EFFECT OF GRAPE SEED EXTRACT UPON PLASMA OXIDATIVE STATUS AND ALVEOLAR BONE, IN LIGATURE INDUCED PERIODONTITIS

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ABSTRACT
In this study, we evaluated the effects of two different regimens of dietary supplementation with grape seed extract (GSE) based on serum total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI) and osteocalcin (OC) in experimental periodontitis. The investigation was performed at the Department of Animal Experimentation at Erciyes University, Kayseri, Turkey, from May 2011 to June 2011. Experimental periodontitis was induced by placing 5.0 silk sutures around the maxillary first molars. Twenty-seven adult male wistar rats were divided into four study groups as follows: Healthy control (HC; N = 7); Ligature only (LO; N = 6); Ligature-induced periodontitis plus GSE 50 mg/kg (GSE 50; N = 8); Ligature-induced periodontitis plus GSE 100 mg/kg (GSE 100; N = 6). GSE administration was performed for 14 days following induction of experimental periodontitis. On day 15, serum samples were obtained and rats were sacrificed. Serum samples were analyzed for TAS, TOS, OSI and OC. Defleshed jaws were analyzed morphometrically for alveolar bone loss. The results showed that placing 5.0 silk sutures around maxillary first molars resulted in statistically significant bone loss compared to the HC group (P < 0.05). None of the GSE administrated groups showed evidence that GSE was effective in preventing ligature-induced alveolar bone loss. The GSE 100 and GSE 50 groups had a significantly higher TAS compared to the HC group. No significant differences were seen in TOS, OSI and OC levels. As a whole, GSE administration does not seem to influence TAS, TOS and OC. The lack of a therapeutic benefit of GSE in this study is difficult to explain, and further studies are required to fully assess the potential role of GSE in periodontal treatment.

Introduction
Human periodontal diseases are inflammatory disorders that give rise to tissue damage and tooth loss, resulting from the complex interactions between pathogenic bacteria and the host’s immune response (19). The accumulation and persistence of periodontopathogenic bacteria initiate periodontal tissue destruction either directly by releasing specific bacterial products including lipopolysaccharides (LPS), or indirectly by activating host immune defense systems (20, 44). When stimulated by bacterial pathogens, host cells (e.g. polymorphonuclear leukocytes) release reactive oxygen species (ROS) as part of the immune response (53). Excessive production of ROS in polymorphonuclear leukocytes is one of the pathologic features of the periodontal lesion (21), and it leads to damage of the periodontal tissue by oxidizing DNA, lipids, and proteins (14).

Some studies have reported that the excess production of reactive oxygen species (ROS) leads to damage in gingival tissues, periodontal ligament and alveolar bone (6, 7, 60, 63). The search is, therefore, for an antioxidant that could be used to control these diseases, and polyphenolic compounds are likely candidates (32).

Grape (Vitis vinifera) seeds are an important source of proanthocyanidins, which are oligomers of monomeric flavan-3-ol units. The major flavan-3-ols identified in grape seeds are catechin, epicatechin, and epicatechin-3-O-gallate (51). Grape seed extract (GSE) has been reported to possess anti-inflammatory, anti-arthritic, and anti-tumor properties (55). GSE is also known to be effective in protecting macrophages against oxidative stress induced by the lipopolysaccharide (LPS) of periodontopathogens (32). Grape seed proanthocyanidins may exert their beneficial effects via their anti-oxidative and radical scavenging properties. Oxidative stress arises within tissues when the normal balance between ROS generation and antioxidant defense shifts in favor of the former, a situation arising from either an excess of ROS and/or a depletion of antioxidants (40). There is still debate as to whether antioxidant depletion is a cause of disease or a consequence of the tissue damage that accompanies disease progression. Hypothetically, as ROS do have a role, good tissue antioxidant status may then help to reduce tissue injury or damage (38).

The measurement of different antioxidant and oxidant molecules separately is not practical, and their antioxidant and oxidant effects are additive. Measuring the total antioxidant status (TAS) and the total oxidant status (TOS) of a sample can provide a practical approach (22, 23). The percentage ratio of the TOS to the TAS gives the oxidative stress index (OSI), an indicator of the degree of oxidative stress (24).
Osteocalcin (OC) is a calcium-binding protein of the bone and the most abundant non-collagenous protein in mineralized tissue (37). The serum level of OC is considered a marker of bone formation (17, 27, 56). Increased serum concentrations of OC have been proposed to indicate an increase in bone turnover (37). Periodontitis patients have been reported to have lower serum levels of OC than healthy subjects, suggesting lower osteoblastic activity and bone formation ability (54).

