Resveratrol Derivative-Rich Melinjo Seed Extract Induces Healing in a Murine Model of Established Periodontitis

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A short running title: Resveratrol induces healing of periodontitis in mice

A one-sentence summary: Resveratrol derivative-rich melinjo seed extract, that have

antioxidant and anti-inflammatory properties, accelerated healing of periodontal bone and tissue destruction and that these effects are related to decreases in oxidative stress and osteoclast activity.

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ABSTRACT

Background: Products of internal defense systems, like pro-inflammatory cytokines, reactive oxygen species, and leukocytes, are released which attack periodontal bacteria in periodontitis, but at the same time, lead to tissue destruction as well. We hypothesize that resveratrol derivative-rich melinjo seed extract (MSE), an edible plant extract that has antioxidant properties, should promote healing of periodontal bone loss and modulating immune-inflammatory systems that leads periodontal tissue destruction.

Methods: We used an experimentally induced periodontitis (EP) model in mice. Ligatures were placed first for development of EP (15 days). MSE was intraperitoneally administrated (0.001% (w/w)) to reverse bone loss that had already occurred in established EP and mice were then sacrificed (day 17, 20 and 22).

Results: Morphometric outcomes revealed lower bone-loss in the MSE groups compared to control. Immunohistochemistry assays demonstrated lower oxidative stress in MSE groups. MSE also inhibited M-CSF/sRANKL mediated osteoclast formation and down-regulated osteoclast activity.

Conclusions: Treatment with MSE in EP actually caused healing of bone and that these effects are probably related to decreases in local oxidative damage and osteoclast activity. Given MSE's positive effects on osteodifferentiation as well, these findings suggest that MSE could be a useful therapeutic agent for the management of periodontitis.

KEY WORDS

Alveolar bone loss; osteoclasts; oxidative stress; periodontal diseases; periodontitis; reactive oxygen species; resveratrol.

INTRODUCTION

Oxidative stress through a process called oxidative burst that has been suggested as a key mechanism in periodontal disease pathogenesis and periodontal tissue damage.[1, 2] A few studies have demonstrated consistent and highly significant increases in intracellular reactive oxygen species (ROS) production by peripheral blood polymorphonuclear neutrophils (PMNs) as well as oral PMNs in patients diagnosed with chronic periodontitis compared to healthy controls.[3, 4] Previous work in our laboratory* has demonstrated a specific proinflammatory PMN phenotype, found during chronic periodontal disease, characterized by exaggerated ROS production[3] *versus* healthy patients. Furthermore, inhibition of antioxidant production through down-regulation of the master regulator of anti-oxidants nuclear factor erythroid 2-related factor 2 (Nrf2) pathway in oral PMNs, is associated with severe periodontitis.[5] The existence and stimulation of osteoclasts, the main bone resorbing cells, are required for periodontal bone loss.[6] Osteoclasts are regulated by the interplay of receptor activator of nuclear factor κ-B ligand (RANKL) and osteoprotegerin.[7] Bone resorption is stimulated by several mediators, such as interleukin (IL)-1 and tumour necrosis factor (TNF)-α, ionized calcium and endothelial cells.[6, 8] It is also known that the production of ROS is required for recruitment and differentiation of osteoclasts.[9]

As suggested above, ROS play a critical role in the initiation and progression of periodontal bone loss and tissue destruction. Excessive ROS can cause oxidative stress within tissues and bring about direct damage to cellular proteins, DNA and lipids.[10] In

