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Resveratrol attenuates oxidative stress during experimental periodontitis in rats exposed to cigarette smoke inhalation

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Objectives: This study aimed at investigating the effect of the systemic administration of resveratrol (RESV) on oxidative stress during experimental periodontitis in rats subjected to cigarette smoke inhalation.

Material and Methods: Experimental periodontitis (EP) was induced in 26 male Wistar rats by the insertion of a ligature around one of the first mandibular and maxillary molars. The animals were assigned randomly to the following groups: cigarette smoke inhalation (CSI; 3 times/d, 8 minutes/d) + resveratrol (10 mg/Kg), that is, SMK + RESV (n = 13) and cigarette smoke inhalation + placebo, that is, SMK + PLAC (n = 13). The substances were administered daily for 30 days (19 days prior and 11 days following EP induction), and then, the animals were euthanized. The maxillary specimens were processed for morphometric analysis of bone loss, and the tissue surrounding the first maxillary molars was collected for mRNA quantification of Sirtuin 1 (SIRT1) by real-time PCR. The gingival tissues surrounding the mandibular first molars were collected for quantification of superoxide dismutase 1 (SOD1) and nicotinamide adenine dinucleotide phosphatase oxidase (NADPH) using an ELISA assay.

Results: Reduced bone loss was demonstrated in animals in the SMK + RESV group as compared to those in the SMK + PLAC ($P < 0.05$) group on the basis of morphometric analysis. Resveratrol promoted higher levels of SIRT and SOD ($P < 0.05$) as well as reduced levels of NADPH oxidase ($P < 0.05$) were found in tissues derived from animals in the SMK + RESV group when compared to those in the SMK + PLAC group.

Conclusion: Resveratrol is an efficient therapeutic agent that reduces exacerbation of bone loss found in animals with EP that were also exposed to smoke. The results suggest that its effects could be mediated, at least in part, by its antioxidant and anti-inflammatory properties which attenuate the effects of oxidative stress on EP in the presence of cigarette smoke.

KEYWORDS
oxidative stress, periodontitis, resveratrol, smoking

1 | INTRODUCTION

Periodontitis is a chronic inflammatory disease triggered by increased microbial load and colonization of pathogens and mediated by host response, resulting in periodontal tissue destruction and tooth loss.[1] During the inflammatory process, T lymphocytes, macrophages, and polymorphonuclear neutrophil (PMNs) infiltrate the affected sites with PMNs representing the majority of the infiltrate. The latter produce proteases and reactive oxygen species (ROS)[1-5] which can be useful for management of microbial invasion but in excess can also cause periodontal tissue damage. Additionally, other cells such as fibroblasts, vascular endothelial cells, and osteoclasts also produce ROS.[6] The imbalance between ROS and antioxidant factors results in a metabolic state defined as oxidative stress, which is considered to be harmful as alluded to above.

It has been suggested that ROS play an important role in the progression of other inflammatory diseases besides periodontitis. It is also known that ROS production is increased by smoking while antioxidant production is reduced thereby leading to magnification of the deleterious effects of ROS on tissue.[6-8] Oxidative stress is known to cause DNA damage, peroxidation of lipid membranes, and protein inactivation.[6] These factors likely explain why smoking is considered to be a major risk factor for periodontitis increasing both its prevalence and its severity.[9-15] Not only does smoking exacerbate periodontitis, it also obtunds treatment effects for this condition.[16-20] More specifically in relation to periodontitis, it has been shown that smokers with this condition have lower levels of naturally produced antioxidants and higher levels of ROS when compared to non-smokers. This is, of course, consistent with smoking-induced increases in disease prevalence and severity as well as poor response to treatment.[7,8,21]

Furthermore, cigarette smoke itself is an exogenous and major source of ROS (10^8 organic-free radicals per puff in the gas phase and 10^{19} free radicals per gram in the tar phase).[22-24]

