ORIGINAL ARTICLE

Effects of alpha-tocopherol on gingival expression of inducible nitric oxide synthase in the rats with experimental periodontitis and diabetes

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Abstract

Background: The aim of this study was to investigate effects of α-tocopherol and/or insulin on the number of gingival inducible nitric oxide synthase (iNOS) positive cells in rats with experimental periodontitis with or without streptozotocin (STZ)-induced diabetes.

Materials and Methods: A total of 60 Sprague-Dawley rats were divided into three groups: Group I: The group without diabetes; Group II: The group with STZ-induced diabetes; Group III: The group with STZ-induced diabetes receiving insulin therapy. All animals received anesthesia, and 3/0 silk suture was inserted around the mandibular molar teeth. All groups were divided into subgroups receiving saline (Groups IA, IIA, IIIA) and α-tocopherol injection (Groups IB, IIB, IIIB). After a period of 3 weeks, all rats were sacrificed, and the number of gingival iNOS positive cells was analyzed using image analysis software.

Results: Applying α-tocopherol suppressed the number of gingival iNOS positive cells in Groups IB, IIB, and IIIB compared to application of saline (Groups IA, IIA, and IIIA) ($P < 0.05$). Numbers of gingival iNOS positive cells were found to be similar in the Groups I and III ($P > 0.05$).

Conclusions: Within limitations of the current study, this is the first study one may suggest that α-tocopherol may reduce oxidative damage in the gingiva of the rats with periodontitis with or without STZ-induced diabetes and increase effects of insulin.

Key words: Diabetes, experimental periodontitis, inducible nitric oxide synthase, α-tocopherol

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Introduction

Periodontitis is a chronic, irreversible condition in which inflammatory changes beginning in the gingiva as a consequence of bacterial impacts result in destruction in the alveolar bone, increased depth of pocket, loss of attachment, mobility, and unless treated loss of tooth.[1] Diabetes mellitus has been defined as a group of metabolic disorders occurring due to decreased secretion and/or effects of insulin and is characterized by impairments in carbohydrate, protein, and lipid metabolism causing several disorders in the body

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and even death, and its frequency has been reported to steadily increase.[2,3] The events during development and occurrence of diabetes have been reported to be related oxidative stress.[4,5]

In 1980, Furchgott and Zawadski reported that vasodilation due to acetylcholine was mediated by an endothelium-derived relaxing factor (EDRF). In 1986, Dr. Furchgott reported that EDRF might be nitric oxide (NO) because of similarities between pharmacological actions of EDRF and NO EDRF was defined as NO molecule in 1987, and in 1988 it was demonstrated that vascular endothelial cells might use L-arginine in NO synthesis.[6,7] The non is synthesized from L-arginine by a member of an enzyme family called NO synthases. This enzyme has three forms: Type-1 neuronal NO synthase, brain enzyme; type-2 inducible NO synthase (iNOS) enzyme, which is found in the macrophages; and type-3 NO synthase, which is endothelial cellular enzyme.[8]

It has been reported that iNOS level is higher in the presence of periodontal disease than in healthy individuals.[9-11] NOS activity has been reported to be higher in the neutrophils from individuals with a diagnosis of localized aggressive periodontitis than those obtained from healthy individuals.[12] However, it was also reported that iNOS secretion in gingival tissue of individuals with chronic periodontitis is higher than healthy controls.[11] It has been reported iNOS secretion is higher in areas of intense inflammation in the periodontal tissues.[13] Furthermore, levels of iNOS and tumor necrosis factor (TNF)-α are reported to increase in gingival tissue of rats with experimental periodontitis.[10]

Vitamin E is an essential liposoluble vitamin that functions as an antioxidant not produced by the human body. Eight substances were found in nature that possess Vitamin E activity: α-, β-, γ- and δ-tocopherol; and α-, β-, γ- and δ-tocotrienol.[14,15] Vitamin E and α-tocopherol have been reported to enhance cellular proliferation and wound healing, suppress iNOS production, and provide protection against oxidative damage.[16-20]

The present study aimed to investigate the effects of systemic application of α-tocopherol and insulin on gingival cells based on the hypothesis that there was a strong relationship between diabetes and periodontal disease, that oxidative stress due to reactive oxygen species such as iNOS produced in the rats with experimental diabetes might be suppressed by α-tocopherol which is an antioxidant and that the present oxidative stress might be reduced by controlling diabetes.

