperscript{11,12} Glutathione (GSH) is an important antioxidant that protects cells against oxidative damage.\textsuperscript{13} Increased GSH is a sign for that cell has been protected from oxidative stress.\textsuperscript{14} As for ascorbic acid (AA) appearing in pretty high amounts on exocrine acinus of salivary glands, there are a great many studies in which its roles in salivary gland functions were researched.\textsuperscript{14,15} AA amount affects the lipid metabolism.\textsuperscript{16} Low AA plays an important role in all chronic inflammatory diseases (CIDs) and in the cases where the lipid peroxidation has increased.\textsuperscript{17}

In our study, it was aimed to reveal the effects of exogenous EGF application on oxidant (TBARS) and antioxidants (GSH and AA) in oral wounds developing in salivary gland.

**Methods**

**EGF formulations:** The beats used in the experiments were prepared at Gazi University, Ankara, Turkey, using pharmaceutical technology. Polyethyleneglycol (PEG) 4000 was first melted and then mixed with EGF solution to prepare the pellets. After the solidification of the molten mass, lumps were used and the EGF concentration in each lump was 40 \( \text{ng/ml} \). The EGF + PEG 4000 mixture was created in pellet-shaped forms and then kept in closed ampoules until analysis.\textsuperscript{6,18,19}

**Animals and wound modeling:** Our study received local ethics committee approval (92-15912). It aimed to show effects of EGF upon oxidant events for salivary gland by applying EGF externally so as to speed up the wound healing. This study is a research article and in experiments, according to power analysis, 42 New Zealand-type male rabbits, 5-month-old, with weight of 2.5 ± 0.4 kg were used. Experimental animals were fed with pellet feed and water before and during the experiment. In our study, TBARS, GSH, and AA were determined according to the days. For this, two experiment groups were chosen, to which incised wounds were made in their oral mucosas and EGF was administered exogenously after incision; oxidant events for salivary gland were investigated on 1\(^{st}\), 3\(^{rd}\), and 5\(^{th}\) days in both groups post-wounding. Three groups were formed:

\begin{itemize}
  \item I. Control group
  \item II. a) Unreated incisional group (\( n = 6 \), on day 1)
  \item b) Untreated incisional group (\( n = 6 \), on day 3)
  \item III. a) EGF therapy group (\( n = 6 \), on day 1)
  \item b) EGF therapy group (\( n = 6 \), on day 3)
  \item c) EGF therapy group (\( n = 6 \), on day 5)
\end{itemize}

In the study, rabbits were sacrificed on days 1, 3, and 5 with high dose of pentobarbital sodium (NA). For creating a wound model, general anesthesia was provided with ketamine (Ketalar 50 mg/kg) injected intramuscularly and xylazine (Rompun, 5 mg/kg). The animal's mouth was then opened, the cheek and tongue were excluded, and a 1.5-cm long posterior-anterior incision in the right and left jaw was performed in a single operation in the middle of the hill between incisors and molar teeth. After incision, the wound lips were eliminated by using surgical techniques until the formulations were placed on the mucous membrane with the periostial elevator in the vestibul and lingual direction. The wound tissues were removed immediately, frozen via liquid nitrogen, and stored at -30 \( ^\circ \text{C} \) until assayed.

**Determination of TBARS:** Lipid peroxidation was determined by measuring TBARS.\textsuperscript{20} The samples were measured at 535 nm in the spectrophotometer. Lipid peroxide levels were expressed as MDA equivalents using a \( 1.56 \times 10^5 \text{ mol-1 cm-1} \) extinction coefficient.

**Determination of GSH:** GSH levels were determined by a modified Ellman method.\textsuperscript{21} Results were expressed as micromoles of GSH per gram of tissue and as micromoles per milliliter of GSH.

**Determination of AA:** The AA levels were assayed by a spectrophotometric method.\textsuperscript{22} After mixture of tissue, perchloric acid/ethylenediaminetetraacetic acid (PCA/EDTA) was homogenized and
centrifuged. Colour reagent was added into, placing standard AA-solution into a tube, and was vortexed. Sulphuric acid (H₂SO₄) was added and the absorbance was read at 515 nm.

Statistical analysis was performed by the SPSS software (version 20, IBM Corporation, Armonk, NY, USA). All data are given as mean ± standard deviation (SD). In statistical analysis, significance level was preferred as 0.05. The number of animals to be included in the study was determined by power analysis. The tests to be used for the analysis of the data obtained after the study were determined according to the normality tests and the number of samples. Normality distribution was investigated via Kolmogorov-Smirnov test. In cases where these assumptions were not met, non-parametric Mann-Whitney U test was used for comparison. When these assumptions were met, parametric analysis of variance (ANOVA) test was used for comparison.

**Results**

When control group data without any application were examined, TBARS, GSH, and AA levels were determined as 53.33 ± 7.77 nmol/g, 11.82 ± 1.07 μmol/g, and 0.51 ± 0.10 mg/g, respectively (Table 1).