addition to direct tissue damage, ROS modulates the immune-inflammatory system, through activation of reduction-oxidation reaction (Redox)-sensitive transcription factors, such as nuclear factor-κB and activating protein-1.[10, 11] These Redox state changes lead to tissue damage secondary to the induction of a pro-inflammatory state.[12] It is also noteworthy that smoking represents one of the most important epigenetic risk factors for the development of periodontitis and contributes to poor response to treatment of periodontitis.[1, 2, 13] We have shown in previous investigations that a major component of cigarette smoke that could be mediating these effects are the aryl hydrocarbons two of which include dimethyl benzanthracene and benzo-a-pyrene. These compounds act as agonists of the cytosolic aryl hydrocarbon receptor (AhR) thereby inhibiting differentiation of osteoblasts, as well as up-regulation of the synthesis of ROS.[14] Resveratrol (3, 4', 5-trihydroxystilbene) is a widely available plant polyphenol, which is thought to have several health benefits.[15, 16] Previous studies on resveratrol (and other related molecules) have demonstrated health benefits including improved metabolic control of diabetes, anti-cancer and anti-inflammatory activities.[17-19] Resveratrol has also been shown to antagonize the deleterious effects of environmental and cigarette smoke-related aryl hydrocarbons by acting as an antagonist of AhR-modulated antioxidant enzyme activity.[20-22] In addition, resveratrol is itself an antioxidant and thus likely to directly inhibit ROS effects.[23] There are various sources of resveratrol and it was decided to focus on the use of melinjo (Gnetum gnemon L.) seed extract (MSE). Melinjo is a small tree native to Southeast Asia and MSE is a readily available dietary

supplement which is also being added to and/or used in a variety of baked goods and foods in the Southeast Asian diet. As alluded to here, MSE contains, among resveratrol itself, several variants of resveratrol including trans-resveratrol, gnetin C (a resveratrol dimer), gnemonoside A and gnemonoside D.[24, 25]

It has been shown in an animal model of experimental periodontitis (EP) that resveratrol almost completely inhibits the progression of periodontal bone loss and decreases the levels of specific proinflammatory cytokines.[26-28] In light of these findings, it has been suggested that resveratrol could conceivably prove to be a useful therapeutic agent for patients who have periodontitis. However, while resveratrol might prevent the initiation and subsequently the progression of periodontitis-mediated tissue loss, it does not necessarily follow that resveratrol, despite its ability to prevent the development of bone loss, can be used for treatment of already established periodontitis. Based on the foregoing then, we proposed to utilize resveratrol compounds, in this case MSE, in the treatment of already established EP in a mouse model. We anticipated that treatment of established periodontitis with MSE (as opposed to the prevention that most studies have focused on) should induce or at least accelerate healing and possibly even promote regeneration of bone and other connective tissues that would otherwise not occur in control animals that have established periodontitis but would not have received MSE.

MATERIALS AND METHODS

Animals

The animal cohort was composed of 89 male 6-7 week old C57BL/6J wild-type mice, weighing 21.6 ± 1.5 g at the beginning of the study. All procedures were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved to the University of Toronto Animal Care Committee (Protocol number: 20011295).

Melinjo seed extract

According to the manufacturer, ** MSE was obtained from melinjo seeds. The seeds were mixed with hydrous ethanol and filtered. The filtered extracts dissolved in dextrin solution were lyophilized. The manufacturer's criteria for quality control were as follows: 1, appearance (light brown powder which has a smell of melinjo and an astringent taste); 2, loss on drying (≤ 5%); 3, resveratrol content (≥ 20%); 4, total plate count (≤ 3000); 5, total coliform (negative). The MSE we used for this experiment contained 24.96% resveratrol, including gnemonoside A (16.44%), trans-resveratrol (0.12%), gnemonoside D (4.90%) and gnetin C, (3.50%). The chemical components of MSE as measured are outlined in (Japan Food Research Laboratories) in Supplemental Tables S1.

Experimental Groups and Ligature Induced Periodontitis

Mice were assigned randomly to one of these 4 groups: group 1, experimental periodontitis (P); group 2, periodontitis treatment (T); group 3, experimental periodontitis with MSE administration (P+MSE); group 4, periodontitis treatment and MSE administration (T+MSE). Experimentally induced periodontitis model time schedule was shown in **Fig. 1**. To induce EP, a 9-0 silk suture was placed in the gingival sulcus of the maxillary left second molar (M2) and tied on the palatal side using a surgeon's knot as previously described[5]. The right side served as control (no ligature). These procedures were performed under general anaesthesia by intraperitoneal administration of a mixture of ketamine hydrochloride (0.05% (v/w)) and xylazine (0.01% (w/w)). Chronic periodontitis was induced and then allowed to progress for a period of 15 days. To compare between the diseased condition and the healing phase, the silk ligatures were removed to simulate 'treatment' in T and T+MSE groups, while ligatures were allowed to remain in placed in the other groups as might be seen in untreated periodontitis in P and P + MSE groups. One day before ligature removal (day14), mice in either the P+MSE or T+MSE groups were injected intraperitoneally with MSE dissolved in Dulbecco's phosphate-buffered saline (PBS) †† and mice in the P and T groups were injected PBS only. MSE was administrated at a dose of 0.004% (w/w) (= 0.001% (w/w) resveratrol) bodyweight. At the end of the experimental period, mice were euthanized by CO_2 inhalation at each time point: 17, 20

