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Research Article / Araştırma Makalesi

Investigation of Effects of Local Vitamin C Application on Inflammatory Response and Periodontal Tissue Destruction in Rat Periodontitis Model

Sıçan Periodontitis Modelinde Lokal C Vitamini Uygulamasının İnflamatuar Yanıt ve Periodontal Doku Yıkımı Üzerine Etkilerinin Araştırılması

Zeliha AYTEKİN[α](ORCID-0000-0002-6743-1994), **Ayşe TORAMAN**[β](ORCID-0000-0001-7988-0765), **Kübra KARAÇAM**[¥](ORCID-0000-0001-5981-5253)

[α]Akdeniz University, Faculty of Dentistry, Department of Periodontology, Antalya, Türkiye
[α]Akdeniz Üniversitesi, Diş Hekimliği Fakültesi, Periodontoloji AD, Antalya, Türkiye
[β]Department of Periodontology, Faculty of Dentistry, Sağlık Bilimleri University, İstanbul, Türkiye
[β]Sağlık Bilimleri Üniversitesi,Diş Hekimliği Fakültesi, Periodontoloji AD,İstanbul Türkiye
[¥]Afyonkarahisar University of Health Sciences, Faculty of Dentistry, Department of Periodontology, Afyonkarahisar, Türkiye
[¥]Afyonkarahisar Sağlık Bilimleri Üniversitesi, Diş Hekimliği Fakültesi, Periodontoloji AD, Afyonkarahisar, Türkiye

ABSTRACT

Background: The present study aims to evaluate the effect of local vitamin C application on inflammatory response and periodontal tissue destruction in rats with experimental periodontitis.

Methods: A total of 21 animals, 7 rats in each group, were used in the study: 1- Non-ligation (NL), 2-experimental periodontitis (EP), and 3- local vitamin C (VtC) groups. Experimental periodontitis was induced in EP and VtC group rats. After 11 days, the ligature was removed. 50 mL of saline solution and vitamin C were injected locally into the vestibule sulcus of the EP and VtC group rats, respectively. After the rats were sacrificed, blood and gingival tissue samples were taken. TNF-α levels were analyzed biochemically in serum and 8-OHdG and MMP-8 were analyzed immunohistochemically in gingival tissue. The mandibular specimens were histologically evaluated for bone destruction and attachment loss.

Results: The numeric density of 8-OHdG and MMP-8 immunopositive cells were found to be statistically significantly lower in the Vtc group than in the EP group. Vitamin C administration significantly reduced attachment loss and bone loss in the VtC group as compared to the EP group. However, there was no significant difference in serum TNF-α level in the VtC group compared to the EP group.

Conclusions: Local application of vitamin C may reduce inflammation-related bone destruction by supporting the inflammatory response with the antioxidant activity of vitamin C and contributing to the resolution of inflammation with its potential immunomodulatory effect. Vitamin C may be a therapeutic agent that can be used in the treatment of periodontitis.

Keywords: Antioxidants, Experimental Periodontitis, Oxidative Stress, Vitamin C

ÖZ

Amaç: Bu çalışmanın amacı deneysel periodontitisli ratlarda lokal C vitamini uygulamasının inflamatuar yanıt ve periodontal doku yıkımı üzerine olan etkisini değerlendirmektir.

Gereç ve Yöntemler: Çalışmada her grupta 7 sıçan olmak üzere toplam 21 hayvan kullanıldı: 1-Ligatür bağlanmayan (NL) 2-deneysel periodontitis (EP) ve 3-lokal vitamin C (VtC) grupları. EP ve VtC grubu sıçanlarda deneysel periodontitis oluşturuldu. Ligatürler 11 gün sonra çıkarıldı. EP ve VtC grubu sıçanların vestibül sulkusuna sırasıyla 50 µL salin solüsyonu ve C vitamini lokal olarak enjekte edildi. Ratlar sakrifiye edildikten sonra kan ve dişeti dokusu örnekleri alındı. TNF-α seviyesi serumda biyokimyasal olarak, 8-OHdG ve MMP-8 düzeyleri ise dişeti dokusunda immünohistokimyasal olarak analiz edildi. Mandibula örnekleri kemik yıkımı ve ataşman kaybı açısından histolojik olarak analiz edildi.