TBARS: The TBARS data we obtained as a result of our experiments are shown in Table 1 and Figure 1. The TBARS levels were measured on the 1st, 3rd, and 5th days. The data on the 1st, 3rd and 5th days were found as 98.81 ± 2.65 nmol/g, 76.00 ± 9.89 nmol/g, and 56.83 ± 10.25 nmol/g in the untreated incisional group (Group 2), respectively (Table 1 and Figure 1). These data were significantly higher than the control (53.33 ± 7.77 nmol/g). When the TBARS levels of the 1st and 3rd days were compared with the control group, a statistically significant difference was observed (P < 0.05). When the TBARS levels of EGF-treated group were compared with the data of both the control and the untreated incisional group, it was found that TBARS levels decreased significantly on the 1st, 3rd, and 5th days (P < 0.05).

**GSH:** GSH levels in the study groups are shown in Table 1 and Figure 2. Salivary gland GSH values were determined to be quite low in the control group on the 1st, 3rd, and 5th days compared to both the untreated incision group and EGF therapy group (Table 1 and Figure 2).

**Table 1.** The effect of exogenous epidermal growth factor (EGF) administration on the thiobarbituric acid reactive substances (TBARS), glutathione (GSH), and ascorbic acid (AA) levels of salivary gland tissue after incisional surgery.

<table>
<thead>
<tr>
<th>Groups</th>
<th>TBARS (nmol/g tissue)</th>
<th>GSH (μmol/g tissue)</th>
<th>AA (mg/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 6)</td>
<td>53.33 ± 7.77</td>
<td>11.82 ± 1.07</td>
<td>0.51 ± 0.10</td>
</tr>
<tr>
<td>Untreated incisional</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1 (n = 6)</td>
<td>98.81 ± 2.65</td>
<td>22.22 ± 5.32</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Day 3 (n = 6)</td>
<td>76.00 ± 9.89</td>
<td>35.42 ± 3.00</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Day 5 (n = 6)</td>
<td>56.83 ± 10.25</td>
<td>24.78 ± 2.90</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>EGF therapy</td>
<td>11.82 ± 1.07</td>
<td>24.07 ± 5.20</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Day 1 (n = 6)</td>
<td>21.86 ± 1.40</td>
<td>24.07 ± 5.20</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Day 3 (n = 6)</td>
<td>20.35 ± 2.76</td>
<td>26.67 ± 5.83</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Day 5 (n = 6)</td>
<td>22.08 ± 4.18</td>
<td>24.07 ± 5.20</td>
<td>0.08 ± 0.01</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation (SD)

EGF: Epidermal growth factor; TBARS: Thiobarbituric acid reactive substances; GSH: Glutathione; AA: Ascorbic acid

TBARS: P < 0.05: a-b, a-c, a-e, a-f, a-g, b-c, c-d, d-e, e-f, f-g, g-h

GSH: P > 0.05

AA: P < 0.05: a-b, a-c, a-d, a-e, a-f, a-g, b-c, e-f, e-g, e-h, f-g, f-h
The fact that the level of GSH in the wounds formed in the untreated incision group was higher than the control group indicates that the level of protection against oxidative damage is high. When the study data were analyzed by day, it was determined that there was a decrease in GSH levels from day 1 to day 5 with a similar ratio in both untreated incisional group and EGF therapy group. This situation was not statistically significant (P > 0.05) between intra-group days. However, statistically significant difference was found between control group-untreated incision group and control group-EGF therapy group (P < 0.05).

**AA:** AA data measured from the experimental groups are shown in table 1 and figure 3. According to the data, it was determined that AA levels of both the untreated incision group and the EGF therapy group were quite less than the control. AA for the untreated group showed the maximum decrease on 3rd day (0.02 ± 0.01 mg/g), reaching again on 5th day (0.09 ± 0.01 mg/g) to equivalent level on 1st day (0.09 ± 0.01 mg/g). However, it was determined that while AA showed the most decrease on 1st day (0.09 ± 0.01 mg/g) in the EGF therapy group, it was at the same level on 3rd (0.08 ± 0.01 mg/g) and 5th days (0.08 ± 0.01 mg/g) and was at higher value than 1st day (0.40 ± 0.01 mg/g) (Table 1 and Figure 3). The statistically significant difference (P < 0.05) was observed between control group-untreated incision group and control group-EGF therapy group. But, no statistical difference was observed in the days examined within both untreated incisional group (group 2) and EGF therapy group (group 3) (P > 0.05).