and 22 days after periodontitis induction, while mice in the P group were sacrificed only for baseline measures on day 15.

Morphometry

Each maxilla was skinned and defleshed in a colony of dermestid beetles (Royal Ontario Museum, Toronto, ON) and freeze-fumigated for 7 days. Dry skulls were stained with methylene blue (1% in water), and images of the buccal aspects of the right (healthy) and left (diseased) M2s were taken at a magnification of X40 using a video camera‡‡ mounted on a stereomicroscope. Horizontal bone loss was measured from the cemento-enamel junction (CEJ) to the alveolar bone crest at the mid-buccal, mesial, and distal roots on the buccal aspect of the M2s on the disease and healthy control sites using µScope Professional x64 software. Alveolar bone loss values were analysed by subtracting control alveolar bone height measurement values from those obtained from the side treated with the ligature.

Micro-computed Tomography Analysis

Micro-computed tomography (µ-CT) was performed using a µ-CT scanner§§ set at a resolution of 9.1 µm and 0.5 mm Al filter. µ-CT was employed to scan the mouse skulls collected 20 days after periodontitis induction and relevant measurements for bone loss were performed using the reconstructed scan using CTAn software v1.13. Three-dimensional images were produced using the computer software imageJ. Proximal

alveolar bone loss was measured on the slices selected in orthogonal views of the stack from mesial and distal CEJ to alveolar bone crest.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from the gingival tissue of the palatal aspects of the right (healthy) and left (diseased) M2s from day 22 mice. RNA was isolated using commercially available kit[¶¶] and stored -80°C until used. Using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), complementary DNA (cDNA) was generated as follows. One microgram of RNA was reverse-transcribed into cDNA using murine leukemia virus reverse transcription[¶¶] and oligo-d(T)$_{18}$ VN primer [##] as described previously.[29] QRT-PCR was performed in triplicate[***]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward, 5-CCTTCCGTGTTCCTACCCC-3; and reverse, 5-CCCAAGATGCCCTTCAGT-3) was used to normalize the expression data of the tested genes (IL-6: forward, 5'-CTGCAAGAGACTTCCATCCAG-3'; reverse, 5'-CGATCACCCCGAAGTTCAGTAG -3'; TNF-α: forward, 5'-CAGGCGGTGCCTATGTCTC-3'; reverse, 5'-CGATCACCCCGAAGTTCAGTAG-3'; IL-1β: forward, 5'- GAAATGCCACCTTTTGACAGTG-3'; reverse, 5'-TGGATGCTCTCATCAGGACAG-3'; IL-17: forward, 5'-GGCCCTCAGACTACCTCAAC-3'; reverse, 5'-TCTCGACCCTGAAAGTGAAGG-3').

Histology

Histology was completed at the Toronto Center for Phenogenomics (Toronto, ON). Maxillae collected 22 days after initiation of the experiment were fixed in 4% paraformaldehyde in PBS (PFA)†††, decalcified in 10% ethylenediaminetetraacetic acid (EDTA)††† for 5 weeks, and embedded in paraffin. To assess ROS damage at sites with periodontitis, 8-hydroxy-2'-deoxyguanosine (8-OHdG) immunohistochemistry, a critical biomarker for oxidative stress,[30] was completed. Coronal sections of healthy and diseased areas were probed with primary goat anti-8-OHdG‡‡‡ and secondary horse anti-goat biotinylated IgG§§§ and stained using an avidin/biotin tissue staining system§§§ and haematoxylin counterstaining. Three regions of interest were defined for each site (ligature and control): alveolar bone crest, sulcular epithelium, and gingival connective tissue. The number of particles for 8-OHdG, per unit area were measured and recorded.[5] Nrf2, a protein responsible for up-regulation of natural antioxidant molecules was identified by immunohistochemistry as well. The sections were probed with rabbit polyclonal antibodies specific for Nrf2‖‖‖, diluted 1: 50 and haematoxylin counterstaining. To quantify bone-resolving osteoclasts at the sites with periodontitis, tartrate-resistant acid phosphatase (TRAP) staining was completed. Commercially available kit††† were used as per the manufacturer's instructions. Assessment of osteoclast coverage area at the alveolar bone around ligated (diseased) and unligated (healthy control) teeth was performed.[29]