Bulgular: 8-OHdG ve MMP-8 immünopozitif hücre yoğunluğunun Vtc grubunda EP grubuna göre istatistiksel olarak anlamlı derecede daha düşük olduğu bulundu. C vitamini uygulaması, EP grubuna kıyasla VtC grubunda ataşman kaybını ve kemik kaybını önemli ölçüde azalttı. Ancak EP grubuna göre VtC grubunda serum TNF-α düzeyinde anlamlı bir fark yoktu.

Sonuç: Lokal C vitamini uygulaması, C vitamininin antioksidan aktivitesi ile inflamatuar yanıtı destekleyerek ve potansiyel immünomodülatör etkisi ile inflamasyonun çözülmesine katkıda bulunarak inflamasyona bağlı kemik yıkımını azaltabilir. C vitamini periodontitis tedavisinde kullanılabilecek terapötik bir ajan olabilir.

Anahtar Kelimeler: Antioksidanlar, Deneysel Periodontitis, Oksidatif Stres, Vitamin C

1. Introduction

Periodontitis is a chronic inflammatory disease of the periodontium characterized by the progressive destruction of tooth-supporting tissues due to complex interactions between periodontopathogens and the host immune response.[1] Polymorphonuclear leukocytes (PMNL) are first-line defense cells that fight bacterial pathogens in periodontitis. When host cells, especially PMNL, are stimulated by periodontopathogenic bacteria, they release reactive oxygen species (ROS) as part of the immune defense.[2]

In a healthy state, there is a critical equilibrium between antioxidant defense and ROS production systems.3 When this balance is disturbed in favor of oxidants, oxidative stress occurs. Evaluating specific footprints of oxidative damage to protein, DNA, and lipid is the most efficient way to determine oxidative stress in a host.[4,5]

8-hydroxy-2′-deoxyguanosine (OHdG) is a product that shows oxidative damage to both mitochondrial and nuclear DNA.[3] Recently, several studies showed an increase in 8-OHdG levels of saliva, serum, and gingival crevicular fluid (GCF) in patients with periodontitis compared to healthy individuals.[6-8] In addition, it has been reported that 8-OHdG can be used as a biomarker to help determine periodontal status since there is a positive correlation between the salivary 8-OHdG level and bacterial load in periodontitis.[9]

During periodontitis, immune response host cells recognize bacteria and their products as danger and then produce proinflammatory mediators, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α).[10] TNF-α is an essential inflammatory mediator that induces an immune response by inducing the secretion of adhesion molecules, matrix metalloproteinase (MMP), and promoting differentiation of osteoclast precursors and subsequent activation of osteoclasts.[11]

Vitamin C which is a member of the body's antioxidant system aids the bactericidal activities of macrophages and PMNLs, and promotes the scavenging of nitrogen species and ROS.[12] Vitamin C has been shown to have a protective and therapeutic role when applied at higher than

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Sorumlu yazar/Corresponding Author: Zeliha Aytekin
E-mail: zelihaaytekin06@gmail.com
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dietary recommendation levels in numerous diseases, including recurrent bacterial or viral infections, cancer, atherosclerosis, diabetes or allergic conditions.[13-15] Previous reports showed a negative relationship between plasma[16] and serum[17] concentration of vitamin C and periodontal attachment loss and the prevalence[4] of periodontitis.

In the light of the aforementioned facts, the present study was conducted to examine the potential ameliorative effects of vitamin C on periodontal tissue destruction in ligature induced experimental periodontitis in rats.