In this study, we investigated the effects of two different doses of GSE in the treatment of experimental periodontitis in a rat model. Our hypothesis was that GSE may provide significant antioxidant effect upon periodontitis patients. The purpose of the present study was to evaluate the possible effects of systemic usage of two different doses of GSE upon serum TAS, TOS, OSI, and OC levels, and the progression of periodontitis in ligature-induced periodontitis.

Materials and Methods

Study design

The experimental protocol of the present study was approved by the Ethics Committee on animal experimentation at Erciyes University. The investigation was performed at the Department of Animal Experimentation at Erciyes University, Kayseri, Turkey, from May 2011 to June 2011. Thirty-two adult male Wistar rats (each weighing from 270 g to 400 g) were purchased from the university’s own facilities, housed in temperature-controlled rooms, and given water and food *ad libitum*.

Experimental periodontitis was induced by placing 5.0 sterile black braided silk thread around the upper first molars under general anesthesia with ketamine (40 mg/kg). Ligatures were placed submarginally and knotted medially. Five rats were lost during the experimental period. The rats were distributed into four experimental groups as follows: 1) healthy controls (HC; N = 7); 2) ligature-induced periodontitis only (LO; N = 6); 3) ligature-induced periodontitis + 50 mg/kg GSE (GSE 50; N = 8) (each rat was orally gavaged daily with 50 mg/kg GSE for 14 days); 4) ligature-induced periodontitis + 100 mg/kg GSE (GSE 100; N = 6) (each rat was orally gavaged daily with 100 mg/kg GSE for 14 days).

On day 15 after baseline, all rats were anesthetized, blood samples were obtained, and the animals were sacrificed by decapitation under ketamine (40 mg/kg) anesthesia. The blood samples were centrifuged for 10 min at 3000 g, separating the serum from the cells. The serum samples were immediately frozen at -80 °C until laboratory analysis. The whole heads were removed and prepared for morphometric evaluations.

Measurement of alveolar bone loss

The molar region of the maxillas were defleshed and stained with aqueous methylene blue (1%) to identify the cement-enamel junction (CEJ). The alveolar bone height was measured under a stereomicroscope (x 40 magnification) by recording the distance from the CEJ to the alveolar bone crest. Measurements were made at 11 points on the buccal and palatal sides to quantify the alveolar bone level (17). The mean alveolar bone level around each tooth was calculated.

Quantification of phenolic compounds by RP-HPLC

Phenolic compounds were evaluated by reversed-phase high-performance liquid chromatography (RP-HPLC), using Shimadzu Scientific Instruments (Tokyo, Japan) equipment. Detection and quantification were carried out with an LC-10A Dvp pump, a Diode Array Detector, a CTO-10Avp column heater, SCL-10Avp system controller, DGU-14A degasser and SIL-10ADvp auto sampler (Shimadzu Scientific Instruments, Columbia, MD). Gallic acid, o-coumaric acid, caffeic acid, chlorogenic acid, protocatechuic acid, p-coumaric acid, ferulic acid, (+)-catechin, (-)-epicatechin, (+)-epicatechin gallate, (-)-epigallocatechin gallate, (-)-epigallocatechin, procyanidin B1, procyanidin B2, procyanidin B3, vanillin, vitexin, rutin, eriodictyol, trans-cinnamic acid, kaempferol and apigenin were used as standards. Identification and quantitative analysis were done by comparison with standards. The amount of proanthocyanidins was expressed as % compound of extract (Table 1).