Resveratrol is a plant-derived molecule with anti-inflammatory[25-27] and antioxidant effects.[28,29] Its antioxidant effects are related to several properties. Firstly, it acts as an antioxidant from a physicochemical perspective thereby quenching ROS when they are present.[30] In addition, resveratrol inhibits the synthesis of CYP450, one of the enzymes responsible for producing ROS,[31] and finally, it is an antagonist of the aryl hydrocarbon receptor,[32] a receptor that is also known to upregulate inflammation and ROS production when activated. These characteristics would explain why resveratrol has been shown to inhibit smoke or aryl hydrocarbon (the latter being found in smoke)-mediated exacerbation of EP.[33] Moreover, we have shown that even in the absence of exposure to cigarette smoke, oxidative stress among other factors plays an important role in the initiation and progression of periodontitis.[26,28,34] Accordingly, RESV also inhibits periodontal disease and progression of experimental periodontitis (EP) in the absence of smoking.[27] Interestingly, Ikeda et al[35] have shown recently reduced bone loss and bone healing with systemic treatment with resveratrol derivative-rich melinjo seed extract through reduced oxidative stress and osteoclast activity.

The inhibitory effects of RESV on systemic levels of oxidative stress have been described.[34] This said, aryl hydrocarbon receptor antagonism does not only interfere with receptor-mediated upregulation of the production of ROS or inflammatory cytokines as described above. It has also been demonstrated that RESV also interferes with aryl hydrocarbon receptor-mediated prevention of osteoblast differentiation and function; another property that would be protective insofar as the periodontium is concerned given that bone is one of the major components of this structure.[31,36-38]

In order to demonstrate more clearly whether or not resveratrol might mediate positive effects on smoking-mediated exacerbation of periodontitis, this study was performed to investigate the effect of systemic administration of RESV on the levels of genes/proteins related to oxidative stress as well as levels of naturally produced antioxidants within periodontal tissues.

2 | MATERIAL AND METHODS

2.1 | Animals

Twenty-six adult male Wistar rats (200-300 g—Butantan Institute, Butantã, São Paulo, Brazil) were used. The rats were acclimatized for 15 days before use, and they were kept in temperature-controlled cages, exposed to a 24-hours light-dark cycle of equal time, and had free access to water and food ad libitum (Labina, Purina, Paulínia, São Paulo, Brazil) in the Bioterium of Paulista University. The experimental procedure was approved by the Paulista University Institutional Animal Care and Use Committee (226/14 CEP/ICS/UNIP).

2.2 | Experimental design

2.2.1 | Treatment groups

The experimental design is observed in Figure 1. The animals were assigned to the following groups: SMK + RESV (N = 13)—rats were subjected to CSI[39] and received daily administration of 10 mg/kg of RESV[26,27]; SMK + placebo (N = 13)—animals were exposed to CSI and received daily administration of a placebo solution. The therapies were administered via gavage for 30 days. A stock solution of RESV (R5010-500MG—Sigma-Aldrich Ltda, São Paulo, SP, Brazil) (molecular weight: 228.2) was prepared in Tween-80 (P4780 – 100 ML, Sigma-Aldrich, São Paulo, SP, Brazil) and further diluted in water for working concentrations. The placebo solution was composed of the same quantities of Tween-80 and water as used in the preparation of RESV. Administration of RESV or PLAC was started 19 days prior to initiation of EP and then for another 11 days following ligature placement.

2.2.2 | Cigarette smoke inhalation model

CSI was initiated 1 week prior to initiation of RESV or placebo administration and was performed until the end of the study. Briefly,

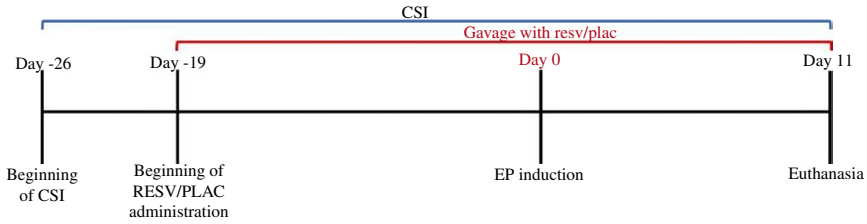

FIGURE 1 Schematic illustration of the experimental design

animals in the CSI group were placed in a cigarette smoke exposure chamber as described previously.[39] The device consisted of a 45 × 25 × 20 cm^3 clear acrylic chamber, an air pump, and two inflow/outflow tubes. Five animals were housed in the chamber at the same time, and the cigarette smoke of 10 cigarettes, containing 1.3 mg of nicotine each, was pumped into the chamber. Thus, the animals breathed the cigarette smoke that contaminated the air for 8 minutes, three times daily, until they were euthanized.

