RESVERATROL AND INSULIN ASSOCIATION REDUCED ALVEOLAR BONE LOSS AND PRODUCED AN ANTIOXIDANT EFFECT IN DIABETICS RATS

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Abstract

Background: The present investigation studied the effects of systemic administration of resveratrol (RSV) on the development of experimental periodontitis (EP) and on the release of markers of inflammation, bone metabolism and oxidative stress in diabetic rats.

Methods: Seventy-five male rats were divided into five groups: DM+PLAC: Diabetes Mellitus + placebo solution; DM+INS: DM + insulin therapy; DM+RSV: DM + RSV; DM+RSV+INS: DM + RSV and insulin; NDM: non-diabetic. Streptozotocin was used to induce DM and EP was induced by the placement of a ligature at the fist mandibular and the second maxillary molars. Euthanasia occurred 30 days after the initiation of the

study and mandible specimens were subjected for morphometric analysis of bone level. Gingival tissues from mandibular molars were collected for quantification of inflammatory and oxidative stress markers by multiplex assay system and ELISA assay, respectively. Maxillary gingival tissues were processed for real-time polymerase chain reaction (real time PCR) assessment of markers of bone metabolism and oxidative stress.

Results: Morphometric analysis revealed greater bone loss in DM+PLAC and DM+INS in comparison to the other treatments ($p<0.05$). RSV used in conjunction with INS reduced the levels of interleukin (IL)-1β, IL-6, IL-17, interferon-gamma (IFN-γ) and superoxide dismutase 1 (SOD) ($p<0.05$). RSV alone reduced nicotinamide adenine dinucleotide phosphatase oxidase (NADPH oxidase) levels, in comparison to DM+INS and DM+RSV+INS ($P<0.05$). All treatments upregulated mRNA levels for osteoprotegerin (OPG) in comparison to PLAC ($P<0.05$). Sirtuin 1 (SIRT) mRNA levels were lower in PLAC when compared to DM+RSV, DM+RSV+INS and NDM ($p<0.05$).

Conclusion: RSV reduced the progression of EP and the levels of NADPH oxidase. Co-treatment with RSV and insulin reduced the levels of pro-inflammatory factors (either proteins or mRNA) and increased the levels of SOD. The data also demonstrated that treatment with RSV and INS alone or in combination had beneficial effects on bone loss.

Key words: Resveratrol; Natural products; Oxidative stress; Periodontitis, Diabetes Mellitus; Experimental model.

INTRODUCTION

Diabetes mellitus (DM) is a metabolic disease characterized by chronic hyperglycemia arising from deficient insulin production, resistance to insulin effects or a combination of both [1,2]. DM is a public health problem because of its high prevalence (451 million people worldwide in 2017, increasing to 693 million in 2045) [3], severe morbidity (cardiovascular disease, retinopathy, nephropathy, neuropathy, periodontitis) and mortality (5 million deaths worldwide) [3,4]. As regards periodontitis, it is a chronic bacterial/inflammatory disease, associated with dysbiosis of microbial flora in the oral cavity, with high prevalence (90% of the world population - and variable degrees of severity) [5, 6] and is mediated largely by the host innate immune response to an oral microbial challenge. This leads ultimately to the loss of hard and soft tissues that support the teeth. Untreated, the condition can progress, thus causing the loss of many or even all teeth [5]. It is also noteworthy that

periodontitis itself has been shown to be correlated to increases in the prevalence of DM and poor glycemic control [7]. In fact as alluded to above, it is now also considered to be one of the known complications of diabetes [8].

As noted above, diabetic patients are at greater risk for the development and/or severity of periodontitis [9-12]. This is exemplified by the presence of increases in alveolar bone resorption, attachment loss, impaired bone formation and increased tooth loss; especially when glycemic control is poor [9-12]. It is also known that hyperglycemia leads also to dysregulation of the balance between reactive oxygen species (ROS) and endogenous antioxidants leading to oxidative stress. This stimulates the progression of periodontitis as well as the development of other known complications of diabetes [5, 13, 14].

The control of periodontal breakdown in diabetic patients may be a challenge due to the altered release of inflammatory factors [12, 15] and to the oxidative stress status [13]. In this context, alternative therapies have been investigated that focus on the concept of host-modulation therapy to alter host response to microbial infection, and more specifically to downregulate oxidative stress with the aim of improving host modulation, specially using natural products [16-20] as well as the production of matrix metalloproteinases [21, 22].