Osteoclastogenesis in vitro Using Isolated Bone Marrow Monocytes

As described previously, bone marrow monocytes (BMMs) were isolated from femurs and tibias from day 20 mice and age-matched unligated mice as control under sterile conditions.[29, 31] Briefly, bone ends were cut and minimum essential medium, alpha modification (α-MEM) culture medium[¶¶¶] was flushed into the bone using a sterile 26-gauge needle, and a single cell suspension was collected. BMMs were cultured overnight in α-MEM to remove fibroblasts/stromal cells. Cells were counted using a Z1 Coulter counter[###] and resuspended in α-MEM containing 1% fetal bovine serum and antibiotics (164 IU/ml penicillin G, 50 g/ml gentamicin and 25% (w/v) fungizone) at a density of 1×10^6 cells/ml. To study osteoclastogenesis in vitro, non-adherent cells from these cultures were seeded onto 8-well Lab-Tek chambered slides, in 400 µl of α-MEM supplemented with 10% fetal bovine serum (FBS)[†††], 20 ng/ml macrophage colony-stimulating factor (M-CSF)[†††], and 0.006 (w/v) recombinant soluble RANKL (sRANKL) for 6 days. Medium was removed and replaced with fresh solutions containing M-CSF and sRANKL every other day during the incubation period. Osteoclast formation was confirmed by TRAP as well as 4′, 6-diamidino-2-phenylindole (0.165 µM in 0.1% Triton X-100/PBS)[†††] staining (to confirm multinucleation).

Statistical Analysis

Statistical analyses were conducted using one-way ANOVA with Tukey's test for morphometric analysis, analyses of µ-CT images, mRNA level, osteoclast analysis, and histological analysis for TRAP staining. The unpaired Student's *t*-test was used for

assessment of histological data. Sample size was estimated based on the results of previous studies.[5, 27] Three to five samples per group were required for detection of a significant difference with an alpha level of 0.05 and a power of 0.80.

All statistical analyses were performed with EZR††††, which is a graphical user interface for R‡‡‡‡.[32] More precisely, it is a modified version of R commander designed to add statistical functions frequently used in biostatistics.

RESULTS

MSE inhibits ligature-induced alveolar bone loss

There was no significant change in body weight among the groups during the experimental periods. There was no statistically significant difference in alveolar bone loss (ABL) of P group mice at each time point. Assessment of ABL by morphometry data (**Fig. 2-A** and **B**) using intragroup analysis revealed effects of MSE on alveolar bone. There were no significant differences in all groups on day 17, while a trend toward lower loss of alveolar bone in T+MSE and P+MSE groups was observed (**Fig. 2-B**). On day 20, significantly lower ABL in P+MSE and T+MSE group's mice compared with P group mice were seen ($p < 0.05$; **Fig. 2-B**). On day 22, ABL values in the T, P+MSE and T+MSE groups were significantly lower than P group ($p < 0.05$; **Fig. 2-B**).

To assess the different amounts of periodontal bone in the various groups in more detail, μ-CT imaging and image analysis was performed at sites with ligature-induced periodontitis in day 20 mice (**Fig. 2-C, D and E**). Statically significant differences in bone content were demonstrated between the various groups as follows. P+MSE and T+MSE group showed lower bone loss (63% less and 53% less respectively, $p < 0.05$) compared with P group in mesial site (**Fig. 2-D**) while T, P+MSE and T+MSE group had lower bone loss (68% less, 62% less and 71% less respectively, $p < 0.01$) compared with P group in distal site (**Fig. 2-E)**.