2.2.3 | Rat periodontitis model

To induce EP, a first mandibular molar and a second maxillary molar in each animal were assigned to receive a cotton ligature (Coats Corrente no. 10, São Paulo, SP, Brazil) knotted subgingivally at the cementoenamel junction (CEJ). The ligatures were kept in position in order to allow biofilm accumulation over 11 days thereby providing for initiation of EP. Contralateral teeth were left unligated so that they could be used as controls. Thirty days after the start of the study, the animals were euthanized by CO_2 inhalation. The maxillas were excised for morphometric analysis, and the gingival tissues surrounding the molars were collected for quantification of various biomolecules. The gingival tissues from the second maxillary molars were collected for quantification of mRNA for Sirtuin 1 (SIRT). The gingival tissues surrounding the mandibular first molars were collected for measurement of superoxide dismutase 1 (SOD1) and nicotinamide adenine dinucleotide phosphatase (NADPH) oxidase using an ELISA assay.

2.2.4 | Linear measurement of alveolar bone loss

The linear measurement of maxillary alveolar bone loss was performed as described previously.[25,26] Briefly, after gingival dissection, the mandibles were de-fleshed by immersing the specimens in 8% sodium hypochlorite for 4 hours. The samples were washed in running water and dried with compressed air. To discriminate the CEJ, 1% aqueous methylene blue solution was applied for 1 minute followed by a wash in running water.[25-27,33] Photographs were taken with a 6.1-megapixel digital camera (EOS 40D; Canon, New York, NY, USA) placed on a tripod to keep the camera parallel to the ground at the minimal focal distance thereby insuring reproducibility of image acquisition. The specimens were stabilized in wax with their occlusal planes parallel to the ground and long axes perpendicular to the camera. Photographs of the buccal aspects were taken, and representative linear alveolar bone loss was assessed on the buccal surface of the lower first molars by measuring the distance of the CEJ from the alveolar bone crest at three equally distant sites using an image analysis system (Image-Pro; Media Cybernetics, Silver Spring, MD, USA). The average CEJ-ABC distance of each tooth was calculated. To validate measurement conversions, all specimens were photographed alongside a millimeter ruler.[25-27] The measurements were performed after intra-examiner calibration by evaluating 10 images not taken for this study. The single examiner (SA) was blinded to the experimental group identities when performing morphometric measurements. The examiner took the linear measurements of all photographs twice within 24 hours. The intra-class correlation was 95.7%.

2.2.5 | Gene expression analysis

The tissues from the second maxillary molars were stored in a stabilization solution (RNAlater®) at −70°C. Evaluation of the mRNA levels for SIRT1 was performed using qRT-PCR, as described previously.[33,40] Total RNA from the biopsies was isolated using the TRIzol method (Gibco-BRL, Life Technologies, Rockville, MD, USA). Total RNA treated with DNase treated to reduce the likelihood of false-positive readings (Turbo DNA-frees, Ambion Inc., Austin, TX, USA), and 1 μg was used for complementary DNA (cDNA) synthesis. The RNA concentration was determined with optical density using a micro-volume spectrophotometer (NanoDrop 1000, NanoDrop Technologies LLC, Wilmington, NC, USA). The reaction was carried out using a cDNA synthesis kit (Roche Diagnostic Co., Indianapolis, IN, USA) following the manufacturer's recommendations. Primers were designed using probe design software (Lightcycler Roche probe design software, Diagnostics GmbH, Mannheim, Germany). The amplification profiles, primer sequences, and lengths of PCR products are demonstrated in Table 1. The qPCRs were performed in a real-time PCR apparatus (LightCycler® 96 Instrument, Roche Diagnostics GmbH, Mannheim, Germany) using a SYBER Green kit (FastStart DNA Masterplus SYBER Green, Roche Diagnostic Co., Indianapolis, IN, USA). The outcomes were expressed as relative amounts of the target gene using GAPDH as the internal reference gene by using the delta-cycle threshold (CT) method. For example, CT values obtained for target genes were normalized against CT values of the reference gene within the same sample. Water was used as a negative control in each run.