For the purposes of this investigation we shall focus on oxidative stress and approaches that could be used to downregulate this damaging pathobiological phenomenon. With respect to inhibition of oxidative stress and its damaging effects on the periodontium, one of the more interesting compounds is resveratrol (RSV). This is a natural product with anti-inflammatory [16, 17] and antioxidant effects [23, 24]. RSV has been shown to possess beneficial effects for both periodontitis and diabetes [16, 17, 18, 23, 25] and this includes also its ability to interfere with the progression of experimental periodontitis-diabetic animal model[26]. Additionally, RSV has been shown to be a scavenger of ROS by way of direct antioxidant activity. In this context, nicotinamide adenine dinucleotide phosphatase oxidase (NADPH oxidase) is an important complex, because it is responsible for initiation of upregulated ROS production [27]. Since RSV has been shown to reduce the expression of NADPH oxidase meaning that RSV can reduce the impact of oxidative stress mediated by ROS by quenching ROS molecules and also reducing their production as well. [28 -34]. Additionally, sirtuin 1 (SIRT1) and superoxide dismutase 1 (SOD) are important molecules responsible for antioxidant activity. SIRT1, is a nicotinamide adenine dinucleotide-dependent class III histone related to AMP-activated protein kinase (AMPK) activation and for mitochondrial biogenesis and RSV antioxidant capacity is related to its effect on the levels of SIRT1 [35]. Superoxide dismutase 1 (SOD) is also an antioxidant enzyme that catalyzes the breakdown of superoxide, which

is an oxygen radical released during inflammation and in this regard RSV also upregulates the levels of SOD causing even more dampening of the development and/or impact of oxidative stress [36, 37].

Although studies have demonstrated beneficial effects of RSV treatment[16, 17, 18] on periodontitis and DM [23, 25] (individually or even in combination) [26], there is still a gap of knowledge relating to the understanding of some of the underlying mechanisms. In light of these effects, we have chosen to focus on the pattern of osteo-immunoinflammatory and oxidative stress markers after RSV treatment in combination with insulin during the progress of experimentally-induced periodontitis in diabetic rats.

MATERIALS AND METHODS

Animals

Fifty-four adult male Wistar rats (200–300 g)[**] were used. The animals were kept in temperature-controlled cages and were allowed to acclimatize for 15 days before the procedures and had access to water and food *ad libitum*[††]. The experimental study approved by the Paulista University Institutional Animal Care and Use Committee (226/14 CEP/ICS/UNIP).

Experimental design

Treatment groups

Animals were divided in 5 groups: DM+PLAC (n=13): DM + placebo solution; DM+INS (n=14): DM + insulin therapy; DM+RSV (n=13); DM + resveratrol; DM+RSV+INS (n=14): DM + RSV and insulin; NDM (n=15): non diabetic.

DM induction was performed by intraperitoneal injection of streptozotocin (STZ; 60 mg/kg)[‡‡] solubilized in citrate buffer (0.01 M, pH 4.5) [38, 39]. An injection of equivalent volume (0.1 mol/L) citrate buffer was administered to the NDM rats. Blood samples were obtained from the tail vein of the rats (after 72 h) and the measurement of glucose was done using a glucometer[§§]. DM was confirmed when glucose levels reached values above 300 mg/dL.

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[‡‡] Sigma-Aldrich, St Louis, MO, USA.
[§§] Accu-Check Active®, Roche Diagnóstica Brasil, São Paulo, SP, Brazil.

Following acclimatization, the experimental protocol was initiated. A stock solution of RSV[***] molecular weight: 228.2) was prepared in Tween-80[†††] and diluted further in water to obtain a working concentration to provide safe and convenient ingestion volume (1 ml) to dose the animals at a level of 10 mg/Kg of RSV [16-20]. The PLAC was prepared with the same volume of Tween-80 and water used in the preparation of RSV. Both solutions were offered daily via gavage. Insulin was administered daily by subcutaneous injection of neutral protamine Hagedorn[‡‡‡] diluted in 0.9% NaCl - 5.5 ml to 3.5 ml at 6:00 pm and at 6 am[20]. All treatments (RSV, PLAC and INS) were performed for 30 days. A period of 19 days previous to EP induction and then for another 11 days after that also is included.