2. Material and methods

2.1. Animals and ethics

A total of 21 male Sprague-Dawley albino rats, weighing 200 to 250 g, were used in the present study. The animals were kept in a temperature and humidity controlled room. (23-25°C and 55-65 relative humidity) The animal procedures used in this study were accepted by the Local Ethics Committee of Atatürk University for Animal Studies. (Permit Number: 2022-5).

2.2. Experimental procedures

The rats were randomly separated into three groups (7 in each group) as: Non-ligation group (NL), experimental periodontitis group (EP), and local vitamin C group (VtC). NL group rats have not received ligation and were used as controls, while 3-0 silk ligatures were tied around the right mandibular first molar teeth at the subgingival level in the EP group and VtC group to cause bacterial biofilm retention.

The entire experimental procedure was performed after being general anesthesia with ketamine hydrochloride* and xylazine hydrochloride**.

After 11 days, the ligature was removed, the VtC group rats were received 50µL commercial vitamin C*** was applied locally [18,19] into the VtC group rats' vestibular sulcus area of mandibular first molar teeth three times at intervals of 2 days with an insulin needle. [20-23] The same local application was administrated to the EP group using 50 µL of physiological saline. Schema of experimental design and time course are demonstrated in **Figure 1**.

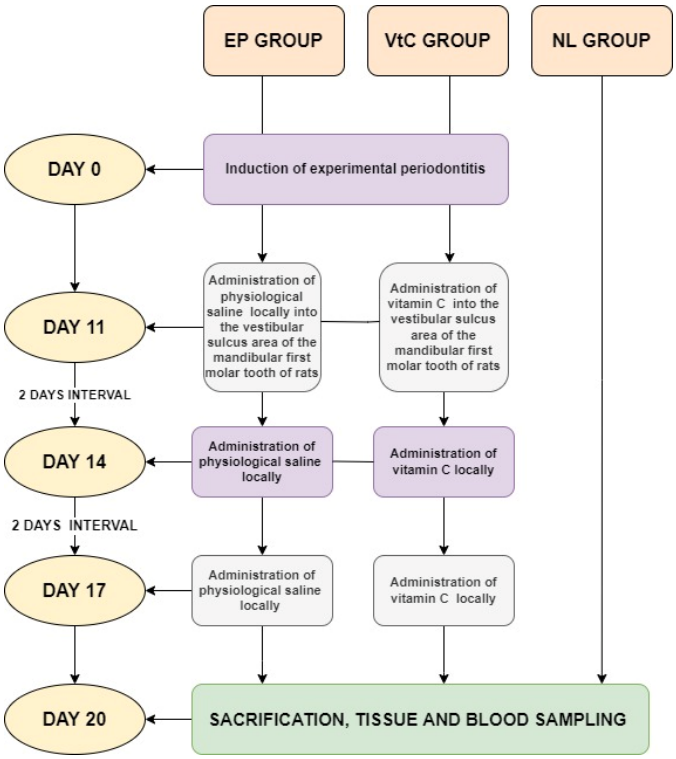

Figure 1.

2.3. Sample collection

After the experimental procedure was completed, the rats were anesthetized with ketamine hydrochloride and xylazine hydrochloride, 10 cc of blood was taken by cardiac puncture for biochemical analysis, and then the animals were sacrificed. The mandibles were removed for immunohistochemical and histological examination. Tissue samples were stored in 10% formaldehyde solution until light microscopic analysis.

The blood samples were transferred into centrifuge tubes and centrifuged at 5000 rpm for 7 min, then serum was stored in a -80°C freezer until analysis day.

2.4. Immunohistochemical evaluation of 8-OHdG and MMP-8

The mandibular specimens which were in formaldehyde solution were prepared for the histological and immunohistochemical analyses. The gingival tissues around the first mandibular teeth were excised from the jawbone and deparaffinized, rehydrated and, sectioned (5-μm) with a microtome $^{\alpha}$ for immunohistochemical analyses. The paraffin-embedded gingival tissue sections were stained with anti-8-OHdG $^{\gamma}$ and anti-MMP8 $^{\mu}$ using the streptavidin-biotin-peroxidase method.