2.2.6 | Immunodetection of superoxide dismutase 1 (SOD1) and nicotinamide adenine dinucleotide phosphatase oxidase (NADPH)

For purposes of immune-enzymatic assay (ie, ELISA testing), the gingival tissue collected was weighed, then cut into small pieces

TABLE 1 Primer sequences for each gene, amplification profiles, and the estimated length of qPCR product for each gene

Gene	Sequence (5′-3′)	Length of qPCR product (bp)	Amplification profile (temperature [°C]/time [s])
SIRT1	GGTCAGGCTGGTTTACAACG	68	95/10, 61/8, 72/10
GAPDH	TGAGTATGTCGTGGAGTCTACTG	159	95/10, 56/8, 72/7

Sirtuin (SIRT1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

(1 mm^3 to 2 mm^3) using scissors and blades,[25-27,33,41] and solubilized in PBS to a final concentration of 100 mg tissue/mL. After extraction on a Vortex mixer for 10 minutes, each sample was centrifuged at 370 ***g*** for 5 minutes, and the supernatant was collected, divided into small portions, and stored at −70°C until use. The levels of SOD1 and NDPH oxidase were measured by using a rat ELISA Kit (MBS2514900; MBS2602768—MyBioSource, San Diego, California, respectively). Samples and standards were prepared following the manufacturer's instructions and analyzed on a fluorescence multiwell plate spectrophotometer.

2.3 | Statistical analysis

Statistical analysis was performed using SAS software (Program Release 9.3; Cary, NC, USA). The data were examined for normality using the Kolmogorov-Smirnov test, and the data that achieved normality were analyzed using parametric methods. Student's *t* test was used to assess for statistically significant intergroup findings while a paired *t* test was performed for intragroup analysis of alveolar bone loss, mRNA, and protein levels. The significance level established for all analyses was 5%.

3 | RESULTS

The animals did not show any signs of systemic illness throughout the study. The rats also did not lose significant weight throughout the experimental period. During the experiment, 1 death was observed in SMK + RESV group and 1 death in SMK + placebo group.

3.1 | Morphometric results

Significantly increased loss of bone was demonstrated about teeth that had been ligated (ie, EP) vs those that were not ligated (ie, healthy) by way of intragroup analysis ($P < 0.05$). As demonstrated previously, intergroup analysis also showed that treatment with RESV reduced loss of bone caused by EP in animals exposed to CSI vs PLAC ($P < 0.05$). There were no measurable differences in bone levels for unligated teeth, in RESV-treated vs PLAC teeth ($P > 0.05$). The morphometric findings are shown in Figure 2.

3.2 | Gene expression levels

Resveratrol promoted higher levels of SIRT in the SMK + RESV group as compared to those found in the SMK + PLAC group ($P < 0.05$) in the ligated side. For unligated teeth, RESV had no effects on SIRT ($P > 0.05$), nor were there any statistically significant differences found using intragroup analysis ($P > 0.05$; Table 2).

3.3 | Gingival tissue SOD1 and NADPH oxidase levels

Higher levels of SOD were observed in SMK + RESV compared with SMK + PLAC in the ligated side ($P < 0.05$; Figure 3). Considering the levels of NADPH oxidase, reduced levels were detected in the SMK + RESV group as compared to the SMK + PLAC group, also on the ligated side ($P < 0.05$; Figure 4). No difference was observed between the groups for the non-ligated side ($P > 0.05$). There was no difference between ligated and non-ligated sides for both proteins ($P > 0.05$).