Rat periodontitis model

EP was induced by the placement of a cotton ligature[§§§] knotted subgingivally at a cervical position of the first mandibular molar and a second maxillary molar in each animal. Contralateral teeth did not receive ligatures and were used as controls. Euthanasia by CO_2 inhalation was carried out 30 days after the initiation of the study. The mandibles were removed for morphometric analysis and the gingival tissues from the molars were collected for quantification of an array of biomarkers as described below. Gene expression analysis for Sirtuin 1 (SIRT), receptor activator of NF-KB ligand (RANKL) and osteoprotegerin (OPG) was evaluated in the gingival tissues surrounding the second maxillary molars using real-time PCR. Quantification of superoxide dismutase 1 (SOD1) and nicotinamide adenine dinucleotide phosphatase (NADPH) oxidase in the gingival tissues adjacent to the mandibular first molars was performed by ELISA as were interleukin (IL)-4, IL-1β, IL-6, interferon gamma (IFN-γ), IL-17 and tumor necrosis factor (TNF)-α. were determined with immune-enzymatic assay.

Linear measurement of alveolar bone loss

Given that loss of supporting alveolar bone about teeth affected by periodontitis is one of the most important hallmarks of this condition, bone loss was assessed using a linear measurement method, as described in detail previously [16-20]. Briefly, the mandibles were de-fleshed through immersion in sodium hypochlorite (8%) for 4 h and then were washed in running water and dried with compressed air. Methylene blue solution (1%) was used to discern the cemento-enamel junction (CEJ). Photographs were taken with a 6.1-megapixel

[***] R5010-500MG – Sigma-Aldrich Ltd, São Paulo, SP, Brazil.
[†††] P4780 – 100 ML, Sigma-Aldrich, São Paulo, São Paulo, SP, Brazil.
[‡‡‡] NPH insulin; Biohulin NU-100, 100 IU/ml.
[§§§] Coats Corrente no. 10, São Paulo, SP, Brazil.

digital camera[****] parallel to the ground. Linear alveolar bone loss was measured on the buccal surface of the lower first molars at three equally distant sites using image analysis software[††††16-20]. A single blinded examiner (AMM) performed morphometric measurements after intra-examiner calibration by evaluating 10 images not used for this study twice within 24 hours with an intra-class correlation of 95.7%.

Quantification of superoxide dismutase one (SOD1) and nicotinamide adenine dinucleotide phosphatase oxidase (NADPH) and Immune-enzymatic assay

The collected tissues were placed into sterile tubes containing 400µl phosphate buffered saline (PBS) with 0.05% Tween-20, as described previously [16-20]. Briefly, the samples were stored at -20 °C and then the tissue was weighed, cut into small pieces (1 mm^3 to 2 mm^3), and solubilized in PBS to a final concentration of 100 mg tissue/ml and then placed onto a vortex mixer for 10 min. After this, the levels of IL-4, IL-1β, IFN-γ, IL-17 and TNF-α were determined using the multiplex assay system (Luminex/MAGpix system[‡‡‡‡]) and commercially available kits[§§§§] for the various analytes. In all cases, the manufacturers' instructions were followed. The quantification of SOD1 and NADPH oxidase was performed using a rat ELISA kit[*****] (450 nm). Manufacturer's instructions were followed and the reactant mixtures were analyzed on a fluorescence plate reader (UV/Vis absorbance).

Gene expression analysis

The tissues destined for mRNA quantification for SIRT, RANKL and OPG using real time PCR were stored in RNAlater at −70 °C [19, 20]. Total RNA from the biopsies was isolated using the trizol method[†††††]. DNase was used to treat total RNA to eliminate contamination with DNA that would have altered data obtained for mRNA measurements using PCR[‡‡‡‡‡] , and then complementary DNA (cDNA) synthesis was performed. Specific primers were designed by the use of probe-design software[§§§§§]. The details of PCR products are shown in Table 1. The (real-time) qPCR reactions were performed in a real-time PCR device[******] with a Syber Green