The immunolabelled cell density in gingival tissue was visualized with a high-power light microscope $^{\beta}$. The numerical intensity values of immunopositive cells in the gingival sections were analyzed and counted utilizing a modified light microscope£ and stereology software$^{¥}$ by an examiner with no prior knowledge of the experimental design. The unbiased counting frame-fractionator combination stereology method was used for counting immunopositive cells.[24]

2.5. Histological evaluation

The mandibles were decalcified with 10% EDTA, dehydrated, and then embedded in paraffin and the specimens were sectioned bucco-palatally into 5-μm-thick serial sections using a microtome, and stained with hematoxylin and eosin (H&E).

The histomorphometric analysis of H&E stained tissue specimens was performed with a high-power light microscope $^{\beta}$. Measurements of the mean distance of the cementoenamel junction to the junctional epithelium (CEJ-JE) and CEJ to the alveolar bone crest (CEJ-ABC), were used for the detection of clinical attachment loss (CAL), and alveolar bone loss (ABL) respectively. [25] (**Figure 2**). CAL and ABL were measured at the mesial and distal sides of the right mandibular first molar tooth.

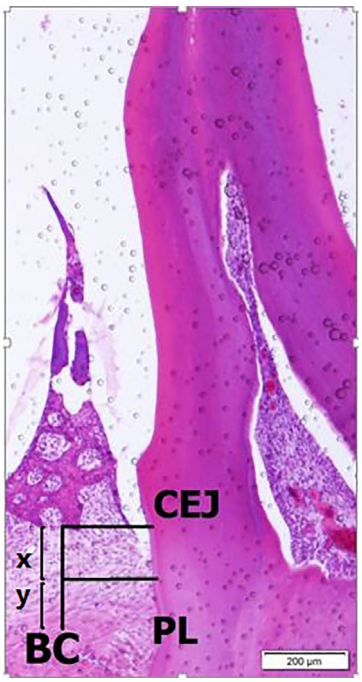

Figure 2.

2.6. Biochemical evaluation of TNF-α

Serum TNF-α concentration was measured using an ELISA kit specific with rat TNF-α kit€ according to the manufacturer's recommendations. The outcomes are presented as the mean (pg/ml) ± standard deviation (SD) of the serum concentration.

2.7. Statistical analyses

All statistical analyses were conducted in IBM SPSS Statistics, Version 23.0 (IBM Corp., Armonk, NY, USA). All data were considered statistically significant at $p < 0.05$. Kolmogorov Smirnov test was performed to choose whether the data of each parameter expressed normal distribution in all groups. Levene's homogeneity test was used to determine the homogeneity of data. Since the data did not show normal distribution, the numerical density values of MMP-8 positive stained cells were examined with the Kruskal-Wallis test. Furthermore, since the 8-OHdG positive cells' numerical density values, serum TNF-α values, amount of ABL, and CAL have presented normal distribution the statistical difference between the groups was examined by Tukey test and ANOVA analysis.

3. Results

3.1. Biochemical results

Biochemical results and comparisons between the groups are presented in **Table 1**. Serum TNF-α levels were statistically significantly higher in the EP group compared to NL group ($p < 0.05$), and local vitamin C application slightly reduced serum TNF-α levels in VtC group but this was not statistically significant ($p > 0.05$).

Table 1. Comparison of biochemical and immunohistochemical results among groups.

GROUPS	8-OHdG (tissue; nmol n/μm²)	MMP-8 (tissue; nmol n/μm²)	TNF-α level (serum; pg/m
NL	0.114±0.42[a]	0.0535±0.0357[a]	41.13±2.62[a]
EP	0.314±0.77[b]	0.2112±0.052[b]	61.04±1.68[b]
VtC	0.182±0.41[a]	0.0856±0.0405[a]	54.19±4.79[b]

Values are expressed as means ± standard deviation. Letters (a,b,c) in the same column indicate significant differences between groups; (n=7) p<0.05. For statistical analysis, differences between groups were determined by Kruskal-Wallis test, Tukey test and ANOVA analysis.

