Local Application of Melatonin Into Alveolar Sockets of Beagle Dogs Reduces Tooth Removal–Induced Oxidative Stress

Antonio Cutando,* Carlos Arana,† Gerardo Gómez- Moreno,* Germaine Escames,† Ana López,† María J. Ferrera,* Russel J. Reiter,‡ and Darío Acuña-Castroviejo†§

Background: The antioxidant and anti-inflammatory hormone melatonin is secreted by saliva into the oral cavity, where it may protect the mucosal and gingival tissues from radical damage. To date, no studies have addressed the potential beneficial role of melatonin in the acute inflammatory response that follows oral surgical interventions, especially tooth extractions. The aim of this study was to determine whether tooth extraction induces changes in plasma oxidative stress levels, and whether melatonin treatment may counteract these changes.

Methods: Maxillary and mandibular premolars and molars of 16 adult Beagle dogs were extracted under general anesthesia. Eight dogs were treated with 2 mg melatonin placed into the alveolar sockets, whereas the other eight dogs received only vehicle. Lipid peroxidation (LPO) and nitrite plus nitrate (NOx) levels were determined in plasma, whereas glutathione (GSH) and glutathione disulfide (GSSG) levels and glutathione peroxidase (GPx) and reductase (GRd) activities were measured in red blood cells before and 24 hours after tooth extraction.

Results: Removal of the premolars and molars caused a significant rise in plasma LPO and NOx levels and in the erythrocyte GSSG/GSH ratio, whereas melatonin treatment restored the normal values of these parameters. Also, melatonin slightly increased erythrocyte GRd activity without changing GPx activity.

Conclusion: For the first time to our knowledge, the results show that during the immediate postoperative period following tooth extraction, there is a significant increase of oxidative stress, which is counteracted by the administration of melatonin into the alveolar sockets. J Periodontal 2007;78:576-583.

KEY WORDS
Antioxidant; free radicals; mouth; oral surgery; oxidative stress.

R eactive oxygen species (ROS), including superoxide anion radical, hydrogen peroxide, and the hydroxyl radical, and reactive nitrogen species (RNS), including nitric oxide (NO) and the peroxynitrite anion, are common byproducts produced by normal aerobic metabolism of oral cavity cells or by inhalation of oxidizing agents in tobacco smoke and other air pollutants.1-4 Moreover, activation of the immune system by inflammatory processes, such as chronic periodontitis, increases ROS-RNS generation.3 Although ROS are necessary for defense of the host, they also expose oral tissues to oxidative damage.2 The mucosal barrier is the first line of defense against flora growing in the oral cavity. In mucosal cells, the production of NO by the expression of inducible NO synthase (iNOS) serves as a chemical barrier to limit bacterial plaque invasion. However, iNOS expression by oral epithelial cells is associated with diminished cell viability, which may depend on the peroxynitrite formation.4,5 These reactive species are involved in the pathogenesis of several oral processes, including recurrent aphthous ulceration,6 leukoplakia,7 lichen planus,8 and especially in oral cavity cancer and periodontal inflammatory disease.9-12
Periodontal disease in its destructive phase is considered to be initiated and perpetuated by Gram-negative bacteria that colonize the subgingival area. When stimulated by periodontal pathogens, host cells release proinflammatory cytokines, whereas massive polymorphonuclear cell migration to the gingival crevicular fluid leads to abnormal spreading of ROS. Additionally, macrophage infiltration to the periodontal tissues increases iNOS and NO, with the latter being related to the pathogenesis of periodontitis and subsequent bone loss. (Usually, an increase in free radical production coexists with a decrease in the antioxidant defense system. The imbalance between the prooxidant and antioxidant systems may lead to further oxidative damage of periodontal tissues.}