[****] EOS 40D; Canon, New York, NY, USA.
[††††] Image-Pro; Media Cybernetics, Silver Spring, MD, USA.
[‡‡‡‡] MiraiBio, Alameda, CA, USA.
[§§§§] RCYTOMAG-80K - Millipore Corporation, Billerica, MA, USA
[*****] MBS2514900; MBS2602768 - Mybiosorce, San Diego, California; respectively.
[†††††] Gibco BRL, Life Technologies, Rockville, MD, USA.
[‡‡‡‡‡] Nanodrop 1000, Nanodrop Technologies LLC, Wilmington, NC, USA.
[§§§§§] Light-Cycler Roche probe design software, Diagnostics GmbH, Mannheim, Germany.
[******] LightCycler® 96 Instrument, Roche Diagnostics GmbH, Mannheim, Germany.

kit[††††††]. The results were expressed as relative amounts of the target gene using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the inner reference gene using a relative quantification tool. As the experiment progressed, expression of GAPDH was observed to be stable under the different test conditions confirming that it could be used to normalize mRNA expression data.

Statistical Analysis

The data were analyzed using a statistical software package[‡‡‡‡‡‡]. First, the data were examined for normality using the Shapiro-Wilk curve. Inter-group analysis was performed using analysis of variance (ANOVA) and Tukey's test was used for statistical analysis of alveolar bone loss, NADPH, SOD and mRNA levels. The paired Student's t-test was used for intra-group comparisons between ligated and unligated teeth.

The level of significance of was established for all analyses at 5%.

RESULTS

No differences in the weight of the animals in any of the groups were observed at the beginning of experiment ($p>0.05$). Diabetes and glycemic oscillations lead to loss of some animals during the experimental phase. At the end of the study there were 13, 14, 13, 14, and 15 animals in the DM+PLAC, DM+RSV, DM+INS, DM+RSV+INS, and Non-DM groups, respectively. In order for an animal to have been considered as having developed DM, serum glucose had to reach or exceed 300 mg/dL [40]. When insulin was administered to the appropriate group in the morning, normalization of serum glucose levels was observed within about 8 hours. Throughout the study, consistently elevated blood levels of glucose were demonstrated in the DM+PLA group.($p<0.05$, Table 2) whereas animals with DM that were also treated with INS, RSV or INS+RSV experienced a significant reduction in hyperglycemia at 30 days as compared to baseline ($p<0.05$, Table 2). Non-DM rats maintained normal glucose levels ($p>0.05$, Table 2) throughout the study period. At baseline, lower glucose levels were observed in Non-DM group compared to the diabetic groups ($p<0.05$, Table 2). At 30 days, significantly increased levels of glucose were demonstrated in animals with DM (PLAC group) when compared to the other groups ($p<0.05$, Table 2). In addition, as of 30 days, lower glucose levels were noted in animals in both the INS or RSV as monotherapy and in the non-NDM group when compared to the animals in the diabetic group ($p<0.05$, Table 2).

[††††††] FastStart DNA Masterplus Syber Green, Roche Diagnostic Co., Indianapolis, IN, USA.
[‡‡‡‡‡‡] Statistical Analysis System (SAS) software Program Release 9.3; Cary, NC, USA.

Morphometric results

Intragroup analysis revealed significant differences in alveolar bone loss between the sites that received ligatures and the sites that did not receive ligatures in all groups ($P < 0.05$), showing that periodontitis could be induced by use of the EP ligature model. Intergroup comparison revealed that significantly higher levels of bone loss were demonstrated when comparing alveolar bone loss in the DM+PLAC group vs. the other groups ($p<0.05$). In animals with DM treated with RSV (with or without INS), it was demonstrated that the levels of bone loss were identical to those observed in the control non-diabetic animals ($p>0.05$). The inter-group analysis of unligated sites showed no significant differences between groups ($p>0.05$). The morphometric results are represented in Figure 1.

Gingival tissue inflammatory markers levels

Assessment of markers of the immuno-inflammatory response in the various groups demonstrated lower levels of IL-1β in the DM+RSV+INS group as compared to the DM+PL, DM+INS e DM+RSV groups ($p<0.05$). Additionally, lower levels of IL-6 were found in the DM+RSV+INS group as compared to the findings obtained in the DM+PL group (similar to control/NDM) ($p<0.05$). As regards the levels of IFN-γ and IL-17, these were reduced in the DM+RSV+INS group compared to DM+PL group ($p<0.05$). There were no differences in the levels of IL-4 and TNF-α ($p>0.05$) amongst any of the groups. Data pertaining to the assessment of markers of the immune-inflammatory response are shown in Figure 2.