**Figure 2.** The effect of exogenous epidermal growth factor (EGF) application on salivary gland glutathione (GSH) activity

**Figure 3.** The effect of exogenous epidermal growth factor (EGF) application on salivary gland ascorbic acid (AA) activity

**Discussion**

This study was designed to reveal the changes on oxidant TBARS and antioxidant GSH and AA levels of exogenous EGF applied on oral wounds formed in the submandibular gland. Many studies have shown that EGF and many growth factors induce the EGFR signal, thereby increasing the proliferation and pluripotency of stem cells. In addition, studies have emphasized the importance of EGF in the early phase of wound healing, so researchers emphasized the importance of additional use of EGF in the first phase of wound repair. In our study, TBARS, GSH, and AA were determined according to the days. Two experiment groups were chosen, to which the incisional wounds were made in their oral mucosas and EGF was administered exogenously after incision; oxidant events for submandibular gland were investigated on 1st, 3rd, and 5th days in both groups post-wounding. In our study, TBARS level indicates that oxidative damage is high in the wound area; and also, these data show that exogenous EGF administration reduces lipid peroxidation of salivary gland tissue. In some studies, it has been shown that applied EGF decreased MDA for gastric mucosa, reduced ulcer content, and showed an antioxidant feature in cases changing within the
mouth.\textsuperscript{8,25,26} Mohanty and Pradhan demonstrated the effectiveness of EGF in wound healing and it has been stated that it should be evaluated with many combinations.\textsuperscript{27} Some studies have shown that various levels of pharmacological concentrations of EGF trigger the necessary biological actions for wound healing.\textsuperscript{28} Basso et al. reported that EGF, a potent growth factor, promoted epithelial cell proliferation and division, improved collagen structure, and regulated protein synthesis, thereby accelerating wound healing.\textsuperscript{29} Another working group, Ojalvo et al., evaluated the effects of EGF application on wound healing. They found that EGF application was related with marked improvement in oxidative stress and antioxidant indicators. In addition, EGF has been reported to contribute to wound healing.\textsuperscript{30} In another study, Garcia-Ojalvo et al. performed a similar study to our study. They demonstrated that EGF was effective in oxidative stress and antioxidant markers and created a significant oxidative balance in intra-lesional application. In addition, the physiological efficacy of EGF was demonstrated between healing and oxidative balance. Also, they showed that local infiltrated EGF had a systemic effect on the restoration of a pro-physiological redox balance and was effective in stress level.\textsuperscript{31} Similarly, Kalay and Cevher showed that EGF could act as an antioxidant in wound tissue. They also demonstrated that EGF might contribute to wound healing at earlier stages and would play a potentially effective role in antioxidant treatments, especially up to day 5.\textsuperscript{32} In a study, they determined that EGF had an effect on the development of intussusceptive angiogenesis on blood vessels, especially dermal wound healing.\textsuperscript{33} In Erkasap et al. study, EGF administration with ethanol has been shown to reduce TBARS levels in stomach tissue in rats.\textsuperscript{34} When various studies researching antioxidant features of the saliva have been examined, it has been reported that the saliva significantly contains enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), GSH, and GSH transferase.\textsuperscript{35,36} In our study, GSH was determined from antioxidants which were considered as taking active role in protecting against oxidative damage. When GSH in salivary gland in general was examined in both the untreated incision group and the EGF-administered group, it was observed to be at pretty higher rates than that of the control. Similarly, Akbulut et al. reported that GSH did not play an important role in wound healing. They reported that GSH values were not changed in ulcer healing after EGF application in their study.\textsuperscript{37} In accordance with our study, Gulec Peker et al. reported that EGF administration in the oral mucosa did not cause any change in GSH levels.\textsuperscript{8} In addition, in Gupta et al. study, GSH levels were shown to decrease during wound healing process for 14 days in rats with suppressed immune system.\textsuperscript{11} According to our results, despite the decreasing TBARS both in the untreated group and in the EGF group in the days following the injury, GSH did not show any change, suggesting that other antioxidant defense systems were in place instead of GSH antioxidant. Therefore, in another study to be carried out in parallel with this experiment, studying of SOD and CAT activities will be appropriate. The fact that no change occurred in GSH in all groups may make us think that vitamin C, which is a strong antioxidant, may have been used in trying for oxidative damage to be removed. AA must be present in these tissues in sufficient amount for the function of collagen. AA, which is another antioxidant, significantly increased in the control group when compared to the all groups. This finding may suggest that AA is used as an antioxidant during injury and that neutrophils use vitamin C during respiratory burst. AA of the EGF-administered group meaningfully increased on 3\textsuperscript{rd} and 5\textsuperscript{th} days in comparison to 1\textsuperscript{st} day. Using of EGF caused no effect on decreasing vitamin C levels for the tissue. Low AA plays an important role in
all CIDs where lipid peroxidation increases. Vitamin catabolism increases during inflammation and peroxidation and the metabolism is constantly under oxidative stress. Consistent with our study, Kilicaslan et al. reported that EGF treatment applied to the wound created on the oral mucosa did not change AA levels.33

**Conflict of Interests**

Authors have no conflict of interest.

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**References**