Materials and Methods

The present study was approved by Ethics Committee of Center of Research and Application of Experimental Medicine of Selcuk University (2008/54). A total of 60 male Sprague-Dawley rats (mean weight: 300 g) from a single center were included in the study. They were put in cages as groups of five and fed only on standard rat feed and water.

In order to create a control group, experimental diabetes was not induced in 20 of the rats (Group I). The remaining 40 rats were reserved to induce experimental diabetes. In order to induce irreversible experimental diabetes, 50 mg/kg of streptozotocin (STZ, Sigma-Aldrich, USA) was injected intraperitoneally (IP). Three days after injection, blood samples were taken from the rats' tails and plasma levels of glucose were determined to be above 300 mg/dl and the rats were considered as diabetic. Half of the 40 rats with experimental diabetes constituted the group of "uncontrolled diabetes" (Group II, $N = 20$), and the other half of the 40 rats constituted the group of "controlled diabetes," receiving insulin treatment (Group III, $N = 20$). One day after induction of diabetes, 20 rats (Group III) received 2 units (U) of insulin (100 U/ml of insulin containing 30% soluble insulin aspartate and 70% insulin aspartate protamine crystals) (Novo Nordisk, France) twice daily for 7 days via intramuscular (IM) route and success of glycemic control was evaluated. Ten days after initiation of the study, 3/0 silk suture was inserted subgingivally on the first right mandibular molar teeth of all rats for ligature-induced periodontitis[21,22] under general anesthesia (ketamine 30 mg/kg IM and Rompun 5 mg/kg IM). Then, half of each group (Groups IA, IIA and IIIA) received saline solution (0.9% NaCl) once daily for 21 days and the other half of each group (Groups IB, IIB, and IIIB) received α-tocopherol (40 mg/kg) IP.

For immunohistochemically examination, lower jaws of the rats decapitated by injecting a high dose of anesthetic substance were removed and put in 10% of formaldehyde solution. Immunohistochemically analysis of the specimens from the rats was carried out in the pathology laboratory at Meram Medical School of Selcuk University. All samples were decalcified first in 10% solution of formic acid. After the process of decalcification lasting for about 2 days, samples of about 5 mm in thickness were taken from the mandibles to cover the suture area between the first and second molar teeth. These samples were cassetted and subjected to a process of routine tissue follow-up in Autotechnicon instrument (Leica ASP 300). The samples for which process of tissue follow-up was completed were embedded in paraffin blocks.

Samples 5 μm in thickness were taken using microtome from the paraffin blocks prepared for immunohistochemically examination. All pictures were transferred to a computer and evaluated by image analysis system (Clemex Vision Lite 3.5). First, length was calibrated by the photogram (Nikon Stage Micrometer Type A [MBM11100] photogram).[1]

[1] Nikon, Nikon Corp., Tokyo, Japan

Number of the cells positively stained with iNOS in the connective tissue under the junctional epithelium was evaluated. Similar areas were chosen in each subject and an area of 0.1 mm^2 was marked by image analysis system. The cells positively stained with iNOS on this area were marked and counted automatically by the image analysis system. The damaged cells were excluded from the analysis. The reader performed the evaluations without knowing features of the subjects and the stained marker.

Statistical analysis of the data

The statistical analysis was performed using commercially available software (SPSS v.17.0, IBM, Chicago, IL, USA). Two-way analysis of variance was used to evaluate the inter-group differences. Data for which inter-group difference was significant were evaluated using one-way analysis of variance and Tukey-highly significant difference test. $P < 0.05$ were considered as statistically significant.

