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The Effects of Aminoguanidine on the Antioxidant Mechanisms and Nitrate Levels in Incisional Oral Mucosal Wound Healing Process

[Aminoguanidinin ağız mukoza kesi yara iyileşmesinde antioksidan mekanizmalar ve nitrat düzeylerine etkisi]

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ABSTRACT

Purpose: We aimed to investigate the effects of either local application of aminoguanidine to oral mucosal wound or systemic administration application of aminoguanidine on the oxidant and anti-oxidant parameters of wound tissue and plasma.

Materials and Methods: New-Zealand rabbits (n=18) were used in the study. A standard incision was applied to the oral mucosa of rabbits. Rabbits were divided into three groups as: Untreated incisional group (Group I), local polyethylene glycol + aminoguanidine (12.7 mg) bead (Group II), and subcutaneous aminoguanidine administrated group (100 mg/kg 3 day) (Group III). Oral wound tissue nitric oxide, malondialdehyde, and glutathione levels and plasma thiobarbituric acid reactive substances, nitric oxide and total sulfhydryl group levels were measured.

Results: In histological analysis, a significant reepithelization in the wound region was seen in aminoguanidine treated rabbits. The rabbits in Group III had significantly increased fibroblasts and collagen fibers. Aminoguanidine administration significantly decreased the malondialdehyde and plasma thiobarbituric acid reactive substances levels of wounded-tissues ($p<0.05$). Although not significant, the glutathione levels of wounded tissues were increased in the aminoguanidine treated groups compared to non treated group ($p>0.05$). Plasma total sulfhydryl group levels were significantly increased after subcutaneous aminoguanidine administration ($p<0.05$).

Conclusion: In conclusion, our results suggested that subcutaneous/local administraiton of aminoguanidine might improve the wound healing by reducing the levels of oxidant products.

Key Words: Rabbit, incision, wound healing, nitric oxide, aminoguanidine, oxidative stress, glutathione

ÖZET

Amaç: Ağız yara dokusuna lokal ya da sistemik uygulanan aminoguanidinin plazma ve yara dokusunda oksidan ve antioksidan parametreler üzerindeki etkilerini araştırmayı amaçladık.

Materyal ve Metod: Bu çalışmada Yeni Zelanda tavşanlar (n=18) kullanıldı. Tavşanların oral mukozalarına standart bir insizyon uygulandı. Tavşanlar üç gruba ayrıldı. Grup I: tedavi edilmeyen insizyon yarası grubu, Grup II: yara+lokal polietilen glikol boncuk içinde + aminoguanidin (12.7 mg), Grup III ise, yara+subkutan aminoguanidin (100 mg/kg x 3 gün) olarak tanımlandı. Oral yara dokusu nitrik oksit, malondialdehit ve glutatyon düzeyleri ile plazma tiobarbitürik asit reaktif maddeleri, nitrik oksit ve total sülfidril grup düzeyleri ölçüldü.

Bulgular: Histolojik analizde aminoguanidinle tedavi edilen tavşanların yara bölgesinde önemli bir reepitelizasyon görüldü. Grup III'deki tavşanlarda fibroblastlar ve kollajen lifleri önemli ölçüde artmışdı. Aminoguanidin uygulaması yara dokusu ve plazmada malondialdehit düzeylerini anlamlı olarak azalttı ($p<0.05$). Aminoguanidinle tedavi edilen grupta yara glutatyon düzeyleri, tedavi edilmeyen gruba kıyasla artmakla birlikte, bu artış istatistiksel olarak anlamlı bulunmadı ($p>0.05$). Plazma total sülfidril grup düzeyleri, subkutan aminoguanidin tedavisinden sonra önemli derecede artış gösterdi ($p<0.05$).

Sonuç: Bu çalışmadan elde edilen bulgular, aminoguanidinin subkutan/local uygulamasının oksidan ürünlerin düzeylerini azaltarak yara iyileşmesini geliştirebileceğini göstermektedir.