Previous studies reported that oral inflammatory processes, such as periodontitis, can trigger signals that increase not only plasma melatonin (aMT) levels but also aMT levels in the oral cavity, where the indolamine may exert an antioxidant role. The direct action of aMT as a free radical scavenger of both ROS and RNS is complemented with an indirect stimulatory effect of the antioxidant enzymes, including glutathione peroxidase (GPx) and reductase (GRd), superoxide dismutase, and catalase. Due to its stimulatory effect on GRd, aMT favors the recycling of glutathione (GSH) from glutathione disulfide (GSSG), maintaining a high GSH/GSSG ratio. aMT also promotes the de novo synthesis of GSH by promoting the activity of glutamyl-cysteine synthetase. Additionally, aMT is also capable of reducing NO and peroxynitrite generation because of its ability to inhibit iNOS activity and expression, which increase the tissue damage that accompanies inflammation. aMT also may exert an immunoenhancing role in the oral cavity because patients with severe periodontal status with bone damage and gingival involvement show concomitant high interleukin-2 and aMT levels, which may stimulate CD4 lymphocytes in response to periodontal disease.

It is now well established that the acute inflammatory response of gingival tissue during the first 24 to 48 hours postextraction causes an important polymorphonuclear leukocyte infiltration, which is responsible, in part, for the increase in ROS and RNS generation. Thus, the aim of this study was to test whether plasma changes reflect the acute inflammatory response caused by tooth extractions in Beagle dogs, and whether aMT treatment could modify any observed changes.

**MATERIALS AND METHODS**

**Chemicals**

GSH, GSSG, GRd, nicotinamide adenine dinucleotide phosphate (NADPH), cumene hydroperoxide, ophthalaldehyde, N-ethylemaleimide, methanol, N-(1-naphthyl) ethylenediamine dihydrochloride, sulphanilamide, trichloroacetic acid, and phosphoric acid were purchased. All other reagents were of the highest purity available.

**Animals, Surgery, and Treatment**

The study was performed in 16 male Beagle dogs obtained from the Veterinary Faculty, University of Córdoba, Córdoba, Spain. The animals were maintained in the University’s facility in individual kennels in a 12:12 light-dark cycle (lights on at 7:00 am) at 22°C ± 2°C with regular chow and tap water. Animals were 14 months of age at the time of the study and weighed 16 to 18 kg. All experiments were approved and performed according to the Spanish Government Guide and the European Community Guide for animal care.

Both upper and lower maxillary and mandibular premolars and molars of the 16 Beagle dogs were extracted under general anesthesia. The anterior group of teeth was conserved so that the dogs could maintain an appropriate masticatory function. All interventions were supervised by the veterinarian of the Animal Experimentation Service of the University of Granada. Fifteen minutes before general anesthesia, the animals received an intramuscular injection of 0.5 to 1 mg/kg acepromazine maleate, an anxiolytic. General anesthesia included ketamine plus chlorbutol, 5 to 8 mg/kg intravenously; 0.5 to 1 mg/kg acepromazine maleate as coadjuvant; and 0.05 mg/kg atropine. Dexamethasone isonicotinate (2 ml intramuscularly) and amoxicillin (2 ml intramuscularly) were administered at the end of surgery and every 2 days for a total of 4 days.

After the tooth extractions and before suturing, eight dogs received aMT applied into the extraction wounds and gingival tissue surrounding the premolar and molar area. The following groups of dogs were included: 1) control group (Con), consisting of all 16 dogs sampled 1 hour before tooth extractions; 2) vehicle-treated group (Veh), consisting of eight dogs with tooth extractions but without postextraction treatment; and 3) aMT-treated group (aMT), consisting of eight dogs receiving 2 mg powder aMT into the alveolar sockets and surrounding gingival tissue after surgical removal of the tooth.

Blood samples were taken from the vena cephalica antebrachii 1 hour before tooth extraction (control samples) and 24 hours after the surgical procedure. Blood was rapidly transferred to cold EDTA-K-containing tubes and centrifuged at 3,000 × g for 10 minutes at 4°C. Plasma aliquots were stored at −80°C for lipid peroxidation (LPO) and nitrite plus nitrate (NOx) determination. GSH and GSSG levels and GPx and GRd activities were determined in red blood cells.
Melatonin and Oral Oxidative Stress

The cells were separated from the plasma and washed two times with 0.9% sodium chloride solution. Red blood cell aliquots were stored at −80°C until assays were performed.