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>%</th>
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<tbody>
<tr>
<td>Total proanthocyanidins</td>
<td>30.98</td>
</tr>
<tr>
<td>Monomers</td>
<td>29.16</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>15.35</td>
</tr>
<tr>
<td>(-)-Epicatechin</td>
<td>6.35</td>
</tr>
<tr>
<td>(-)-Epicatechin gallate</td>
<td>4.51</td>
</tr>
<tr>
<td>(-)-Epigallocatechin gallate</td>
<td>1.09</td>
</tr>
<tr>
<td>(-)-Epigallocatechin</td>
<td>1.86</td>
</tr>
<tr>
<td>Dimers</td>
<td>1.81</td>
</tr>
<tr>
<td>Procyanidin B1</td>
<td>0.53</td>
</tr>
<tr>
<td>Procyanidin B2</td>
<td>0.39</td>
</tr>
<tr>
<td>Procyanidin B3</td>
<td>0.89</td>
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Total antioxidant status

Serum TAS was determined using a commercially available kit developed by Erel (23). In this assay, the antioxidative effect of the sample against a potent free-radical reaction initiated by the hydroxyl radical produced, is measured. The results are expressed as millimoles of Trolox equivalent per liter (mmol Trolox equiv/L).

Total oxidant status

Serum TOS was determined using a commercially available kit, developed by Erel (23). The assay is calibrated with hydrogen peroxide, and the results are expressed in micromolar hydrogen peroxide equivalent per liter (μmol H₂O₂ equiv/L).

Oxidative stress index

The percentage ratio of the TOS to the TAS gives the OSI, an indicator of the degree of oxidative stress. To perform...
the calculation, the result unit of TAS, millimole of Trolox equivalent per liter, was converted to micromole equivalent per liter, and the OSI value was calculated by the formula OSI = [(TOS, µmol/L)/(TAS, µmol Trolox equivalent/L) × 100].

Osteocalcin
The levels of OC in serum samples were analyzed by ELISA. Specific ELISA kit (AD-12F1, IDS, UK) was used for quantitative analysis of OC.

Statistical analysis
All data analysis were performed using statistical softwares SPSS 15.0 (Statistical Package for the Social Sciences for Windows, Chicago, IL) and SigmaStat 3.5 (Jandel Scientific, San Rafael, CA). Shapiro–Wilk’s test was used for the normality of the test parameters. For parameters that passed the normality test, comparisons between the four study groups were performed using one-way analysis of variance. The Kruskal–Wallis test was used for parameters which failed the normality test. Spearman rank correlations were used to examine the relationships between the serum parameters and the amount of alveolar bone loss. The Tukey test was used for multiple comparisons of mean groups in one-way analysis of variance which show the homogen variance. Dunn’s test was used for multiple comparisons of mean groups in Kruskal–Wallis Analysis. P-values of <0.05 were considered statistically significant.

Results and Discussion
The hypothesis of the study was that the antioxidant feature of GSE may have an effect upon bone metabolism in the experimental periodontitis model. With this, it was aimed to create a new point of view in the treatment of periodontitis, by modulating host defence.

A decrease in cancer incidence and mortality has been reported to be higher in periodontitis patients compared to the healthy group (47). These studies show that blood oxidative stress is increased in periodontitis patients. Oxidative stress arises from the imbalance between the production of ROS and antioxidant protection (8, 14). The evaluation of the relationship between periodontitis and blood oxidative stress should include the evaluation of blood antioxidant levels. Konopka et al. (34) and Baltacıoğlu et al. (4) observe that the blood antioxidant levels are lower in periodontitis patients compared to healthy people. In the light of these data, serum TAS, TOS, and OSI level were used to evaluate the possible changes in blood as a result of periodontitis.

Rats were used to develop the ligature-induced acute periodontitis model. The rats were preferred because feeding is easy, they are convenient for accommodation and to work on, due to their small size, and they tolerate very well the stress burden the study brings about. Periodontitis was induced by placing 5.0 silk sutures around the neck of the maxillar first molars. In this model, the ligature disrupts the tissue integrity by causing trauma in the dentogingival area, and causes intense host-plaque interaction. The ligature increases the plaque retention in the area. Defleshed jaws were analyzed morphometrically for alveolar bone loss. The results showed that placing 5.0 silk sutures around maxillary first molars resulted in statistically significant bone loss (P < 0.05) compared to the HC group (Table 2).

TABLE 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Alveolar Bone Loss * (mm)</th>
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<tbody>
<tr>
<td>Healthy Control</td>
<td>0.37 ± 0.051†</td>
</tr>
<tr>
<td>Ligature Only</td>
<td>0.45 ± 0.054</td>
</tr>
<tr>
<td>GSE 50</td>
<td>0.46 ± 0.039</td>
</tr>
<tr>
<td>GSE 100</td>
<td>0.47 ± 0.027</td>
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* All values are mean ± SD;
† Significantly lower than the other study groups (P < 0.05).