MSE regulates IL-1β level at sites with periodontitis

The expression levels of proinflammatory cytokines (IL-6, TNF-α, IL-1β and IL-17) in gingival tissue around M2s from day 22 mice were evaluated. Compared with the P group, the mRNA expression levels of IL-1β were significantly lower in the P+MSE group as compared to P group ($p < 0.05$) and a trend toward lower levels in T+MSE group and T group were observed ($p = 0.06$ and 0.06 respectively). There were no significant differences of IL-6, TNF-α and IL17 levels among the groups (**Fig. 3**).

MSE inhibits M-CSF/sRANKL mediated osteoclast formation

Following six days of induction of osteoclastogenesis using marrow cells from day 20 mice in the presence of M-CSF and sRANKL, the number of nuclei within TRAP positive multinucleated osteoclasts were counted in six random fields of views respectively (**Fig. 4-A**). There was a statistically significant reduction in the number of multinucleated cells (i.e. osteoclasts) in cultures of marrow cells derived from the P+MSE, T+MSE and control groups as compared to the number of osteoclasts counted in cultures of marrow cells derived from animals in either the P or T groups ($p < 0.05$; **Fig. 4-B**). These results demonstrate that MSE treatment effectively inhibited with M-CSF/sRANKL mediated induction of fusion as needed for the

production of osteoclasts and that this was even observed in the absence of treatment (i.e. ligature not removed).

MSE down-regulates osteoclast activity at sites with periodontitis

Osteoclast coverage of the alveolar bone was assessed by TRAP staining (**Fig. 5-A**). TRAP staining of individual osteoclast cells in coronal sections of ligated and control molars showed T+MSE group had significant lower TRAP positive area compared to P group and a trend toward lower area in P+MSE group were observed ($p = 0.07$). MSE administration decreases osteoclast coverage area (**Fig. 5-B**).

MSE promotes down-regulation of oxidative stress through Nrf2 pathway at sites with periodontitis

To assess ROS damage at sites with periodontitis (**Fig. 5-C**), we quantified 8-OHdG-positive cells and found decreased numbers ($p < 0.05$) around ligated molars of P+MSE group mice compared with P group mice (**Fig. 5-D**). Positive cells identified at the control sites were considered as being representative of a physiological background level of DNA oxidation, so the number of cells at sites with periodontitis was normalized by dividing the total counts by the number of cells measured at the control sites of the same tooth. Nrf2 antibody staining was more

intense in P+MSE group mice compared to P group mice (**Fig. 5-E**). These data suggest that MSE promoted the down-regulation of oxidative stress through the activation of the endogenous Nrf2 antioxidant defence pathway. Of course, it must also be emphasized that resveratrol within MSE is itself an antioxidant from a physicochemical standpoint and therefore, ROS not neutralized by NrF2 were likely neutralized even more by the antioxidant characteristics of MSE (particularly the resveratrol monomers and dimers).

DISCUSSION

To the best of our knowledge and for the first time, the current study demonstrates the effect of MSE on the healing of experimentally induced EP in mice which is more or less representative of actual treatment as opposed to prevention of disease which has been demonstrated in several other studies. In this regard, in prior studies EP is induced concurrently with or even after initiation of treatment with resveratrol or resveratrol containing agents and so any beneficial effects might be seen as being preventive as opposed to actual treatment of established EP. To reiterate, in the studies described here, MSE was used to 'treat' established EP, even whilst retaining the ligatures which would be akin to introducing treatment of periodontitis in the absence of root debridement. In general, our results showed that MSE reduced the levels of pro-inflammatory cytokines, reduced osteoclast formation and activity gauged by reduced TRAP activity, reduced bone resorption and reduced ROS production respectively. Together these actions actually induced a substantial amount of healing even when 'treatment' had not been done (i.e. no removal of the disease-inducing ligature). We base our conclusions on the notion that *EP was established for 15 days before the ligatures were removed*. After 15 days of ligation, P group mice showed stably increased levels of ABL, while treatment with MSE appeared to stimulate more regrowth of periodontal bone that had been lost as compared to the amount of bone that had been regenerated

following mere removal of the ligature and as noted above. Even more importantly, it was demonstrated that MSE could induce periodontal bone regeneration *even when the ligature was left in place* (as indicated above this is tantamount to not performing any sanative therapy). This is an important finding since the down-regulation of inflammation and healing or periodontal bone occurred even in the presence of gross microbial load. This suggests that MSE has a rather potent impact on inflammation-induced bone loss initiated by applying a large microbial challenge in the periodontium.