**LPO Determination**

Malonaldehyde and 4-hydroxyalkenals concentrations provide a convenient index of lipid peroxidation. These lipid peroxidation products were determined with a special kit. The kit takes advantage of a chromogenic reagent that reacts with malonaldehyde and 4-hydroxyalkenal (4HDA) at 45°C yielding a stable chromophore with maximal absorbance at the 586-nm wavelength. Plasma LPO levels were expressed in nanomoles per milliliter.

**NOx Determination**

Levels of NOx were measured in plasma previously treated with nitrate reductase. Then, pretreated plasma aliquots were incubated with 100 μl of Griess reagent (0.1% N-[1-naphthyl] ethylenediamine dihydrochloride; 1% sulfanilamide in 5% phosphoric acid; 1:1) at room temperature for 20 minutes. The absorbance at 550 nm was measured with a spectrophotometer. NOx concentrations were calculated by comparison to the absorbance of a standard solution of known sodium nitrite concentration and expressed in nanomoles per milliliter.

**Measurement of GSH and GSSG**

Both GSH and GSSG were measured by a fluorometric method, which was slightly modified. Aliquots of saline-washed red blood cells were thawed and hemolized (1:20) with 10 mM phosphate buffer, 1 mM EDTA-Na₂, pH 6.5, at 4°C yielding a stable chromophore with maximal absorbance at the 586-nm wavelength. Plasma LPO levels were expressed in micromoles per gram of Hb.

**RESULTS**

Plasma levels of LPO and NOx are shown in Figure 1. LPO levels in plasma increased highly significantly in dogs without postextraction treatment with aMT compared to the plasma samples collected before surgery (20.04 ± 0.89 versus 6.89 ± 0.73 nmol/ml, respectively; \( P < 0.0001 \)). Treatment with aMT reduced plasma LPO levels to control values (\( P < 0.0001 \)).

Plasma nitrite levels were also significantly increased in the vehicle-treated dogs compared to the preextraction levels (72.88 ± 5.39 versus 36.05 ± 3.465 nmol/ml, respectively; \( P < 0.0001 \)). The dogs that received aMT had significantly lower levels of nitrite compared to control preextraction rates (17.66 ± 2.31 versus 36.05 ± 3.465 nmol/ml, respectively; \( P < 0.0001 \)), and very significantly reduced values.

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1 Bioxytech LPO-568, Cayman Chemical, Ann Arbor, MI.
3 Bio-Tek Instruments.
4 Shimadzu Deutschland, Duisburg, Germany.
5 Shimadzu Deutschland.
relative to those in dogs that received vehicle as the postextraction treatment ($P<0.0001$).

Figure 2 shows the activities of GPx and GRd in the preextraction and postextraction plasma samples. The vehicle-treated dogs after tooth extractions exhibited a significant increase in GPx compared to plasma samples collected before teeth extraction (85.82 ± 3.56 versus 74.38 ± 3.465 μmol/minute/g Hb, respectively; $P<0.05$); however, no significant change in GRd activity was measured (1.73 ± 0.19 versus 1.328 ± 0.13 μmol/minute/g Hb). Treatment with aMT significantly increased the activity of GRd, but not that of GPx, in dogs compared to the preextraction levels (1.98 ± 0.13 versus 1.328 ± 0.13 μmol/minute/g Hb, respectively; $P<0.005$).

Figure 3 summarizes the levels of total glutathione, GSH, GSSG, and GSSG/GSH ratio in red blood cells. Although there were no significant differences in the levels of total erythrocyte glutathione between animals of the three groups, tooth extractions without aMT treatment caused a significant rise in GSSG levels (2.36 ± 0.13 versus 1.69 ± 0.129 μmol/g Hb; $P<0.005$). The GSSG/GSH ratio also increased very significantly in erythrocytes of vehicle-treated dogs compared to the preextraction values (0.70 ± 0.03 versus 0.430 ± 0.04; $P<0.0001$). Treatment with aMT increased the levels of GSH significantly compared to values before teeth removal (3.93 ± 0.16 versus 4.47 ± 0.16 μmol/g Hb; $P<0.05$) and compared to the vehicle-treated dogs and to the control group without treatment (3.43 ± 0.22 versus 4.47 ± 0.16 μmol/g Hb; $P<0.005$). aMT also significantly reduced GSSG levels compared to those in vehicle-treated dogs ($P<0.05$); thus, the GSSG/GSH ratio was lower in aMT-treated animals (0.70 ± 0.03 versus 0.451 ± 0.02; $P<0.0001$).