Tomofuji et al. (58) and Sanbe et al. (50) have reported that the antioxidant properties of vitamin C can prevent the progression of periodontitis. In studies investigating the effect of proanthocyanidins in vitro, it was concluded that tea catechins (42), epicatechene gallate and epigallocatechin gallate (18), and cranberry extract (11), can prevent the destruction in the periodontal tissues. Vu Dang La et al. (62) have reported that GSE prevents the secretion of MMP 1, 3, 7, 8, 9, 13 from the macrophages, and consequently prevents the damaging of type 1 collagen and gelatin, caused by MMP 1 and 9. Houde et al. (32) have shown that grape seed proanthocyanidins extract decreases nitrite oxide and ROS production in the macrophages and decreases iNOS expression. All these studies show that GSE has the potential of developing new host modulation strategies in vitro.

Studies on the in vivo effect of proanthocyanidins have shown a moderate reverse correlation between green tea...
consumption and periodontitis (30, 36, 43). No study was found to have investigated the in vivo protective effect of GSE upon periodontal diseases. Hence, we evaluated the effect of serum oxidant–antioxidant state upon the morphometry of alveolar bone and the relationship to serum osteocalcin level, under the influence of GSE in an experimental periodontitis model. Osteocalcin is the most abundant non-collagenous protein in mineralized tissues, and binds the calcium in bones (37). It is secreted by osteoblasts, odontoblasts and chondrocytes (26). Serum osteocalcin levels have been reported to be lower in periodontitis patients compared to healthy people (54). This has been explained by the decrease in osteoblastic activity and bone formation capacity (54). In our study, the mean OC serum levels were 110.43 ng/mL, 115.50 ng/mL, 117.50 ng/mL, 104.33 ng/mL for the HC, LO, GSE 50, GSE 100 groups, respectively. No significant differences were found between any of the study groups, regarding the serum OC levels (P > 0.05) (Fig. 1). While Yoshihara et al. (64) reported a correlation between serum osteocalcin levels and clinic attachment in elderly Japanese population, Özçaka et al. (45), similar to our results, found no statistically significant difference in the plasma osteocalcin levels between individuals with chronic periodontitis and healthy individuals.

**Fig. 1.** Serum osteocalcin levels in the study groups. HC: healthy controls; LO: ligature-induced periodontitis; GSE 50: ligature-induced periodontitis + 50 mg/kg GSE; GSE 100: ligature-induced periodontitis + 100 mg/kg GSE. No significant difference between any of the groups (P > 0.05).

In 11480 individuals who participated in NHANES III (National Health and Nutrition Examination Surveys) and were re-evaluated, a reverse correlation was found between serum vitamin C, and bilirubin levels, and total antioxidant capacity and periodontitis (high serum antioxidant levels were protective against periodontitis). It has also been reported that, in individuals with severe periodontitis, this relationship is even more pronounced (16). Pavlica et al. (48) reported a significant negative correlation between gingiva inflammation in dogs and serum total antioxidant capacity. In our study, the serum TAS was significantly higher in the GSE 100 (40 mmol Trolox equiv/L) and GSE 50 (38.89 mmol Trolox equiv/L) groups than in the HC (28.79 mmol Trolox equiv/L) group (P < 0.05). The serum TAS in the LO (31.45 mmol Trolox equiv/L) group was lower than that in the GSE 100 and GSE 50 groups but these values were not statistically significantly different (P > 0.05) (Fig. 2).

**Fig. 2.** Serum TAS levels in the study groups. HC: healthy controls; LO: ligature-induced periodontitis; GSE 50: ligature-induced periodontitis + 50 mg/kg GSE; GSE 100: ligature-induced periodontitis + 100 mg/kg GSE. * Significantly higher than the HC group (P < 0.05).