Even considering that the microbial element is still a critically important factor insofar as the development and progression of periodontitis is concerned it must be understood that one cannot 'sterilize' the oral cavity or subgingival crevices or pockets.[33] Furthermore, even without any attempt to reduce microbial load we have shown that by down-regulation of inflammation and oxidative stress by the use of MSE (and therefore other phenols[34, 35] including of course resveratrol), can promote dramatic healing in this model of EP. Therefore, we suggest that that utilization of MSE, or perhaps more specifically resveratrol itself, for management of periodontitis might be effective for many patients, particularly those who don't appear to respond to conventional therapy. As regards the latter population we also refer to patients with refractory periodontitis who have exaggerated levels of

oxidative stress due to hypersensitive PMNs which express powerful oxidative bursts. Hence although such patients do not necessarily respond well to debridement, antimicrobials or surgery, they do seem to respond well to host modulation therapy including subantimicrobial dose doxycycline (which in addition to its ability to block matrix metalloproteinases is also an antioxidant) that is sometimes also coupled with low dose non-steroidal anti-inflammatory treatment.[36] Such patients might also respond even more robustly well to treatment with MSE (either in combination with the other medications or singly).[3] We recognize and appreciate that MSE was administered intraperitoneally in our studies and therefore the effects might have been magnified due to better systemic absorption of its components including resveratrol. Yet for human clinical trials and treatment it is likely that resveratrol will be administered orally thus potentially reducing its effects due to reduced absorption. However, others including Ribeilo et al. have reported previously that resveratrol (pure, as opposed to MSE) inhibited periodontal bone loss when it was administered via oral gavage. Hence whatever is absorbed orally is quite effective. Moreover, there is no reason to believe that other methods of administration will be developed to enhance delivery of resveratrol to diseased periodontal tissues and therefore we conclude that the route of administration might not be as critically important as might be thought since biological effects have been demonstrated using various routes, including

oral.[37]

Past studies saw the effects of resveratrol in experimental periodontitis consecutively administrated resveratrol at least several weeks.[26, 38] However, we injected MSE only once (after periodontitis was established). Yet it was still possible to demonstrate highly significant effects of treatment when comparing the P+MSE group to the P group. Pharmacokinetic studies have shown that trans-resveratrol is absorbed quickly, but speedily metabolized within 48 hours.[39, 40] On the other hand, gnetin C (resveratrol dimer) is maintained in plasma for over 96 hours and this could be at least part of the explanation as to why a single dose of MSE were observed with only one treatment and up to 8 days after its injection.[40]

ABL is a key clinical symptom of periodontitis and osteoclasts are the principal bone resorptive cells.[6] Proinflammatory cytokines, such as IL-1, -6, -11 and -17, TNF-α, leukaemia inhibitory factor and oncostatin M play essential roles in osteoclast stimulation and local stimulation of osteoclast activity is a crucial requirement for periodontal bone loss.[41] In particular, it is demonstrated that both IL-1β and TNF-α promote local osteoclast activity.[42, 43] In the current study, we considered local expression of proinflammatory cytokines and osteoclast activity in bone marrow and alveolar bone. We showed MSE utilization and/or clinical treatment modulated the production of IL-1β in gingival tissue, whereas other three cytokines, Il-6, -17 and TNF-α, had no significant difference among groups.