**DISCUSSION**

Free radicals have been implicated in many pathophysiologic processes of the oral cavity. The...
relationships among aMT, the immune system, and oral status are not well defined. Reduced oral health (with advanced periodontal processes with gingival tissue damage and bone loss) serves as a trigger for increases in salivary aMT levels, which in turn stimulates the CD4 lymphocytes. However, there are no published reports related to aMT oxidative stress interactions during surgical oral interventions, including after tooth extractions. For the first time to our knowledge, the current results show the existence of significant oxidative stress during the immediate postoperative period following tooth extraction, with the changes being counteracted by local aMT application into the alveolar sockets after tooth removal.

One day after oral surgery, the dogs that did not receive aMT exhibited a significant increase in parameters of plasma oxidative stress as a consequence of the damage and the inflammatory process that follows surgical intervention. After tooth removal, bacteria of the oral cavity colonize the surface of the blood clot that covers the alveolar socket, granulation tissue, wound epithelium, and the adjacent gingival tissue. These events cause an acute inflammatory response of the gingival mucosa, which surrounds the blood clot. Thus, during the first 24 to 48 hours postextraction, edema and vasodilatation are observed in the periphery of the alveolar socket with a marked infiltration of polymorphonuclear leukocytes. Gingival tissue infiltration by polymorphonuclear leukocytes and monocytes, whose principal function after tooth extraction is phagocytosis of bacteria, is also responsible for the generation of ROS. Besides the inflammatory process, other mechanisms, including breakdown of the gingival fibers, damage to periodontal vessels, and the mechanical mutilation to oral tissues as a consequence of tooth extraction, also participate in the oral damage after tooth removal. Together, these events participate in promoting oxidative stress generated by the inflammatory process. In turn, the increased ROS stimulate the production of proinflammatory cytokines, transcription factors, such as nuclear factor-kappa B (NF-κB), and vascular cell adhesion molecules, thereby increasing the progression of the inflammatory process and the synthesis of RNS such as NO and peroxynitrite.

Both ROS and RNS locally generated in the oral cavity after tooth removal can enter the circulation. In fact, plasma levels of LPO and NOx, which reflect the increased production of ROS and RNS, respectively, were significantly higher 24 hours after tooth extraction. Overproduction of lipid hydroperoxides and aldehyde products causes depletion of GSH, disrupting mucosal turnover. Our results document these changes because GSSG levels and the GSSG/GSH ratio were significantly elevated in the vehicle-treated dogs. Thus, the observed differences in the cellular pool of GSH in the vehicle-treated animals reflect a generalized oxidative stress. These alterations may also produce changes in the GSH redox cycling enzymes. In fact, the increase in GPx activity after tooth extraction likely reflects the activation of the antioxidant machinery. However, the measured rise in GSSG was not adequately metabolized to GSH because of only a slight increase in GRd activity.

Recently, an inverse relationship between salivary aMT levels and periodontal status was found. This study supported a protective role of aMT against free radicals produced by inflammatory periodontal diseases. Herein, we found that the application of aMT into the alveolar sockets after tooth removal reduced significantly the oxidative stress parameters in both the plasma and the erythrocytes. Increased levels of LPO caused by tooth removal were counteracted by aMT at 24 hours after surgery. The ability of aMT to efficiently reduce the oxidation of lipids under a variety of conditions where free radicals are generated is well established. It is likely that aMT achieves this high degree of lipid protection by neutralizing the radicals (i.e., hydroxyl radical and peroxynitrite) that initiate the process of lipid breakdown. aMT positions itself among the membrane lipids in such a way as to impede the oxidation of the polyunsaturated fatty acids.

