Original

Antioxidant effects of melatonin in heart tissue after induction of experimental periodontitis in rats

Muhsin Özdem¹⁾, Fatma Y. Kırzioğlu²⁾, Hacı R. Yılmaz³⁾, Hüseyin Vural⁴⁾,
Özlem Fentoğlu²⁾, Efkan Uz⁴⁾, Ahmet Koçak⁵⁾, and Ayşe Yiğit⁶⁾

¹⁾Department of Periodontology, Faculty of Dentistry, Abant İzzet Baysal University, Bolu, Turkey

²⁾Department of Periodontology, Faculty of Dentistry, Süleyman Demirel University, Isparta, Turkey

³⁾Department of Medical Biology, Faculty of Medicine, Mevlana University, Konya, Turkey

⁴⁾Department of Medical Biochemistry, Faculty of Medicine, Süleyman Demirel University, Isparta, Turkey

⁵⁾Department of Histology and Embryology, Faculty of Medicine, Dumlupınar University, Kutahya, Turkey

⁶⁾Department of Medical Biology, Faculty of Health Sciences, Süleyman Demirel University, Isparta, Turkey

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Abstract: The aim of this study was to evaluate the effects of melatonin on the oxidative stress in heart tissues after induction of experimental periodontitis in rats. Thirty Wistar Albino male rats were divided into four groups as follows: healthy + saline solution (Hs, $n = 7$), healthy + melatonin (Hm, $n = 7$), periodontitis + saline solution (Ps, $n = 8$), and periodontitis + melatonin (Pm, $n = 8$). Experimental periodontitis was induced using a ligature placed at the gingival margin of the maxillary second molars. Melatonin was applied intraperitoneally (10 mg/kg) every day for 2 weeks. After sacrificing the rats, serum levels of malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) levels, and melatonin levels were evaluated. The Pm group exhibited lower alveolar bone loss than the Ps group. Melatonin levels increased in the periodontitis groups, and the Pm group had lower MDA levels and higher GSH-Px levels than the Ps group. These findings suggest that melatonin administration reduces MDA and increases GSH-Px levels in heart tissue, and these effects may be due to its antioxidant properties.

Further studies are needed to understand the effects of melatonin on the association between periodontitis and cardiovascular disease.

Keywords: melatonin; oxidative stress; antioxidants; experimental periodontitis.

Introduction

Periodontitis is a chronic inflammatory disease caused by dental plaque microorganisms, and is characterized by the destruction of supporting tissues (bone and connective tissue) and loss of teeth (1,2). The progression of periodontal disease depends on the host's response to the bacteria and bacterial products, and this often leads to systemic inflammation (3). The effect of periodontitis on systemic disease is still relatively unclear, and low-level bacteraemia caused by it may affect vessel wall structure, platelet function, and coagulation (4). It is well known that the periodontal health status affects the onset or progression of heart disease (Cardiovascular disease—CVD) through certain inflammatory mechanisms (5). Empirical evidence also indicates the presence of an association between periodontitis and CVD (6).

Recently, the importance of oxidative stress in the relationship between periodontitis and CVD has been highlighted (7), and studies have reported an increased risk of developing CVD among patients with chronic periodontitis (8). Although the mechanisms responsible for this relationship are still unclear, it is believed that

Correspondence to Dr. Muhsin Özdem, Department of Periodontology, Faculty of Dentistry, Abant İzzet Baysal University, Bolu, Turkey
Fax: +90-374-2700066

E-mail: ozdemmuhsin@yahoo.com, muhsinozdem@ibu.edu.tr

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the microorganisms reach the vessel walls through the systemic circulation and increase cytokine levels (9,10).

Periodontitis initiates a temporary and systemic vascular inflammation, and causes deterioration of blood vessels characterized by increased endothelial vasoconstriction. This often acts as a prelude to CVD. All of this is associated with increased systemic inflammation, changes in lipid metabolism, and occurrence of oxidative stress (11).

The imbalance between pro-oxidant and antioxidant systems often lead to further oxidative damage and destruction of periodontal tissue, potentially leading to CVD (12). Reactive oxygen species (ROS) resulting from polymorphonuclear leukocyte infiltration can lead to structural and metabolic changes in the biomolecules of cells, along with the production of malondialdehyde (MDA) as a final product of lipid peroxidation (LPO). MDA can inhibit protein synthesis and inactivate the antioxidant enzymes (13,14).