3.2. Histological results

Histological results and comparisons among the groups are presented in **Figure 3**. The microscopic visualizations displayed the periodontal tissue destructions, including gingival tissue inflammation related mesial and distal ABL and, mesial and distal CAL in the EP group when compared with those in the NL group ($p < 0.05$). However, statistically significant lower periodontal destructions were observed in the vitamin C treatment group compared to the EP group ($p < 0.05$).

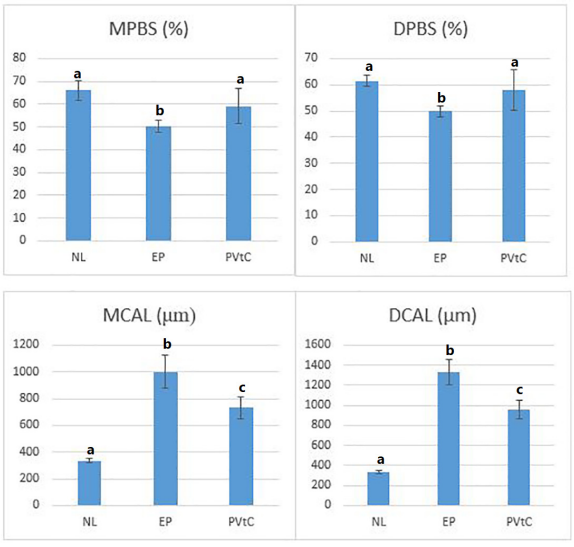

Figure 3.

3.3. Immunohistochemical results

The comparisons between groups in the numerical density values of anti-8OHdG and anti-MMP-8 immunopositive cells were presented in **Table 1**.

The immunohistochemical results of 8-OHdG and MMP-8 showed statistically significantly higher immunolabeling patterns in the EP group compared with the NL group in the gingival tissue sections. Local vitamin C administration significantly decreased the anti-8-OHdG positive stained cells counts in VtC group compared with the EP group ($p < 0.05$). Furthermore, the VtC group showed significantly lower immunostaining in terms of MMP-8 as compared with that in the EP group ($p < 0.05$). The immunohistochemical stainings were shown in **Figure 4** and **Figure 5**.

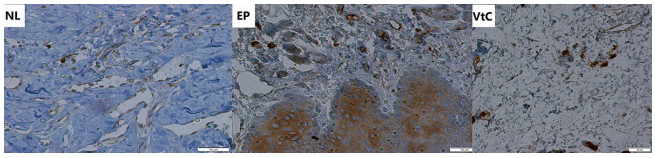

Figure 4.

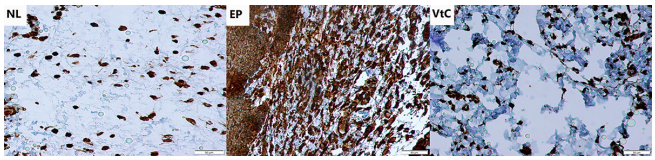

Figure 5.

4. Discussion

To the best of the authors' knowledge, this is the first study to examine the immunomodulatory and antioxidant effects of local vitamin C application on periodontal destruction in experimental periodontitis in rats using gingival 8-OHdG, MMP-8, and serum TNF-α parameters. Results of the present study showed that local vitamin C application decreased alveolar bone loss, attachment loss, and gingival tissue levels of 8-OHdG and MMP-8 after ligation induced periodontitis in rats.