4 | DISCUSSION

Periodontal tissue destruction is related to the imbalance between pro- and anti-inflammatory markers and to the metabolic state of oxidative stress. Smoking is a recognized risk factor for periodontitis and is related to higher levels of ROS and lower levels of naturally produced antioxidants. Resveratrol has been shown anti-inflammatory and antioxidant activity. The present study evaluated the effect of resveratrol on the levels of genes/proteins related to oxidative stress in rats exposed to CSI and induction of EP. Our results showed reduced alveolar bone loss and improvement of the

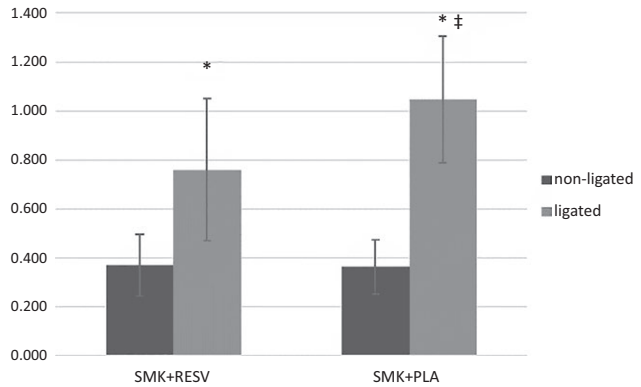

FIGURE 2 Mean ± SD of alveolar bone loss (millimeters) for ligated and unligated teeth. ‡ Represents significant difference between groups (Student's *t* test; $P < 0.05$). * Represents significant intragroup difference (Student's *t* test; $P < 0.05$)

TABLE 2 Mean (±SD) of the relative levels of mRNA for SIRT (mRNA gene/mRNA GAPDH)

	Ligated	Non-ligated
SMK + RESV	2.374 ± 3.912*	2.049 ± 2.570
SMK + PLAC	0.850 ± 1.299	1.162 ± 1.802

*Significant difference between groups by Student's *t* test, $P < 0.05$.

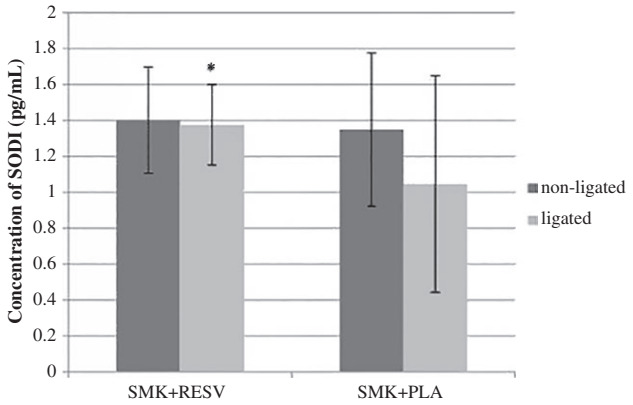

FIGURE 3 Means ± SD of superoxide dismutase 1 (SOD1) gingival levels (U/mL) measured by ELISA assay. * Represents statistical intergroup difference (Student's *t* test, $P < 0.05$)

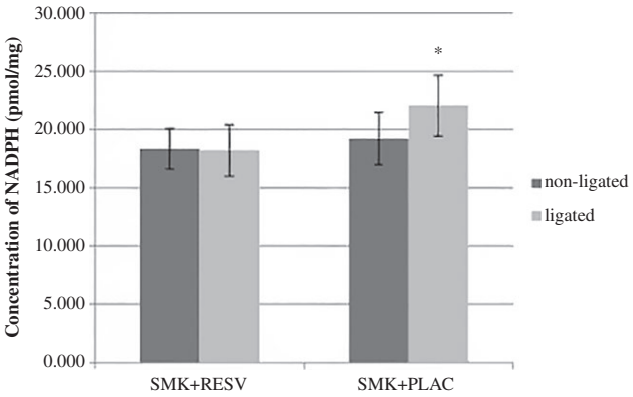

FIGURE 4 Means ± SD of nicotinamide adenine dinucleotide phosphatase oxidase (NADPH oxidase) gingival levels (U/mL) measured by ELISA assay. * Represents statistical intergroup difference (Student's *t* test, $P < 0.05$)

levels of gene/proteins related to oxidative stress following systemic administration of resveratrol.