Melatonin has various biological effects, including antioxidant and immunity-enhancing ones (15-18). It exhibits high lipophilicity, and easily reaches the cell membrane and mitochondria. It can also be found in high concentrations in other cellular compartments. Physiological and pharmacological doses of melatonin increase enzyme activity and the gene expression of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (14). It also has a direct neutralizing effect on ROS (19) and MDA (14,20). Melatonin may also contribute to the antioxidative defence by directly affecting free radicals or indirectly preventing production of ROS (14,21). It also exhibits indirect effects by increasing the effectiveness on SOD and GSH-Px, possibly via epigenetic mechanisms (13,22). The direct antioxidant abilities of melatonin have been demonstrated in vitro and in vivo, and include inactivation of free radicals and/or induction of DNA repair enzymes (22,23).

Several studies have provided evidence of the positive contributions of melatonin and its physiological and pathological implications in the oral cavity (24-27). Gölle et al. (27) reported that melatonin ameliorated periodontal inflammation-induced organ injuries in rats. However, there is no information in the literature on the effects of melatonin on the oxidative status in heart injuries caused by periodontitis. Thus, in this study, we aimed to evaluate melatonin, MDA, SOD, and GSH-Px levels after induction of experimental periodontitis in the heart tissues of rats.

Materials and Methods

Experimental animal

Thirty Wistar Albino male rats (180-220 g) were obtained from the Süleyman Demirel University Experimental Animals Research Center and divided into four groups, as follows: healthy + saline solution (Hs, $n = 7$), healthy + melatonin (Hm, $n = 7$), periodontitis + saline solution (Ps, $n = 8$), and periodontitis + melatonin (Pm, $n = 8$). This study was approved by the Süleyman Demirel University Ethics Committee, Animal Experiments Council (Date: 2009, number: 12/03).

Experimental design

Experimental periodontitis was induced under anesthesia by intraperitoneal injection of ketamine hydrochloride (25 mg/kg, Eczacıbaşı, Istanbul, Turkey) and xylazine (Xylazinbio 2%, Bioveta, Czech Republic). Ligatures (Silk suture 3/0, Doğan, Istanbul, Turkey) were placed at the gingival margin of the second molars (28-31), and melatonin was administered intraperitoneally (i.p.) at a dose of 10 mg/kg (dissolved in ethanol/saline solution 1%, MerckSchuchardt, Hohenbrunn, Germany) per day for 2 weeks (32). During this period, two rats in the Hs and Ps groups died.

After the second week, the remaining rats were anaesthetized and sacrificed, and blood samples were then collected from each animal. The serum samples were separated by centrifugation for 10 min at 1,500 rpm, and then stored at -20°C until required for analysis. In addition to the heart tissues, maxillary samples were taken by decapitating the rats and separating the maxilla into halves along the sutura palatina after dissecting the muscles and soft tissues. The maxilla samples were then fixed with 10% formalin (33).

Evaluation of alveolar bone

The maxillary halves were immersed in 3% H_2O_2 for 1 day. After staining the samples with 1% methylene blue (1 g/100 mL, diluted water) for 1 min, the limit of the cemento-enamel junction was determined (Figs. 1, 2).

The distance between the enamel-cementum border (ECB) and alveolar crest peak (ACP) was measured using a stereomicroscope (SZ-PT STU1, Olympus C3040-ADL, Japan; magnification 40%). The measurement process was performed at three points on the buccal surface of each tooth (34), and the mean values were then calculated. A digital camera was used to determine the alveolar bone destruction area (ABDA), and the obtained images were evaluated using the "Image J (National Institutes of Health in the USA)" program on a computer.

Fig. 1 Staining of (1% methylene blue) samples in the periodontitis groups.

Fig. 2 Staining of (1% methylene blue) samples in the healthy groups.

Homogenization process of heart tissues

The heart tissues were washed immediately upon removal with cold isotonic water, wrapped in aluminum foil, and stored at -85°C in the freezer until required for biochemical tests. The tissues were homogenized at 16,000 rpm, and MDA levels in the obtained homogenates were measured.

The homogenates were placed in a refrigerated centrifuge (at 3,220 rpm for 30 min, $+6^{\circ}\text{C}$), and the GSH-Px and protein levels in the separated supernatant were recorded. The supernatant 1/1 (v/v) mixture of chloroform/ethanol (3/5, v/v) (35) was placed in glass tubes, vortexed, and then centrifuged at 3,220 rpm for 40 min at $+4^{\circ}\text{C}$. Protein and SOD enzyme activity was determined using the upper part of the ethanol phase.

Biochemical analysis

Antioxidant enzymes and MDA levels were studied using the spectrophotometric method.