Gene expression levels in ligated sites

Evaluation of intra-group gene expression for ligated sites indicated that in the DM+PLAC and NDM groups higher gene expression for RANKL was found ($p<0.05$, table 3).

Lower levels of mRNA for OPG were observed in the DM+PLAC group compared to the DM+INS, DM+RSV and DM+RSV+INS groups ($p<0.05$, table 3). Additionally, lower levels of mRNA for SIRT were found in the DM+PLAC group as compared to all other treatment groups ($p<0.05$, table 3).

There were no intra and intergroup differences found for these biomolecules in non-ligated sites ($p>0.05$, table 3).

Gingival tissue SOD1 and NADPH oxidase levels

Lower levels of SOD were observed in DM+PLAC and DM+INS groups, when compared to DM+RSV+INS, RSV and NDM groups in ligated teeth ($p<0.05$, Figure 3A). Higher levels of SOD were found

in the NDM group as compared to the other groups in unligated teeth ($p<0.05$, Figure 3A). Intragroup comparison revealed statistically significant differences between the ligated and unligated sites in the NDM group ($p<0.05$, Figure 3A).

As regards the levels of NADPH oxidase, intergroup evaluation showed lower levels of this protein in the DM+RSV group compared to DM+RSV+INS and DM+INS groups at the ligated sites ($p<0.05$, Figure 3B). No significant difference between the groups ($p>0.05$, Figure 3B) was noted for non-ligated (i.e. EP) sites. Intragroup analysis revealed higher levels of NADPH oxidase at the ligated sites, when compared to the non-ligated sites in animals from the DM+PLAC group ($p<0.05$, Figure 3B).

DISCUSSION

DM and periodontitis are inflammatory diseases and accordingly there are several parallels insofar as the underlying pathophysiological mechanisms are concerned. For instance, both can be associated with similar immune-inflammatory functional disturbances. Moreover, purely on the basis of statistical relationships, it has been demonstrated clearly that the prevalence and severity of periodontitis are greater in patients with DM. Furthermore in patients with DM, treatment of periodontitis is also less effective in comparison to patients who do not have DM. As alluded to above and along similar lines it is also recognized that patients who have periodontitis often have more severe DM (along with increased prevalence of DM).

Resveratrol is a natural product that has several beneficial effects on host response modulation during periodontitis progression with or without DM. It has also been demonstrated to provide benefits for patients suffering from DM [28, 29]. This underscores why this study focused on the evaluation of the effects of RSV on the progression of EP and in the release of osteo-immuno-inflammatory and oxidative stress markers during EP in rats with DM. Reduced levels of alveolar bone loss, reduced levels of IL-1β, IL-6, IFN-γ, IL-17 and higher levels of SOD levels when either or both RSV and insulin treatment (DM+RSV+INS) was provided were demonstrated. Co-treatment with RSV and insulin led to upregulation of the mRNA levels for OPG. The levels of mRNA for SIRT were upregulated by all treatments while sole treatment with RSV induced reductions in the levels of NADPH oxidase, which was not seen in other treatment groups.

The morphometric findings of this study showed that association of insulin and RSV treatment (DM+RSV+INS) caused reductions in the loss of alveolar bone vs. placebo and was probably the result of reduced levels of interleukins among other things. Immunoenzymatic analyses revealed lower levels of pro-inflammatory cytokines (IL-1β, IL-6, IFN-γ and IL-17) in association with insulin and RSV treatment

(DM+RSV+INS) further contributing to reduced loss of bone. The reduction in bone loss mediated by RSV agrees with other studies [16-19, 30] where reduced levels of IL-17, IFN-γ, IL-4 and TH17/TH2 response with RSV treatment have been shown [16, 17, 19].