Oxidative stress has been associated with tissue destruction in periodontitis[4], as in many diseases.[26-28] During inflammation, the tissue antioxidant levels drop rapidly, while the production of free radicals increases at the periodontal lesion.[29,30] There is a negative relationship between the severity of periodontitis and systemic antioxidant defense.[4] Therefore, antioxidant support may be beneficial in the amelioration and/or prevention of periodontitis.[31]

Vitamin C is an important reducing agent as an antioxidant in the body.[32] In our previous study [22], it has been shown that local vitamin C supplementation to the inflammation side has a therapeutic effect on reducing lipid peroxidation related tissue damage by oxidative stress. Toraman et al.[23] evaluated the therapeutic effects of vitamin C comprehensively in diabetic rats induced experimental periodontitis model and reported that local vitamin C administration suppressed inflammatory alveolar bone resorption; this study also revealed that local vitamin C administration significantly decreased anti-8-OHdG immunostaining cell count in gingiva. Ekuni et al. [29] reported a 173% increase in aortic 8-OHdG level in periodontitis-induced rats compared to the unligated control group. However systemic vitamin C treatment induced a 38% decrease in this level in the vitamin C group compared to the periodontitis group. Furthermore, Tomufuji et. al.[33] found that the systemic increase in plasma vitamin C decreased gingival 8-OHdG levels. These findings are consistent with the results of the present study, which showed that local vitamin C application decreased 8-OHdG levels in the periodontal tissues.

No cytotoxic effect was observed in studies using vitamin C at higher doses than the dose used in our study.[34,35] In addition, in our previous studies, the dose used in this study was applied to rats without any side effects. [22,23]

Oxidative stress contributes to impaired cellular differentiation function, which stimulates osteoclast differentiation [10] and the overproduction of proinflammatory cytokines such as IL-1, IL-6, and TNF-α [36]. TNF-α inhibits bone formation by reducing osteoblastic activity and induces downregulation of the osteocalcin gene.[37] Previous studies have found significantly increased TNF-α levels in serum, saliva, and GCF in periodontal disease. [37,38] It has been reported in the literature that vitamin C administration decreases the production of proinflammatory cytokines TNF-α and IFN-γ and increases the formation of IL-10 from lipopolysaccharide (LPS) stimulated peripheral blood lymphocytes. [39] However, the results of our study showed that vitamin C administration did not cause a significant effect on serum TNF-α levels. The reason for this may be that the amount of cytokine produced due to inflammation in the gingiva, which is a very small tissue, is too low to be detected systemically in the blood.

MMP-8 is one of the main components responsible for the degradation of collagen in the physiological remodeling of periodontal tissues in both health and periodontal disease.[40] In several studies, it has been shown that MMP-8 is a collagenase whose secretion is increased in gingival tissues in periodontal disease.[22,41] In this study, immunohistochemical analysis revealed a strong immunopositivity of MMP-8 in the EP group than in the NL group. This result is in line with the results of previous studies.[40,41] Furthermore, it was found weaker immunolabelling of MMP-8 in rats' tissue sections in VtC group than in the periodontitis group. This result may be associated with the role of vitamin C in collagen formation and its potential immunomodulatory ability to the mediation of the resolution of inflammation.

Histomorphometric analysis performed in this study revealed a significant alveolar bone loss and apical migration of junctional epithelium in the ligature placed area in the EP group compared to NL group. This finding is in line with previous research.[25,30] During the immune response to bacterial stimuli in periodontitis, bone and soft tissue destruction occur due to ROS and proinflammatory mediators. In previous research, it has been reported that vitamin C improves phagocyte function by 1) stimulating neutrophil migration to the infection side, 2) increasing ROS production and microbial killing of phagocytes. Besides, vitamin C is increased caspase-dependent neutrophil apoptosis and clearance by macrophages. Hereby, vitamin C not only promotes inflammation but also supports the resolution of inflammation and protects host tissues from excessive tissue destruction with inflammation.[42] In addition, in-vitro studies showed that vitamin C suppressed osteoclast differentiation but supported osteoblastic differentiation.[43,44] However, in a clinical study, it was shown that systemic vitamin C supplementation in addition to non-surgical periodontal treatment did not have an additional effect on clinical parameters and plasma antioxidant levels in patients with periodontitis.[45] In contrast to this study, Yussif et al.[46] demonstrated that local application of vitamin C after nonsurgical periodontal therapy is an effective adjunctive therapy in reducing the clinical and histological manifestations of chronic gingivitis. We may infer that local application of vitamin C decreased alveolar bone loss and periodontal tissue damage by suppressing oxidants and supporting the host modulation to the resolution of inflammation.