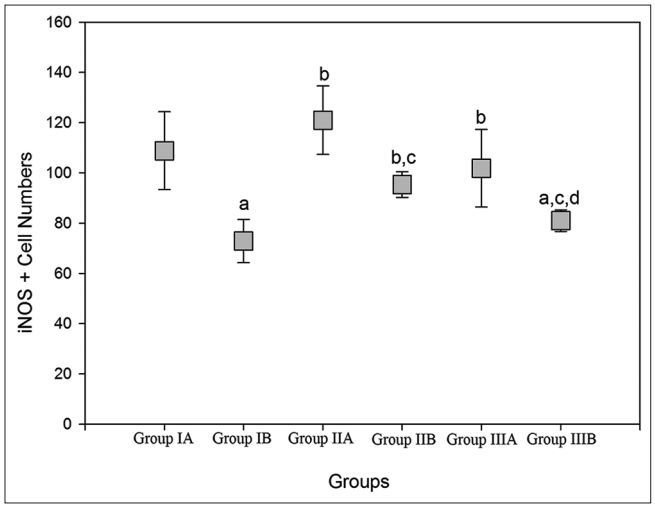

Figure 1: Statistical analysis of inducible nitric oxide synthase + cells in the groups. For statistical comparisons, two-way analysis of variance, one-way analysis of variance, and Tukey-highly significant difference test were used.(a) Significantly different than the Group IA.(b) Significantly different than the Group IB. (c) Significantly different than the Group IIA.(d) Significantly different than the Group IIIA

Results

Sixty rats were included in the current study, and one rat from the Group IB, two rats each from Groups IA, IIB, IIIA and IIIB and three rats from Group IIA were lost, and the study was completed with 48 rats. Samples for which immunohistochemically measurement was not made were not statistically analyzed.

Based on results of the two-way analysis of variance, iNOS expression levels were found to be different for Groups I, II, and III ($P < 0.05$) [Table 1, Figure 1]. It was found that number of iNOS positive cells in the sections from the rats in the uncontrolled diabetes group (Group II) was higher than those from the rats in the Groups I and III ($P < 0.05$). Number of the cells positively stained with iNOS in all rats

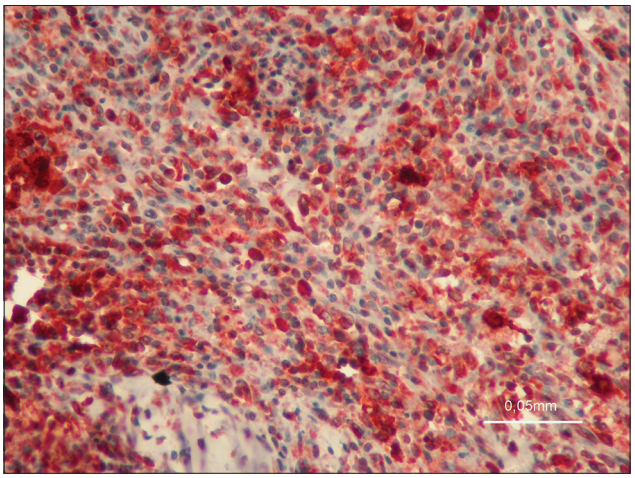

Figure 2: Immunohistochemical staining of inducible nitric oxide synthase + cells in gingival tissue samples from Group IA

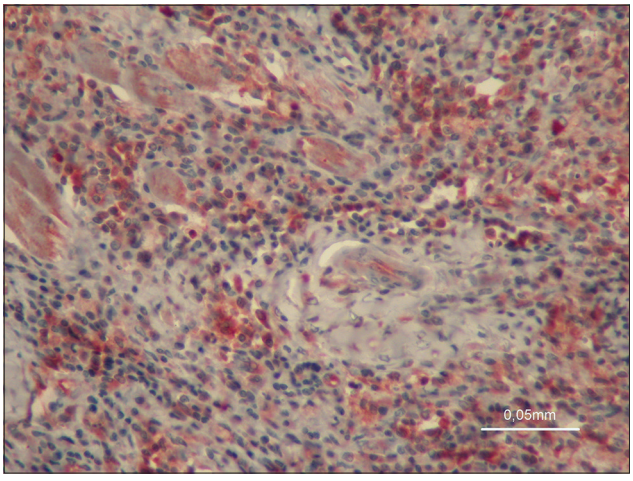

Figure 3: Immunohistochemical staining of inducible nitric oxide synthase + cells in gingival tissue samples from Group IB