Resveratrol mediated inhibition of CSI-induced increases in bone loss seen in EP agree with previous studies done by our research group[25,27,33] and others.[34,35,42,43] Reduced periodontal damage related to use of resveratrol is high if not solely related to its effects on levels of IL-17, IFN-γ, IL-4, and TH17/TH2 response.[25,27,33] Although in this study, the levels of inflammatory interleukins were not measured, this has been demonstrated in other investigations.[25,27,33]

In our investigation, reduced levels of NADPH oxidase were demonstrated following systemic treatment with resveratrol. NADPH oxidase is an enzymatic complex responsible for the formation of the first ROS from which others are derived.[44-47] The subunits derived from NADPH oxidase are called NOX (NOX 1-5), and they catalyze the reduction in oxygen to superoxide using NADPH as an electron donor.[48] It is known that NOx and nitrotyrosine (residues of protein nitrosylated by ROS formed by the combination of nitric oxide and superoxide) are increased significantly in periodontal tissue during EP.[49,50] In vitro and in vivo studies have also demonstrated that NOX production and activation can be induced by CSI[51-53]; findings that have also been shown in human studies.[54,55] In this regard, a recent study demonstrated reduced activation of NADPH oxidase/ROS/NF-κB pathways in human fibroblast-like synoviocytes after resveratrol treatment, leading to the suppression of particulate matter air pollution-induced COX-2 expression and PGE2 release.[56] Other studies have demonstrated the regulatory effect of resveratrol on the expression of NADPH oxidase.[57-60] Therefore, the observed reduction in NADPH oxidase levels demonstrated with resveratrol is consistent with reduced inflammatory bone loss caused by CIS.

With regard to natural antioxidant activity, it is known that both SIRT1 and SOD are important antioxidants in their own right. Since smoking can reduce the levels of these proteins, it follows that the development of oxidative stress is upregulated with CSI.[7,8] In the present study, systemic administration of resveratrol prevented the downregulation of mRNA for SIRT1 caused by CSI. This result agrees with previous studies that observed the activator effect of resveratrol on Sirt.[61,62] SIRT1, a member of the sirtuin family (SIRT1-SIRT7), is a nicotinamide adenine dinucleotide-dependent class III histone deacetylase which activates AMP-activated protein kinase (AMPK). This protein is responsible for mitochondrial biogenesis, and resveratrol antioxidant capacity is related to its effect on the levels of SIRT1.[63,64] SIRT1 also is responsible for several cellular activities including gene expression, metabolism, stress resistance, and apoptosis.[65-69] Interestingly, SIRT1 also downregulates TH-17 cytokines,[70] which are responsible for tissue destruction through the induction of the production of pro-inflammatory[71-73] and osteoclastogenic factors.[74-76] Previous studies done by our group[27] demonstrated reduced levels of IL-17 during experimental periodontitis progression as well as regulation of Th17/Th2 balance (even when exposed to CSI) with resveratrol systemic treatment, and this finding is consistent with the known effects of TH-17 cytokine regulation by SIRT. Alternatively, smoking reduces the expression of SIRT.[77] Thus, it can be suggested that SIRT1 upregulation, even in the presence of cigarette smoke, reduces periodontal tissue damage by reducing the local levels of oxidative stress and the levels of important molecules related to tissue loss (eg, matrix metalloproteinases).[44]

SOD is an antioxidant enzyme which catalyzes the breakdown of superoxide, an oxygen radical released during inflammation. It has been shown that smoking downregulates SOD levels in gingival crevicular fluid.[8,21,78] Cigarette smoking reduces the levels of SOD through the elevation of hydrogen peroxide which inactivates SOD. Since resveratrol elevates the levels of SOD, this would reduce

oxidative stress and attendant destruction of tissues related to this condition.[79-83] Resveratrol has also been shown to upregulate production of the transcription factor, Nrf2 (nuclear factor erythroid 2-related factor 2). Nrf2 is an important modulator of the antioxidant response which regulates the expression of antioxidant genes including SOD. It is noteworthy that in our investigation, resveratrol upregulated levels of SOD despite exposure to CSI (CSI lowers SOD production).

In conclusion, resveratrol reduced periodontal tissue destruction and attenuated oxidative stress seen in EP in animals submitted to CSI through the reduction in NADPH oxidase levels and upregulation of SIRT1 and SOD. This study supports the concept that resveratrol could be used therapeutically, at least because of its antioxidant properties, for inhibition of periodontitis, particularly in smokers.

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