MDA analysis

The amount of MDA was measured by the Draper and Hadley method (36). Briefly, the MDA and thiobarbituric acid (TBA) reaction caused coloration, and this was evaluated using spectrophotometric measurements at 532 nm against a blank reagent.

SOD analysis

The total SOD was studied using the Sun method (Nitro blue tetrazolium reduction [NBT] principle) (35), where colored formazon is formed by the reaction between O_2^- and NBT in the medium. This complex provides the maximum absorbance value at 560 nm.

GSH-Px analysis

GSH-Px was studied according to Paglia (37), and the absorbance value of GSH-Px was determined at 340 nm.

Measurement of melatonin

The amount of melatonin in the serum was evaluated with ELISA (ELISA Kit for Rat melatonin [MT], USCN Life Science, Wuhan, PR China).

Statistical analysis

All statistical analyses were performed using the SPSS software (version 15.0, SPSS, Chicago, IL, USA). Nonparametric test groups were evaluated using One-sample Kolmogorov-Smirnov Test, while one-way ANOVA test was used to compare the groups. LSD (post hoc multiple comparison test) test was also employed, and $P \leq 0.05$ was considered statistically significant.

Results

Histomorphometric results

The ECB-ACP and ABDA measurements for the Ps and Pm groups have been shown in Table 1. Although the values were smaller for the Pm group, they were not statistically significant ($P > 0.05$).

Serum melatonin levels

The highest mean value of serum melatonin was found in the Pm group, whereas the Hs group presented the lowest values (Table 2). Increased serum melatonin levels were observed in the periodontitis groups, and the differences between the Hs and Hm group, Hs and Pm group, and the Ps and Pm groups were significant ($P < 0.05$).

Table 1 Histomorphometric measurements of the periodontitis groups

	Groups	<i>n</i>	mean $\pm$ SD (min-max)	<i>P</i> value
ECB-ACP (mm)	Hs	6	1.436 $\pm$ 0.353 (1.077-2.137)	0.865
	Hm	7	1.410 $\pm$ 0.156 (1.130-1.609)	
ABDA (mm ²)	Ps	7	9.340 $\pm$ 3.816 (4.276-17.059)	0.538
	Pm	8	8.365 $\pm$ 1.486 (6.976-11.255)	

Hs: Healthy + saline solution administration, Hm: Healthy + melatonin administration, Ps: Periodontitis + saline solution administration, Pm: Periodontitis + melatonin administration, ECB: Enamel-cementum border, ACP: Alveolar crest peak, ABDA: Alveolar bone destruction area, SD: standard deviation, min-max: minimum-maximum. Statistically significant difference between the groups ($P < 0.05$).

Table 2 The levels of melatonin and oxidative stress parameters in the heart tissue (mean $\pm$ standard deviation)

Groups	Melatonin (pg/mL)	SOD (U/mg protein)	GSH-Px (U/g protein)	MDA (nmol/g protein)
Hs (<i>n</i> = 6)	8.86 $\pm$ 1.24* [¶]	0.56 $\pm$ 0.16	31.7 $\pm$ 2.9 [¶] _γ	18.7 $\pm$ 3.1 [¶] _γ
Hm (<i>n</i> = 7)	10.01 $\pm$ 1.09 [¶]	0.57 $\pm$ 0.71	33.0 $\pm$ 3.6* [¶] _§	16.9 $\pm$ 4.2* [¶] _§
Ps (<i>n</i> = 7)	9.40 $\pm$ 0.56 [†]	0.54 $\pm$ 0.28	31.4 $\pm$ 0.1 [†] _§	20.2 $\pm$ 5.5 [†] _§
Pm (<i>n</i> = 8)	10.16 $\pm$ 0.84* [†]	0.54 $\pm$ 0.01	49.5 $\pm$ 7.1* [†] _γ	12.9 $\pm$ 3.7* [†] _γ

Hs: Healthy + saline solution administration, Hm: Healthy + melatonin administration, Ps: Periodontitis + saline solution administration, Pm: Periodontitis + melatonin administration, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, MDA: Malondialdehyde. *, [¶], [†], [‡], [§], _γ Statistically significant difference between the groups ($P < 0.05$).

Heart MDA, SOD, and GSH-Px levels

The MDA, SOD, and GSH-Px levels have been shown in Table 2. The SOD levels were similar between the groups ($P > 0.05$), whereas the MDA in the Ps group was higher than that in the Hs group. Additionally, the GSH-Px levels were higher and the MDA levels were lower in the Hm rats compared to the Hs rats. The Pm group had lower MDA levels and higher GSH-Px levels compared to the Ps group ($P < 0.05$).