Additionally, the reduced bone loss mediated by RSV through reduction of pro-inflammatory cytokines agrees with findings reported in another study[26] that demonstrated reductions in levels of IL-1β, IL-6, IL-8, TNF-α, and TLR4 in gingival tissues of diabetic mice treated with RSV. These cytokines are important inflammatory markers but they're not merely 'markers'. They play an important functional role in the progression of the destruction of the periodontium as well as the development of the other long-term complications of DM. In fact it has been suggested in a consensus statement released by the European Federation of Periodontology and the International Diabetes Federation that high levels of IL- 1β, TNF- α, and IL- 6 are likely to be considered as playing an important role in the mechanisms that ostensibly link periodontitis and diabetes [6].

We also noted that all treatments promoted reductions in blood glucose levels. In fact, combined treatment with RSV and insulin promoted the development of glucose levels similar to those in the NDM group. Alternatively, if either RSV or insulin were administered individually, the DM groups still had higher levels of glucose compared to animals in the NDM group. Our results are in accordance with the results of several animal studies that show beneficial effects of RSV on reduction of blood glucose levels [41,42], particularly since it is known that hyperglycemic status enhances and sustains the levels of cytokines such as IL-6 and TNF-α [43, 44]. It is noteworthy that although significant reduction of glycemic levels were seen in comparison to baseline (0 vs 30 days) and also in comparison to placebo control (DM+PLAC) at 30 days, glycemic levels in DM+INS animals were still significantly different than those found in animals with NDM. These findings could explain, at least in part, why metabolic regulation of diabetes, even with insulin treatment, can be difficult and is not always predictable. Thus, the reduction of hyperglycemia to levels of normality caused by both insulin and RSV could have potentiated the modulatory effect of RSV on inflammatory factors and might be a clinically significant finding in regard to therapeutics for either or both diseases.

We also demonstrated that treatment with RSV exerted positive effects on the levels of OPG, regardless of the presence of insulin. These results could contribute positively to the modulation of EP in rats with DM possibly favoring osteoblastogenesis even in the face of ongoing periodontal disease. Other investigations carried out *in vitro* demonstrated that treatment with RSV upregulated OPG; consistent with a good environment for osteoblastogenesis as noted above [45]. All treatments upregulated mRNA levels for SIRT,

which is related to antioxidant activity and could be beneficial in relation to DM. The protein SIRT is related to biogenesis in the mitochondria and for cellular activities including stress resistance, gene expression, metabolism and apoptosis [46, 47] and is associated to reductions in factors that lead to osteoclastogenesis such as of TH-17 cytokines [48].

Is it important to discuss that in the current study we used molecular techniques for assessment of mRNA and ELISA for protein levels to elucidate further the impact of RSV on expression or production of osteo-immunoinflammatory and oxidative stress mechanisms in diabetic rats with EP. Not all biomarkers could be analyzed by the use of immune-enzymatic assays but it was possibly to assess these other markers by the use of assays for gene expression. Although the correlation between mRNA levels and the corresponding protein levels has been debated in the literature for many years, with some contradictory findings [49-51], we believe that all analyses performed in the present study provide additional information concerning the objectives proposed by the investigation and that mRNA changes reported here are in all likelihood consistent with expected changes in the proteins for which the mRNA encodes. This conclusion is corroborated by the actual biological and clinical outcomes reported this study.

SOD is an important antioxidant enzyme responsible for the catalysis of superoxide, an oxygen radical released during inflammation. Some studies showed reduced levels of SOD in patients with DM which could play an important role in the development of oxidative stress seen in these patients [52, 53]. Similarly this could also play a role in the reduction of hyperglycemia as well as other deleterious pathobiological phenomena such as glucose autoxidation, non-enzymatic glycation of proteins and activation of protein kinase C, leading to even more overproduction of oxidants like superoxide, hydroxyl radicals and hydrogen peroxide. SOD functionality might also be reduced in patients with DM because this protein, as with others, can become glycated in the presence of hyperglycemia. Our results also show reduction in antioxidant capacity in the presence of DM through the reduction in SOD levels at the unligated sites of all diabetic groups compared to the non-diabetic animals. Furthermore, in the present study, higher levels of SOD were found in animals treated with RSV; whether or not insulin was also used. Therefore, it might be postulated that RSV reduced the production of ROS due to direct action of RSV on the levels of SOD [36, 37] and it could also be related to the upregulation of the transcription factor, Nrf2 (nuclear factor erythroid 2-related factor 2), which regulates the expression of the gene for SOD. Interestingly, treatment with insulin alone did not increase the levels of expression for SOD. Perhaps

then, although insulin effectively reduces hyperglycemia, reductions in hyperglycemia alone are not sufficient to produce protective increases in antioxidant activity in animals suffering from both DM and periodontitis.