Anahtar Kelimeler: Tavşan, kesi, yara iyleşmesi, nitrik oksit, aminoguanidin, oksidatif stres, glutatyon

Introductıon

General purpose of the studies related to wound healing is to define the conditions and factors for transition to normal tissue. Wound healing is a process including the phases such as hemostasis, inflammation, proliferation and remodeling as well a lot of biochemical and cellular mechanisms [1]. Reactive oxygen species (ROS) are associated with all stages of the healing process. ROS are produced by the inflammatory cells and play an integral role during this process. Stimulation of the immune system is associated with the situations such as chronic peridonditis and with the formation of ROS and reactive nitrogen species (RNS) during the inflammatory process. The tissues those exposed to oxidative damage of ROS is correlated with the intent of host defence [2]. But the increase in the ROS production concludes the non-healing wounds. Neutrophils and macrophages constitute most of the ROS sources. Increasing of the free oxygen radicals causes tissue necrosis and disruption of the cellular integrity [3]. Severity of the oxidative damage depends on the balance of antioxidant–oxidant system. Antioxidant administration is beneficial for healing effects [3-6]. Nitric oxide (NO) and superoxide (O_2^-) form peroxynitrite ($ONOO^-$) radical which is quite cytotoxic and lead to lipid peroxidation [7]. NO is necessary for the wound healing [8]. It reacts quickly with superoxide radicals in the presence of the transition metals such as iron and copper. Therefore oxidative and nitrosative free radicals formation is mentioned and lipid peroxidation is suggested to be increased in the wound healing [9]. Malondialdhyde (MDA) is relatively unchanged final product of lipid peroxidation [3]. Aminoguanidine (AMG) is an antioxidant substance which inhibits the activity of nitric oxide synthase (iNOS) that could be selectively induced [10]. AMG can be used to reduce local and systemic inflammations [11]. There are some studies suggested that AMG decreases the periodontitis [12]. In addition, some studies show that colecistisis [13] can help to treat acute lung injury [14] and complications related to diabetes [15]. AMG administration has a facilitating effect on the healing of the burned tissue and ulcer wounds caused by nonsteroid anti-inflammatory (NSAI) drugs [16, 17]. Local formulation applications of drugs gained importance recently because of the controlled drug release. Local application of the drug may also decrease the possible systemic side effects.

Therefore, we have planned to compare the effects of local and systemic administration of AMG on the oxidant and anti-oxydant parameters and histological changes of wound tissue and plasma.

Materıals and Methods

This study was conducted at Gazi University, Medical School, Department of Physiology after ethics committee decision dated 27.12.2007 and coded G.U. ET-07.068

Polyethylene Glycol and Aminoguanidine Formulation

Polyethylene glycol (PEG 6000) polymers are carriers of active ingredients and they used for tissue adhesion. These polymers are used as local distributors of the administered substances. PEGs have low toxicity and they have been allowed to be added to animal feeds and drinking waters. PEGs can be used as a vector to release and carry L-N (G)-nitro arginine methyl ester (L-NAME) [18].

Aminoguanidine hemisulphate (Sigma Chemical Co. [St. Louis, MO]) was mixed with melted polyethylene glycol (PEG 6000) then took into insulin injector and moulded as a bead [19, 20]. Each bead contains 12.7 mg of drug. Our previous study on the PEG formulation which was used for prolonged release was also available [18].

New-Zealand Rabbits total of eighteen were used in the study. A standard incision was applied to the oral mucosa of rabbits. Rabbits were divided into three groups as: Group I: untreated incisional group (control group), Group II: local Polyethylene glycol (PEG) +AMG (12.7 mg) in a bead, and Group III: subcutaneous (sc) AMG administrated group (100 mg/kg for 3 day). During the surgical procedures, sample collection and oral hygiene care was sensitively controlled. Animals were anaesthetised with intramuscularly injected ketamine HCl (35 mg/kg, Ketalar, Parke-Davis Co. Eczacıbası, İstanbul) following sedation with xylazin HCl (5 mg/kg Rompun, Bayer Co., İstanbul).

On the third day of oral incision, wound tissue strips and plasma were obtained.