Discussion

LPO starts with oxidation of polyunsaturated fatty acid by radicals, continues in the form of a chain reaction, and results in the production of demolition products such as MDA, alkenes, alcohols, epoxy fatty acids, and hydroxy fatty acids (38,39). Overall, LPO products lead to increased immune response, fibrosis or inflammation, inactivation of thiol-containing enzymes, gene transcription, or triggered apoptosis due to higher molecular activity, and are associated with CVD (11). The role of LPO in CVD and periodontitis has been confirmed by studies examining the association between periodontitis and impaired lipid metabolism (5,40,41). Increased LPO in serum and periodontal tissue was observed in case of experimental periodontitis (42). Additionally, Tomofuji

et al. (31) reported increased oxidative damage associated with LPO in the internal organs of rats after periodontal inflammation.

Recently, host modulation therapies in inflammatory diseases have become increasingly popular. In many extant studies, the effects of antioxidants such as coenzyme Q₁₀, propolis and N-acetylcysteine, vitamin C, cocoa, proanthocyanidine, and statins have been evaluated in experimental periodontitis (30,43-46). However, no work has been done thus far on the antioxidant activity of melatonin upon heart tissue in the presence of periodontitis.

Various experimental periodontitis models have been proposed in the extant literature (27-29,47). Ligatures placed in the dentogingival area increase plaque formation and the amount of bacteria, and induce effective plaque-host interactions (43,48). In the present study, experimental periodontitis was induced in rats using 3/0 silk sutures for 2 weeks. Melatonin 10 mg/kg per day was given (32).

Our results showed that the periodontitis group (Ps) had higher MDA levels than the periodontally healthy controls, and this was in line with Kara et al. (20) who reported increased MDA levels in the gingival tissue and serum using an experimental periodontitis model. Some

studies also indicate the relationship between severity of periodontal disease and MDA levels (14,49-51), whereas others suggest that periodontal disease has a significant effect on ROS activity in systemic inflammatory conditions (39).

Studies on the association between periodontitis severity and antioxidant enzyme levels have yielded inconsistent results (51-53). While Panjamurthy et al. (52) reported significantly higher SOD levels in periodontitis compared to controls, Akalin et al. (54,55) reported lower SOD in periodontitis patients. In our study, the decreases in SOD levels in rats affected by periodontitis were not statistically significant. Currently available results regarding GSH-Px in periodontitis are also contradictory (52,56,57). For example, Wei et al. (51) and Panjamurthy et al. (52) observed higher levels of GSH-Px in patients with periodontitis compared to healthy individuals. In contrast, Sobaniec et al. (56) suggested that periodontitis patients have lower levels of GSH-Px than controls. In our study, GSH-Px levels were similar between the Hs and Ps groups.

Several authors have reported the presence of different melatonin levels in the serum, GCF, plasma, or saliva of patients with periodontitis (2,24,25). Cutando's study (23) showed that plasma melatonin increased with the value of the Community Periodontal Index. In the present study, melatonin levels increased in periodontitis. Additionally, the periodontitis group that received melatonin had lower alveolar bone loss than those affected by periodontitis and treated with saline. However, the results were not significant. It is thought that melatonin reduces the alveolar bone loss by affecting the inflammatory host response (20).

According to our results, melatonin application leads to a decrease in heart MDA levels. Additionally, GSH-Px levels increased in the groups that received melatonin. Our results are supported by other studies that have reported the neutralizing effects of melatonin on ROS, hydroxyl, singlet oxygen, and lipid peroxyl radicals (58,59). Melatonin in the presence of a focal inflammation such as periodontitis might exhibit more binding to receptors in heart tissues and more powerful antioxidant properties. Melatonin is highly lipophilic in nature and can easily reach the cell membrane. Thus, it may protect the cell membrane against LPO. Our results suggest that melatonin may contribute to the antioxidative defence by directly affecting the free radicals, and by indirectly preventing the production of ROS by increasing the amount of antioxidants (59,60).

Finally, the findings of our study also suggest that melatonin administration reduced MDA and increased

GSH-Px levels in heart tissue. These effects may be due to its antioxidant properties, or may be expressed indirectly via enhanced synthesis of the antioxidant enzymes. Further experimental and clinical studies are necessary to explain the role of melatonin in the relationship between periodontitis and CVD, and in the immunomodulatory therapies of both diseases.

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Conflict of interest

The authors declare no conflict of interest.

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