Systemically administered antioxidants may need to be administered at intolerably high doses in order to achieve sufficient therapeutic doses in the relevant inflammatory region.[47] For this reason, the local application method, which can be applied at lower doses and with fewer side effects have been preferred compared to systemic applications.[46]

The present study has some limitations. First of all, although we chose a dose that did not cause any toxic side effects in previous studies [22,23], the lack of dose-related study groups is one of the limitations of our study. Furthermore, ligature-induced experimental periodontitis is another limitation of this study. The anatomy of rat teeth is remarkably similar to humans. However, the soft and hard tissue in rats is very small, and the ligature-induced periodontitis is of very short duration. Moreover, the fact that cytokine production, which is an indicator of inflammatory activity, was not examined locally in the gingival tissue is a limitation of our study.

CONCLUSIONS

The present findings revealed that locally administrated vitamin C minimized alveolar bone loss and attachment loss, and decreased 8-OHdG and MMP-8 immunolabeling in gingival tissue. Despite the limitations of the current study, local application of vitamin C has been shown to reduce periodontal tissue destruction in periodontal disease. In light of this information, it may be beneficial to choose vitamin C as a local release agent in future studies.

FOOTNOTE

* ketamine hydrochloride (Ketalar, Pfizer, Istanbul, Turkey; 40 mg/kg

** xylazine hydrochloride (Rompun Bayer, Istanbul, Turkey; 10 mg/kg

* * * Redoxon amp 500mg/5mL; Bayer Chemical Industry, Istanbul, Turkey

α microtome (LeicaVR RM2125RT; Leica Instruments, Nubloch, Germany)

γ anti-8-OHdG (Santa Cruz Biotechnology, Santa Cruz, CA)

μ anti-MMP8 (Santa Cruz Biotechnology, Santa Cruz, CA)

£ modified light microscope (Leica DM4000B; Leica Instruments)

¥ stereology software (Stereo-Investigator software v.9.0; Microbrightfield, Williston, VT, USA)

β high power light microscope (Nikon Eclipse i50, Tokyo, Japan)

€ TNF-α kit (Invitrogen, Thermo Fisher Scientific, Waltham MA)

Değerlendirme / Peer-Review

İki Dış Hakem / Çift Taraflı Körleme

Etik Beyan / Ethical statement

Bu çalışmanın hazırlanma sürecinde bilimsel ve etik ilkelere uyulduğu ve yararlanılan tüm çalışmaların kaynakçada belirtildiği beyan olunur.

It is declared that during the preparation process of this study, scientific and ethical principles were followed and all the studies benefited are stated in the bibliography.

Benzerlik Taraması / Similarity scan

Yapıldı – ithenticate

Etik Bildirim / Ethical statement

ethic.selcukdentaljournal@hotmail.com

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Çıkar Çatışması / Conflict of Interest

Yazarlar çıkar çatışması bildirmemiştir. | The authors have no conflict of interest to declare.

Yazar Katkıları / Author Contributions

Çalışmanın Tasarlanması | Design of Study: ZA (%40), AT (%40), KK (%20)
Veri Toplanması | Data Acquisition: ZA (%60), AT (%20),KK (%20)
Veri Analizi | Data Analysis: ZA (%50), AT (%50)
Makalenin Yazımı | Writing up: ZA (%60), AT (%40)
Makale Gönderimi ve Revizyonu | Submission and Revision: ZA (%80), AT (%20)

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