Wound tissue was removed, washed in cold 0.9 % NaCl, wiped, weighed and frozen in liquid nitrogen and kept frozen -70ºC until its use. Healthy oral mucosa groups were created from the healthy mucosal of the administration groups.

Blood samples were collected in ethylene diamino tetra acetic acid (EDTA) tubes and centrifuged as soon as possible at 3000×*g* for 10 min at 4ºC. Plasma samples were stored at −70ºC until the analyses.

Determination of Tissue Lipid Peroxidation and Glutathione Levels

Two hundred milligrams of wound tissue including erosions was homogenized by 2 ml ice-cold 10% trichloroacetic acid solution, then the homogenate was centrifugated at 1500x*g* for 10 min and the supernatant MDA levels assayed by the thiobarbituric acid reactive substances (TBARS) formation [21, 22]. The glutathione (GSH) level was measured by the modified Ellman method [23]. As previously reported 750 µl of supernatant was added to an equal volume of 0.67% (w/v) thiobarbituric acid and heated up to 100ºC for 15 min. The absorbance of the samples was measured at 540 nm. The lipid peroxidation level was expressed in terms of the MDA equivalent using an extinction coefficient of 1.56 x 10^5 mol/

cm. To determine GSH levels, 0.5 ml of supernatant was added to 2 ml of 0.3 M $Na_2HPO_4.2H_2O$ solution. Next 2 ml dithiobisnitrobenzoic acid solution (0.4 mg/ml in 1% sodium citrate) was added, and the absorbance at 412 nm was measured immediately after mixing. The GSH levels were calculated usuing an extinction coefficient of 13000 mol/cm.

Determination of Total Nitric Oxide Levels

The NOx levels were obtained using an enzyme-linked immunosorbent assay reader by vanadium chloride (VCl_3)/Griess assay. Prior to NOx determination tissues were homogenized in five volumes of phosphate buffered saline (pH 7.5) and centrifuged at 2000*xg* for 5 min. Then 0.25 ml of 0.3 M NaOH was added to 0.5 ml supernatant. After incubation for 5 min at room temperature 0.25 ml 5% (w/v) $ZnSO_4$ was added for deproteinization. This mixture was then centrifuged at 3000*xg* for 20 min and supernatants were used for the assays [24, 25].

Plasma Thiobarbituric Acid Reactive Substances Levels

Lipid peroxidation was quantified by measuring the formation of TBARS as described previously by Kurtel *et al*. [21] Aliquots (0.5 ml) were centrifuged, and the supernatans were added to 1 ml of a solution containing 15% (w/v) tricarboxylic acid, 0.375% (w/v) thiobarbituric acid, and 0.25 N HCl. Protein precipiate was removed by centrifugation and the supernatans were transferred to glass test tubes containing 0.02% (w/v) butylated hydroxytoluene to prevent further peroxidation of lipids during subsequent steps. The samples were then heated for 15 min at 100ºC in a boiling water bath, cooled and centrifuged to remove the precipitant. The absorbance of each sample was determined at 532 nm. Lipid peroxide levels were expressed in terms of MDA equivalents using an extinction coefficient of $1.56x10^5$ mol^{-1}.

Plasma Total Sulfhydryl Group (RSH) Levels

0.5 ml of each sample was mixed with 1 ml of a solution containing 100 mM Tris-HCl pH 8.2, 1% sodium dodecyl sulfate (SDS) and 2 mM EDTA. The mixture was incubated for 5 min at 25ºC and centrifuged to remove any precipitate. 5, 5-dithiobis (2-nitrobenzoic acid)/ DTNB 0.3 mM was then added to each reaction volume and incubated for 15 min at 37ºC. The absorbance of each sample was determined at 412 nm. The RSH levels were calculated assuming a molar extinction coefficient of 13000 mol^{-1} cm^{-1} at 412 nm [21].

Plasma Total Nitric Oxide Levels

The NOx levels were estimated by the method of Miranda *et al*. [24] and Taşkıran *et al*. [25] Samples were deproteinized with 0.3 M NaOH and 5% (w/v) $ZnSO_4$, centrifuged and supernatants were used for the assays. After loading the plate with samples (100 µl) at room temperature, addition of vanadium III chloride (VCl_3) (100 µl) to each well was rapidly followed by addition of Griess reagents, sulphanilamide (50 µl) and N-(1-naphtyl) ethylenediamine dihydrochloride (50 µl). After incubation (usually 30-45 min), samples were measured at 540 nm using an ELISA reader.