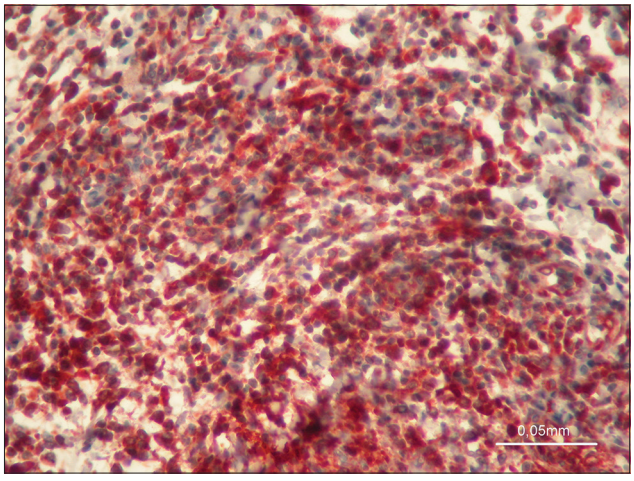

Figure 4: Immunohistochemical staining of of inducible nitric oxide synthase + cells in gingival tissue samples from Group IIA

Table 1: iNOS + cell data (mean±SD, minimum, and maximum) in the study groups

iNOS	Group IA	Group IB	Group IIA	Group IIB	Group IIIA	Group IIIB
Mean±SD	108.8±15.5	72.8±8.6[a]	121±13.6[b]	95.3±5.1[b,c]	101.8±15.4[b]	81±4.2[a,c,d]
Minimum-maximum	96-135	63-87	98-139	91-104	84-129	76-88

For statistical comparisons, two-way ANOVA, one-way ANOVA, and Tukey-HSD test were used. Mean, SD, Minimum, maximum values of iNOS + cells in all groups. [a]Significantly different than the Group IA; [b]Significantly different than the Group IB; [c]Significantly different than the Group IIA; [d]Significantly different than the Group IIIA. ANOVA=Analysis of varience; SD=Standard deviation; HSD=Highly significant difference; iNOS=Inducible nitric oxide synthase

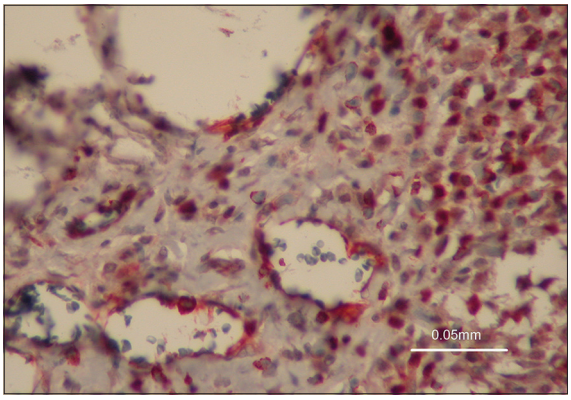

Figure 5: Immunohistochemical staining of of inducible nitric oxide synthase + cells in gingival tissue samples from Group IIB

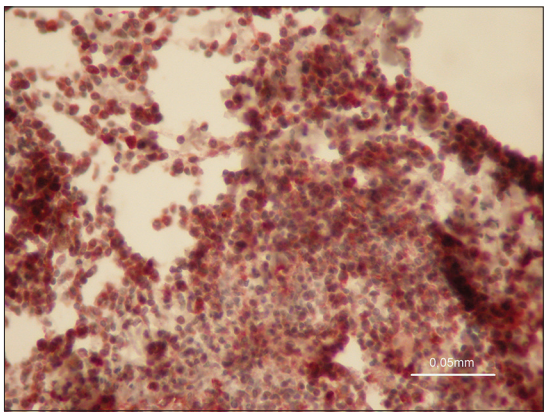

Figure 6: Immunohistochemical staining of of inducible nitric oxide synthase + cells in gingival tissue samples from Group IIIA