In the present study, aMT also counteracted NOx levels that were increased after oral surgery; in fact, the indole reduced NOx concentrations below those measured in plasma before tooth extraction. The effect of aMT on NOx levels may depend, at least in part, on its ability to scavenge nitrite. Furthermore, in vivo studies have documented that aMT inhibits iNOS expression and activity in experimental models of sepsis in rats and mice. Increased iNOS activity and expression are related to several oral mucosal inflammatory diseases; thus, elevated iNOS activity probably contributes to the overproduction of NO and peroxynitrite during the inflammatory process following tooth removal. The inhibition of iNOS by aMT likely reduces NOx levels, thereby diminishing gingival damage and postextraction oxidative stress in the oral cavity. Additionally, aMT could also decrease nitrosative stress in gingival cells by directly neutralizing peroxynitrite.

Besides reductions in plasma markers of oxidative (LPO) and nitrosative (NOx) damage, aMT also reduced significantly the GSSG/GSH ratio, the best index of intracellular oxidative damage in erythrocytes. In addition to the direct scavenging activity of aMT, which reduces GSH consumption, aMT also increased GRd activity, which may account for the reduction of GSSG and increase of GSH levels, thereby providing the cell with additional GSH.
Besides protecting GRd per se from oxidative destruction, the effect of aMT on GRd activity also may depend on a genomic effect of the indolamine to increase the expression of the enzyme.\textsuperscript{24,25} Regulation of the GSH redox cycling is probably of great significance for oral tissue homeostasis, because GSH is a major endogenous antioxidant in the cell. GSH plays an important role in cellular protection from oxidative damage of lipids, proteins, and nucleic acids.\textsuperscript{46} Additionally, GSH regulates the metabolism and activity of other proteins and it interacts synergistically with other components of the antioxidant defense system, such as vitamins C and E and superoxide dismutase.\textsuperscript{47,48}

Although these data support the ability of aMT to reduce oral surgery–dependent oxidative stress, the two-faced character of ROS-RNS should be noted.\textsuperscript{49} Although overproduction of ROS-RNS should be considered a protective response of the immune system to prevent bacteria infection, it also results in oxidative stress and cell damage. By contrast, beneficial effects of these radicals occur at low to moderate concentrations, and involve physiologic roles in a number of cellular signaling pathways.\textsuperscript{49} Thus, the organism tends to prevent an excess of ROS-RNS activating the antioxidant response. In this regard, exogenous administration of aMT should not be considered a treatment to minimize ROS-RNS. Instead, aMT may help in preventing the overproduction of free radicals by maintaining their basal levels. Whether the effects of aMT reported here favor healing reaction is yet unclear and requires further research.

**CONCLUSIONS**

Our results document a significant increase in the levels of oxidative stress, as measured by blood parameters, in the immediate postoperative period following tooth removal. These results suggest that the use of antioxidants, such as aMT, may be a beneficial therapy after surgical procedures in the oral cavity.\textsuperscript{50} Locally, administration of aMT into the alveolar sockets successively counteracted oxidative and nitrative stress in blood, presumably also reflecting the reduction of damage in the oral cavity. Overproduction of ROS-RNS in gingival cells after tooth extraction contributes to postextraction inflammatory and infectious complications. The subsequent increase in the damage to oral tissues may, in turn, delay postextraction wound healing and regeneration of gingival tissue surrounding the alveolar bone. Thus, increased oxidative stress after tooth removal would be related to a poor postextraction prognosis and evolution, mainly when a concomitant inflammatory disease is present in the oral cavity.\textsuperscript{40} Because the antioxidant defense system in the oral cavity counteracts this oxidative stress, stimulation of this defense system aids in wound healing and reduces the recovery. Patients with compromised antioxidant defenses in the oral cavity or with pathologies associated with oxidative stress, such as diabetes, Parkinson’s disease, autoimmune disorders, periodontal disease, or aphthous ulceration, have elevated levels of ROS-RNS, which aggravates the damage to gingival tissue, delaying the regeneration processes. The current results suggest that local application of aMT may be useful in preventing inflammatory and infectious complications induced by oxidative stress after tooth extraction.

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Correspondence: Prof. Dario Acuña-Castroviejo, Department of Physiology, Faculty of Medicine, Avenida de Madrid 11, E-18012 Granada, Spain. Fax: 34-958-246295; e-mail: dacuna@ugr.es.

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