Electron Microscopy

For the transmission electron microscope (TEM) analyses, healthy wound and AMG groups oral mucosa tissue samples were excised and prefixed immediately in 2.5% gluteraldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at room temperature. The tissues were then fixed in a similary buffered solution of 1% osmium tetraoxide (Sigma, St Louis, MO, USA) for 2 h at 48 C. Specimens were dehydrated through a graded series of acetone and then in propylene oxide and embedded in Araldite CY212 and dodecenylsuccinicanhydride (DDSA). One micrometer semi-thin cut sections were stained by toluidine blue to select the region of interest. Ultra-thin sections obtained with an ultramicrotome using a diamond knife were collected on 150 mesh copper grids and stained with uranyl acetate and lead citrate. The sections were examined and photographed using a LEO 906 E TEM.

Statistical Analysis

Data were presented as the mean±SD. Statistical analyses were carried out by Kruskal Wallis test and Bonferroni Correction Mann-Whitney *U*-test (SPSS for Windows 11.5; SPSS, Chicago, IL, USA). $p<0.05$ was taken as significant.

Results

Tissue and Plasma MDA Levels

Group II and Group III wound tissue MDA levels were found significantly decreased compared to Group I (2.13±0.21 nmole/g tissue, 2.20±0.56 nmole/g tissue, and 7.50±3.37 nmole/g tissue respectively, $p<0.05$) (Table 1).

Group II and Group III plasma MDA levels also were found significantly decreased compared to Group I (0.31±0.2 nmole/ml, 0.36±0.15 nmole/ml, and 0.98±0.18 nmole/ml respectively, $p<0.05$) (Table 2).

Tissue GSH and Plasma RSH Levels

Although Group II and Group III wound tissue GSH levels were increased compared to Group I, this increase wasn't significant (0.87±.18 µmole/g tissue, 0.80±.16 µmole/g tissue, and 0.76±.29 µmole/g, respectively, $p>0.05$) (Table 1). However, sulphydryl compounds (RSH) of the Group III were found significantly increased compared to Group I and Group II (476.4±67.2 nmole/ml, 287.1±117.8 nmole/ml, and 359.3±51.1 nmole/ml respectively, $p<0.05$) (Table 2).

Tissue and Plasma NOx Levels

There wasn't any difference between the wound groups in NOx levels. While AMG administration has lowered

Table 1. MDA, GSH and NOx Values of the Local and sc AMG Administration on the Rabbit Wound Tissue and the Control Group (Mean±SD)

	Control group (n=6)	Local AMG group (n=6)	Subcutan AMG group (n=6)
MDA (nmole/g tissue)	7.51±3.37	2.13±0.21*	2.21±0.56*
GSH (μmole/g tissue)	0.76±0.029	0.87±0.18	0.80±0.16
NOx (μmole/g tissue)	10.04±3.03	4.75±1.76	8.84±5.07

*$p<0.05$, as compared to the control group

Table 2. MDA, RSH and NOx Values of the Local and sc AMG Administration on the Rabbit Plasma and the Control Group (Mean±SD)

	Control group (n=6)	Local AMG group (n=6)	Subcutan AMG group (n=6)
MDA (nmole/ml)	0.89±0.18	0.32±0.01*	0.36±0.15*
RSH (nmole/ml)	359.28±51.11	278.11±117.82*	476.43±67.20*
NOx (μM)	50.62±14.78	29.02±8.15*	24.01±11.35*

*$p<0.05$, as compared to the control group

the wound tissue NOx levels, this decrease wasn't statistically significant $p>0.05$) (Table 1).

NOx levels in blood of Group II and Group III were found significantly decreased compared to Group I (29.02±8.15 μM, 24.00±11.35 μM, and 50.62±14.78 μM respectively, $p<0.05$) (Table 2).