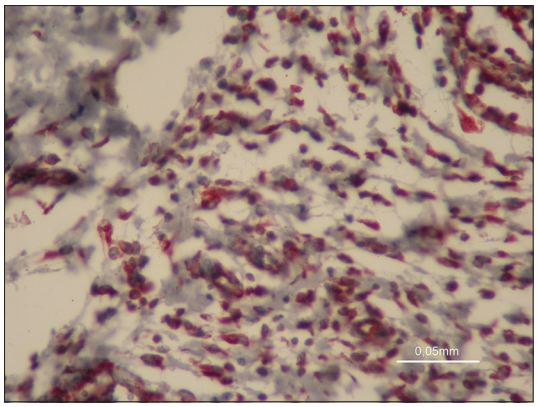

Figure 7: Immunohistochemical staining of of inducible nitric oxide synthase + cells in gingival tissue samples from Group IIIB

receiving α-tocopherol (Group IB, IIB, and IIIB) was found to be different from those receiving saline solution (Groups IA, IIA, and IIIA) ($P < 0.05$) [Figures 2-7].

Discussion

NO is a short-lived free radical affecting molecular, cellular, and physiological processes and is synthesized from L-arginine by an enzyme family called NO synthases[23] iNOS, which is one of three forms of NO synthase and is found in the macrophages.[8,24] It is known that increase in iNOS is a very important marker for inflammatory changes in the body.[25,26] To the best of the authors' knowledge, this is the first study to investigate effects of Vitamin E and α-tocopherol on iNOS mechanism in the treatment of periodontitis and diabetic periodontitis in rats.

Periodontal disease is a chronic inflammatory condition, and it has been found in experimental animal studies that gingival iNOS levels in the ligature-induced periodontitis models are higher than in the healthy controls.[10,27,28] It has been reported in the studies that human gingival expression of iNOS in the presence of gingivitis[9] and chronic periodontitis[11,13] increased parallel to the inflammation. Furthermore, it has been demonstrated that NO and iNOS are expressed in the presence of such pro-inflammatory cytokines from human gingival fibroblasts[29] as TNF-α and interleukin-1β, and that NOS activity in the neutrophils from the individuals with diagnosis of localized aggressive periodontitis is higher compared to healthy individuals.[12] In the present study, we found that gingival iNOS expression was significantly suppressed due to the use of α-tocopherol in all groups. Muià *et al.*(2006) evaluated the effect of "pyrrolidine dithiocarbamate," which was an antioxidant agent in their experimental periodontitis study. Similar to our results, they reported that TNF-α and iNOS levels are increasing in the gingival tissues as a consequence of periodontitis, and pyrrolidine dithiocarbamate is significantly decreasing compared to TNF-α and iNOS levels.[10] Popkov *et al.*(2005) evaluated gingival iNOS activity in experimental diabetes model in rats. They applied "Mexidol" as antioxidant to the rats and showed that iNOS levels decrease in response to the treatment.[30] The present study as well found that gingival iNOS expression decreased in rats in the groups of controlled and uncontrolled diabetes receiving α-tocopherol compared to the group

receiving saline. We showed that alpha-tocopherol given as adjuvant in the treatment of the patients with periodontitis with controlled or uncontrolled diabetes was effective in suppressing iNOS expression. Within the limitations of the current study, it is considered that alpha-tocopherol suppresses gingival inflammation and tissue destruction by decreasing oxidative stress. Furthermore, gingival iNOS expression suppressed due to α-tocopherol in the group of controlled diabetes was significantly lower than the group of uncontrolled diabetes receiving α-tocopherol treatment. Based on the results obtained, controlling diabetes by insulin treatment and giving additional α-tocopherol may be considered as an adjuvant therapy to prevent periodontal tissue destruction.