The disadvantage of using ligation in producing experimental periodontitis is that the destruction developing in chronic periodontitis over a process of years is provoked in a very short period with an acute process, by the trauma and microbiologic accumulation created (19). The pro-inflammatory/anti-inflammatory cytokine balance, and even the ROS/antioxidant balance in the acute process can be expected to be different from those in chronic periodontitis. The fact that we could not find any significant effect of the high serum antioxidant levels in the GSE groups on bone destruction, as compared to the control group, can be due to the development of destruction acutely and in a very short time with the experimental periodontal model. It is suggested to use agents in experimental models aiming to investigate the host modulation, for long periods, in order to reveal their effects (61). There was no significant difference between the LO (31.45 mmol Trolox equivalent/L) and HC (28.79 mmol Trolox equivalent/L) groups, according to TAS. Our results are in agreement with the results from the studies of Brock et al. (12) and Chapple et al. (15), who found no significant difference in the serum and plasma total antioxidant capacity in periodontitis patients and healthy individuals. The median levels of serum TOS were 5.00 µmol H$_2$O$_2$, 3.40 µmol H$_2$O$_2$, 3.40 µmol H$_2$O$_2$, 4.95 µmol H$_2$O$_2$, respectively, for the HC, LO, GSE 50, GSE 100 groups. No significant differences were found between any of the study groups, regarding the serum TOS (P > 0.05) (Fig. 3). This result could be interpreted in two possible ways. One is the interpretation by Vardar-Şengül et al. (61) that experimental periodontitis only modifies the local parameters instead of the serum. Another one is the suggestion made by Koromantzos et al. (35) that there is no relationship between serum oxidative stress levels and the severity of periodontal disease. The median levels of OSI in our experiments were 0.0179, 0.0118, 0.0105, and 0.0123, respectively, for the HC, LO, GSE 50, GSE 100 groups. No significant differences (P > 0.05) were found between any of the study groups, regarding OSI (Fig. 4). Esen et al. (25)
investigated the effect of chronic periodontitis and rheumatoid arthritis on the serum and gingival crevicular fluid oxidant/antioxidant levels and found no difference between the serum OSI values in the studied groups. In the light of this study, it can be hypothesized that, in the evaluation of OSI, the change in local tissue OSI should be evaluated, too, along with that in the serum.

Fig. 3. Serum TOS levels in the study groups. HC: healthy controls; LO: ligature-induced periodontitis; GSE 50: ligature-induced periodontitis + 50 mg/kg GSE; GSE 100: ligature-induced periodontitis + 100 mg/kg GSE. Minimum, first quartiles, median, third quartiles and maximum values are shown. No significant difference between any of the groups (P > 0.05).

Fig. 4. OSI levels in the study groups. HC: healthy controls; LO: ligature-induced periodontitis; GSE 50: ligature-induced periodontitis + 50 mg/kg GSE; GSE 100: ligature-induced periodontitis + 100 mg/kg GSE. Minimum, first quartiles, median, third quartiles and maximum values are shown. No significant difference between any of the groups (P > 0.05).

Antioxidant support and oxidative stress processes have attracted the attention of investigators. Some of these studies have reported negative results (10). In their study, Chapple et al. (16) reported that local total antioxidant capacity decreases in periodontitis, that after phase 1 treatment disease activity and inflammation decrease, and the total antioxidant capacity also decreases, bringing it to the same level as those in healthy controls. Although oxidative stress is thought to play a role in the pathogenesis of chronic diseases, it has been proposed that this state can be the result of the inflammatory lesion, instead of being the factor leading to this inflammatory process. Therefore, using antioxidant substances may not have an effect on the progression of the disease (24).

Chapple et al. (13) investigated, in a double-blind random controlled study, the effect of fruit/vegetable and fruit/vegetable/dried fruits extracts on chronic periodontitis patients receiving phase 1 treatment and compared it to placebo. In the group using fruit/vegetable extract, two months following phase 1 treatment, there was a significant healing in the pocket depth as compared to the placebo group. This difference was not found 5 and 8 months following phase 1 treatment. There was no difference in the clinic attachment levels between the groups at any of the studied periods.

Conclusions

The results from the present study showed that grape seed extract applied to rats for 14 days, in doses of 50 mg/kg and 100 mg/kg by gavage, did enhance an increase in the total antioxidant status but no effect on the destruction in alveolar bones, serum osteocalcin, total oxidant status and oxidative stress index was observed. The antioxidant effect of GSE upon alveolar bones should be evaluated in long-term studies.

Acknowledgements

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Conflicts of Interest

All authors report no conflicts of interest related to this study.

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