Histological Findings

Healthy oral mucosa tissues and experimental groups which made of different applications in semi-thin cut sections that stained by Toluidine blue, the bottom of epithelial cell layer is basale cells with columnar shaped. Poligonal cells with stratified squamous epithelium are in upper section of basale cells. Connective tissue is composed of cell nucleus and collagen fibrils (Figure 1). Electron micrograph of control groups, showing stratified squamous epithelium consist of basale cells which is in the inferior portion of the epithelial cell layer and poligonal cells in upper section of basale cells. Connective tissue consists of collagen fibrils in inferior of stratified squamous epithelium (Figure 2).

In oral mucosa with wounded, epithelium is degraded in photomicrograph and electron micrograph sections. In point of wounds, epithelium degradation is advanced and hemoragic infiltration is observed (Figure 3-4).

Loss of epithelium and minimal reepithelization detected at local AMG group (figure5). Sc AMG group compared with other groups. In subepithelial tissue, fibroblast quantity and activation observed with increased collagen fibrils (figure 6).

Discussion

Recently rapid emerging developments at the cellular and molecular biology provide us to better understanding the wound healing mechanism. ROS are associated with all stages of the healing process. Migration, adhesion, proliferation, neovascularization, remodelling and apoptosis are modulated by ROS during the healing process. ROS should be produced with low doses in the healing process [26].

Increase of the plasma MDA as a result of smoking inhalation was found comparable with the increase of MDA at the lungs and the liver [27]. The tissue MDA progress is parallel with the plasma MDA [28]. Cutando *et al.* [2] shown an increase of plasma lipid peroxidation after teeth extraction.

High concentrations of the GSH are protective against reactive oxygen species and toxins. The redox state of the cells depends on the protection of reduced glutathione [29]. Antioxidants are increase in acute wounds like in chronic wounds. Increased oxidative stress in the acute wounds cause to decrease non-enzymatic antioxidants. Adding antioxidants to the wounds prevents cells from oxidative damage and increase the healing [30].

Increase of the antioxidant enzymes such as glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) in the wound tissue during the periodontal wound healing have been reported [31]. This increase was defined as 100% at third day.

NO has been stated to be freshly secreted in the human saliva [32]. NOS activity has been immunohistochemically identified in the rodents and human dental pulp and periodontal tissue [33-35]. NO plays an important role for regulating bloodstream of the pulp [33]. There is a significant increase at the periodontitis [34]. After NO inhibition which have been increased in periodontitis were inhibit bone destruction and extravasation [35].

The highest levels of NO in tissues in wound healing process are observed at about first week. Intense inflammatory cell infiltration at the wound site is source of the increased NO at this period. Neutrophil and macropha-

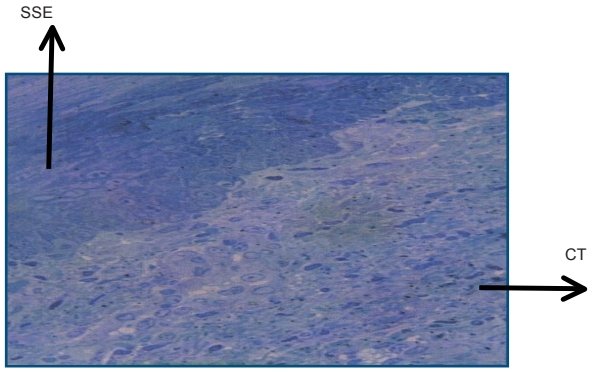

Figure 1. Photomicrograph of healthy oral mucosa group.

Stratified squamous epithelium (upper)(↑) subepthelial connective tissue (lower) (Toluidine blue x4).

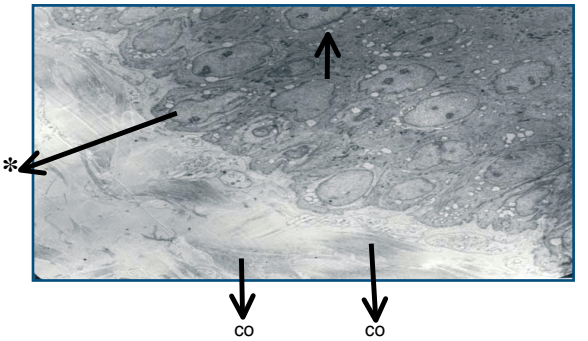

Figure 2. Electron micrograph of tough oral mucosa group.