It was concluded that oxidative stress and tissue destruction due to ROTs such as NO in diabetes and periodontitis might be suppressed by using such anti-oxidants as α-tocopherol might increase effects and activity of insulin.[10,30-33] As far as we know, the current study is the first study to demonstrate that α-tocopherol has suppressive effects on gingival iNOS levels in the conditions of controlled/uncontrolled diabetes and periodontitis and in the case of periodontitis alone. Moreover, in the controlled diabetic group, applying α-tocopherol with insulin created a synergistic effect and suppressed gingival iNOS expression significantly to a higher extent compared to the group receiving saline only. However, it is difficult to translate the results of animal studies to humans. Therefore, studies in human subjects are needed to determine effects of α-tocopherol on the periodontal tissues in the presence of controlled or uncontrolled diabetes and periodontitis, and to evaluate changes in iNOS expression levels with application of α-tocopherol.

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Conflicts of interest
There are no conflicts of interest.

References

1. Flemmig TF. Periodontitis. Ann Periodontol 1999;4:32-8.
2. Diabetes and periodontal diseases. Committee on Research, Science and Therapy. American Academy of Periodontology. J Periodontol 2000;71:664-78.
3. Kim YJ, Jeon JY, Han SJ, Kim HJ, Lee KW, Kim DJ. Effect of socio-economic status on the prevalence of diabetes. Yonsei Med J 2015;56:641-7.
4. Keaney JF Jr, Loscalzo J. Diabetes, oxidative stress, and platelet activation. Circulation 1999;99:189-91.
5. Vincent AM, McLean LL, Backus C, Feldman EL. Short-term hyperglycemia produces oxidative damage and apoptosis in neurons. FASEB J 2005;19:638-40.
6. Moncada S, Palmer RM, Higgs EA. The discovery of nitric oxide as the endogenous nitrovasodilator. Hypertension 1988;12:365-72.
7. Nagano T. Bioimaging probes for reactive oxygen species and reactive nitrogen species. J Clin Biochem Nutr 2009;45:111-24.
8. Chapple IL, Matthews JB. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. Periodontol 2000 2007;43:160-232.
9. Hirose M, Ishihara K, Saito A, Nakagawa T, Yamada S, Okuda K. Expression of cytokines and inducible nitric oxide synthase in inflamed gingival tissue. J Periodontol 2001;72:590-7.
10. Muià C, Mazzon E, Maiere D, Zito D, Di Paola R, Domenico S, *et al.* Pyrrolidine dithiocarbamate reduced experimental periodontitis. Eur J Pharmacol 2006;539:205-10.
11. Pan Z, Guzeldemir E, Toygar HU, Bal N, Bulut S. Nitric oxide synthase in gingival tissues of patients with chronic periodontitis and with and without diabetes. J Periodontol 2010;81:109-20.
12. Shibata K, Warbington ML, Gordon BJ, Kurihara H, Van Dyke TE. Nitric oxide synthase activity in neutrophils from patients with localized aggressive periodontitis. J Periodontol 2001;72:1052-8.
13. Güllü C, Ozmeric N, Tokman B, Elgün S, Balos K. Effectiveness of scaling and root planing versus modified Widman flap on nitric oxide synthase and arginase activity in patients with chronic periodontitis. J Periodontal Res 2005;40:168-75.
14. Schneider C. Chemistry and biology of Vitamin E. Mol Nutr Food Res 2005;49:7-30.
15. Sen CK, Khanna S, Roy S. Tocotrienols: Vitamin E beyond tocopherols. Life Sci 2006;78:2088-98.
16. Kim JE, Shklar G. The effect of Vitamin E on the healing of gingival wounds in rats. J Periodontol 1983;54:305-8.