Regarding osteoclast, MSE administration reduced osteoclastogenesis in bone marrow and a trend toward lower levels of TRAP positive area in MSE administration groups was observed. TNF-α stimulates osteoclast differentiation through a mechanism independent of the RANK-RANKL interaction.[44] These findings are highly relevant to the periodontal bone loss/regeneration results shown here since resveratrol prevents RANKL-induced osteoclast differentiation.[45] Recently, our group* found that the Nrf2 pathway is down-regulated in oral PMNs of patients with severe chronic periodontitis compared with periodontally healthy controls.[5] We showed further that oxidative damage as a result of knocking out Nrf2 leads to more severe loss of bone in a mouse model of periodontitis. The immunohistochemical data showed that periodontal tissue around ligated teeth in mice treated with MSE have less 8-OHdG staining and more Nrf2 staining in PMNs than those not treated with MSE. The role of MSE (i.e. resveratrol monomers and other resveratrol compounds and polyphenols) in activating Nrf2 activity has in fact been shown by others as well.[46] Therefore, our results indicate that treatment with MSE reverses PMN Nrf2 down-regulation , likely through antioxidant-mediated scavenging of ROS.

CONCLUSIONS

Our present study indicates that treatment with MSE, and hence various resveratrol forms in EP actually caused *healing* of bone (even without 'mechanical treatment') as opposed to only prevention of bone loss as has been seen previously[27] and that these effects are probably related to decreases in local oxidative damage and reduced osteoclast differentiation and/or activity. Given MSE's positive effects on osteodifferentiation as well, these findings suggest that MSE, or other resveratrol-like compounds could be useful therapeutic agents for the management of periodontal inflammatory diseases.

FOOTNOTES

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CONFLICT OF INTEREST

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FIGURE LEGENDS

Figure1 Experimentally induced periodontitis model procedures were shown. To induce periodontitis, a silk suture was placed on day 0. MSE or PBS was administrated on day 14. Periodontitis was induced for 15 days and ligature was removed or left in place. Mice were sacrificed at each time point: 15, 17, 20 and 22 days after periodontitis induction.

Figure2 Morphometric analysis and micro-CT image analysis of hard periodontal tissue in mice. **(A)** Representative photographs of buccal aspects of ligated molars demonstrate the bone loss 20 days after ligation and 5 days after ligature removal. **(B)** Alveolar bone loss was measured by morphometry from the cement-enamel junction to the bone crest along the exposed roots on defleshed methylene blue-stained upper jaw. N = 5. **(C)** Micro-CT image of maxillary second molar. Alveolar bone loss (white arrow) was approximately measured by the distance from the cement-enamel junction (white line) to the alveolar bone crest (white broken line). N = 3. **(D** and **E)** The length of bone loss of mesial and distal sites were measured. Data was presented as mean ± standard deviation.

* $p < 0.05$.

† $p < 0.01$.

Figure3 mRNA levels of interleukin (IL)-6, IL-1β, TNF-α and IL-17 were determined using qRT-PCR. N = 3. *$p < 0.05$. Data was presented as mean ± standard deviation.

Figure 4. In vitro osteoclastogenesis was induced by stimulation of BMMs for six days with M-CSF and sRANKL. BMMs-derived osteoclasts were stained with TRAP. **(A)** Representative photomicrographs of TRAP labelled cells were presented. **(B)** Multinucleated osteoclasts were identified as TRAP-positive cells with more than three (include three) nuclei. The mean sum of the nuclei in the multinucleated cells of each field of view was determined. N = 3. Data was presented as mean ± standard deviation.

* $p < 0.01$.

† $p < 0.001$.

Figure 5. Histological assessment of day 22 mice maxillae. **(A)** Osteoclast surrounding ligated tooth was assessed by TRAP staining. **(B)** Osteoclast was assessed in alveolar bone by measuring TRAP positive cells coverage area. **(C)** Reactive oxygen species damage in periodontitis lesions surrounding ligated tooth (black arrowheads) was assessed by immunohistochemistry for 8-hydroxydeoxyguanosine (8-OHdG), a specific marker for oxidative damage to DNA. **(D)** 8-OHdG staining intensity in alveolar bone crest (B), epithelium (Ep) and gingival connective tissue (CT). (**E)** Antioxidant defence pathway was

shown by immunohistochemistry for Nrf2 antibody. N = 3. Data was presented as mean ± standard deviation.

* $p < 0.05$.

† $p < 0.01$.