Basale cells with columnar shaped (lower) (*) epithelium cells transformed in to polygonal shape (↑), subepithelial tissue with collagen fibril (co) (Uranyl acetate and Lead citrate x1293).

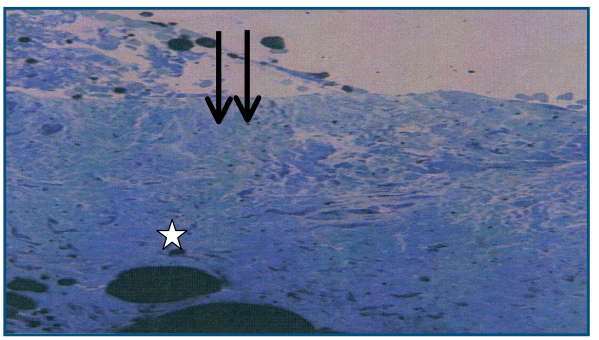

Figure 3. Photomicrograph of wounded oral mucosa group.

Loss of epithelial tissue (↓↓) and hemorrhagic infiltration (*) between the wound regions are observed (Toluidine blue, x4).

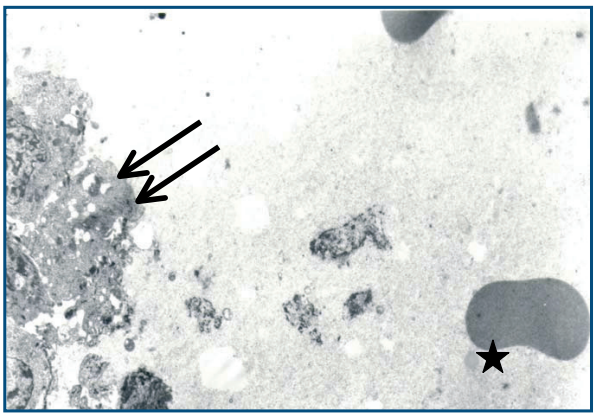

Figure 4. Electron micrograph of wounded oral mucosa group.

Loss of epithelial tissue (↑↑) and hemorrhagic infiltration wounded regions (*) (Uranyl acetate and Lead citrate x2784).

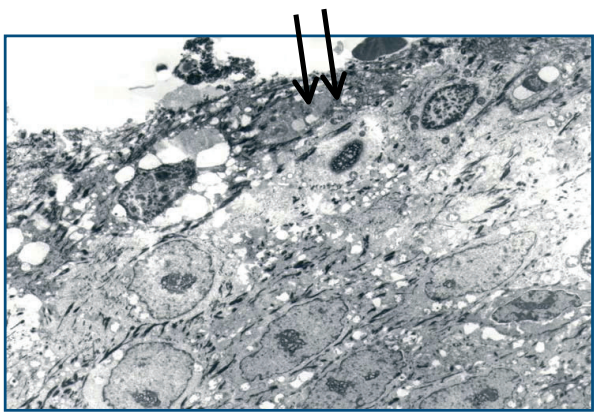

Figure 5. Electron micrograph of local AMG group.

Reepithelialization detected at (↓↓) the upper section of stratified squamous epithelium. (Uranyl acetate and Lead citrate x1670).

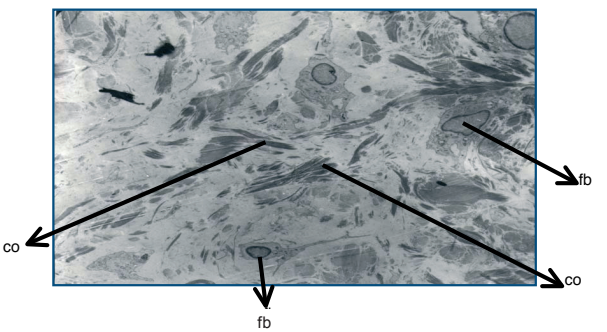

Figure 6. Electron micrograph of sc AMG group.