17. Nishio K, Horie M, Akazawa Y, Shichiri M, Iwahashi H, Hagihara Y, *et al.* Attenuation of lipopolysaccharide (LPS)-induced cytotoxicity by tocopherols and tocotrienols. Redox Biol 2013;1:97-103.
18. Singh N, Chander Narula S, Kumar Sharma R, Tewari S, Kumar Sehgal P. Vitamin E supplementation, superoxide dismutase status, and outcome of scaling and root planing in patients with chronic periodontitis: A randomized clinical trial. J Periodontol 2014;85:242-9.
19. Nizam N, Discioglu F, Saygun I, Bal V, Avcu F, Ozkan CK, *et al.* The effect of a-tocopherol and selenium on human gingival fibroblasts and periodontal ligament fibroblasts *in vitro*. J Periodontol 2014;85:636-44.
20. da Silveira Vargas F, Soares DG, Ribeiro AP, Hebling J, De Souza Costa CA. Protective effect of alpha-tocopherol isomer from Vitamin E against the H2O2 induced toxicity on dental pulp cells. Biomed Res Int 2014;2014:895049.
21. Saglam M, Köseoglu S, Hatipoglu M, Esen HH, Köksal E. Effect of sumac extract on serum oxidative status, RANKL/OPG system and alveolar bone loss in experimental periodontitis in rats. J Appl Oral Sci 2015;23:33-41.
22. Hatipoglu M, Saglam M, Köseoglu S, Köksal E, Keles A, Esen HH. The effectiveness of *Crataegus orientalis* M Bieber.(Hawthorn) Extract administration in preventing alveolar bone loss in rats with experimental periodontitis. PLoS One 2015;10:e0128134.
23. Rizk M, Witte MB, Barbul A. Nitric oxide and wound healing. World J Surg 2004;28:301-6.
24. Grisham MB, Jourd'Heuil D, Wink DA. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites: Implications in inflammation. Am J Physiol 1999;276(2 Pt 1):G315-21.
25. Downey D, Elborn JS. Nitric oxide, iNOS, and inflammation in cystic fibrosis. J Pathol 2000;190:115-6.
26. Dugo L, Marzocco S, Mazzon E, Di Paola R, Genovese T, Caputi AP, *et al.* Effects of GW274150, a novel and selective inhibitor of iNOS activity, in acute lung inflammation. Br J Pharmacol 2004;141:979-87.
27. Lohinai Z, Benedek P, Fehér E, Györfi A, Rosivall L, Fazekas A, *et al.* Protective effects of mercaptoethylguanidine, a selective inhibitor of inducible nitric oxide synthase, in ligature-induced periodontitis in the rat. Br J Pharmacol 1998;123:353-60.
28. Lohinai Z, Stachlewitz R, Virág L, Székely AD, Haskó G, Szabó C. Evidence for reactive nitrogen species formation in the gingivomucosal tissue. J Dent Res 2001;80:470-5.
29. Daghigh F, Borghaei RC, Thornton RD, Bee JH. Human gingival fibroblasts produce nitric oxide in response to proinflammatory cytokines. J Periodontol 2002;73:392-400.
30. Popkov VL, Fil'chukova IA, Lapina NV, Galenko-Yaroshevskii VP, Dukhanin AS. Activity of nitric oxide synthase and concentration of nitric oxide end metabolites in the gingiva under experimental pathological conditions. Bull Exp Biol Med 2005;140:391-3.

31. Paolisso G, D'Amore A, Giugliano D, Ceriello A, Varricchio M, D'Onofrio F. Pharmacologic doses of Vitamin E improve insulin action in healthy subjects and non-insulin-dependent diabetic patients. Am J Clin Nutr 1993;57:650-6.
32. Jain SK, McVie R, Smith T. Vitamin E supplementation restores glutathione and malondialdehyde to normal concentrations in erythrocytes of type 1 diabetic children. Diabetes Care 2000;23:1389-94.
33. Ihara Y, Yamada Y, Toyokuni S, Miyawaki K, Ban N, Adachi T, *et al.* Antioxidant alpha-tocopherol ameliorates glycemic control of GK rats, a model of type 2 diabetes. FEBS Lett 2000;473:24-6.