Increased fibroblast activation (fb) and collagen (co) fibril formation (Uranyl acetate and Lead citrate x1670).

ges are known to have plenty of iNOS expression at the first five days following the injury. Increased NO levels provide differentiation and increase of fibroblasts and keratinocytes so play an important role for transition to the proliferative phase of healing. While NO is synthesized by the cells those involved in the proliferative phase, wound NO levels are gradually reduced in parallel with the decrease at the iNOS expression through the wound healing [15].

In our study effect of local and systemic AMG administration on oral incisional wound was investigated. Local administrated AMG were given in PEG. PEG is a inert substance which has been widely used as a carrier. PEG group without drug was not included in the present

study. Because previous studies showed that PEG group without drug has no statistically significantly effect on the would healing at oxidative parameters [18, 36].

In this study local and subcutaneous AMG adminstration was significantly lowered the MDA levels in wound tissue compared to Group I.

This protective effect of AMG depends on its radical scavenging characteristic. AMG administration in tissue injuries lowered the oxidative stress and lipid peroxidation. AMG administration against paraquat toxicity that induces the oxidative stress was effective on the lowering of the increased MDA levels and increases the decreased total sulphydryl levels in the lung [37]. In experimental liver damage due to obstructive cholestasis, AMG administration decreased MDA levels while increasing the GSH levels in liver [13]. Polat *et al*. [38] have demonstrated that MDA and NO levels induced by gentamisine (GEN) are decreased as a result of AMG administration in rats. GPx, SOD, CAT and GSH levels in renals increased with AMG treatment. There are several studies in the literature which stating the increased GSH levels after AMG administration [13, 37, 38].

In our study, although local and sc AMG administration increased wound tissue GSH levels, it wasn't significant. Increased of plasma RSH levels was significant in sc AMG administrated group.

Ara *et al.* [11] defined that increased NO levels at peritoneum decreased after AMG treatment in the rats.

Periodontitis created in rats, AMG treatment reduced the inflammation and therefore tissue damage. Increased MDA and NO response with ligatures has been reduced after AMG administration [15].

While AMG administration did not have any significant effect on the NOx levels of the wounded tissues, a significant decrease on the plasma NOx levels of AMG treated rabbits were noted compared to nontreated group. Coskun *et al*. [18] have shown that while ip L-NAME had no significant effect on NOx levels in wound tissue, it significantly decreased plasma NOx levels.

Yavuz *et al.* [39] demonstrated that in their diabetic rat wound healing study, to show the collagen structure and TGF-ß1 involvement after AMG administration, granulation tissue and TGF- ß1 involvement in the damaged tissue were quite stronger and collagen fibers were more regular and similar to controls when dyed with type-IV collagen. In our study there was minimal reepithelialization in local AMG administered group, but there was a marked increase for fibroblast activation and collagen fiber production at the sc AMG administered group. This study also has indicated that the role of AMG at wound healing is occured through collagen structuring and organization.

Schaffer *et al*. [15] have indicated that NO is an important mediator for wound healing and NO synthesis is a parallel case with collagen accumulation and depostion in the wound. Therefore AMG as selective iNOS inhibitor could be considered to play an important role for collagen synthesis and deposition through NO production.

Di Paola *et al.* [12] have demonstrated that treating periodontitis which affects alveolar bone, peripheral connective tissues and leads to tooth loss with AMG administration reduces edema and inflammatory cell infiltration at gingivo-mucosal tissue sections.

We observed in our study that cell infiltration and hemorrhage reduced also at AMG group.

Finally; aminoguanidine can be used to reduce local and systemic inflammations. Considering all data it is possible to say that sc and local AMG administration may be effective at wound healing through both lipid peroxidation and NOx levels.

These studies show AMG may be useful for soft tissue, bone, tooth and dental implants. However, we think further studies are required for it's use, such as to observe it's effects on the different phases of wound healing at the next stage will be valuable.

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