It was intriguing to note that treatment with RSV alone caused reductions in levels of NADPH oxidase, an enzymatic complex upon which the first ROS response is reliant [27] NADPH oxidase is composed of subunits called NOX (NOX 1-5), which are significantly increased during EP [54]. As well, increased levels of NADPH oxidase have been described in the presence of DM and this too would lead to overproduction of ROS [55]. The results of this investigation corroborate the results of other studies, showing that RSV has a regulatory effect on the expression of NADPH oxidase [31]. Alternatively and as alluded to above, when insulin was present, regardless of the presence of RSV, the levels of NADPH oxidase were not reduced. One explanation for this result might be the requirement of some level of ROS to induce an effective insulin signaling cascade. After all, constitutive production of ROS is likely important for maintenance of normal metabolism. It is known that with respect to the insulin signaling cascade, insulin-stimulated tyrosine kinase and NADPH oxidase activation lead to generation of an oxidized environment that is localized along the plasma membrane and this environment inhibits phosphatase activity and facilitates insulin signaling [56]. When the levels of insulin decline, NADPH oxidase activity is reduced and the antioxidant systems act, restoring phosphatase activity and changing the insulin signaling cascade at a basal state. Paradoxically, some evidence suggests that insulin resistance is caused by elevated levels of ROS [57, 58]. Thus, it may be suggested that insulin treatment elevates the levels of NADPH oxidase in the beginning of the insulin cascade while RSV could not decrease the levels of this complex in the presence of insulin.

CONCLUSION

Within the limits of this study, it can be concluded that the concomitant use of RSV and insulin reduced periodontal breakdown and led to decreased levels of markers for osteo-immuno-inflammation as well as markers of oxidative stress in rats with both EP and DM. DM might reduce anti-oxidant capacity by causing a reduction in SOD and SIRT levels. The data also demonstrated that treatment with RSV and INS alone or in combination had beneficial effects on bone loss.

This study suggests then, that a combination of both RSV and insulin treatment for DM could be more beneficial than treatment with insulin alone, or indeed either agent alone possibly for both DM as well as EP. Clinical application of these findings could also help to yield better outcomes of treatment for periodontitis in patients with DM.

CONFLICT OF INTEREST STATEMENT

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

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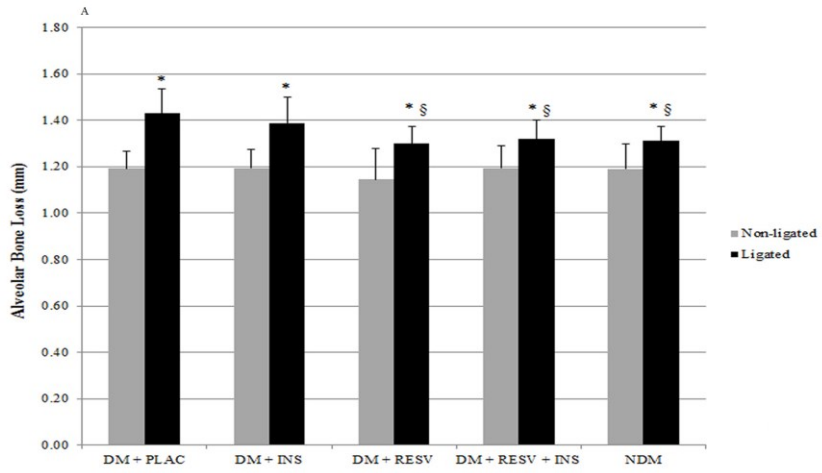
FIGURES

Figure 1. A-Means and standard deviation of linear alveolar bone loss (mm) observed on molars for ligated and non-ligated teeth. *Represents significant intra-group difference (Student T Test; $p<0.05$). § Represents significant inter-group difference when compared to DM+PLAC group (ANOVA/Tukey; $p<0.05$). **B–K)** Representative photographs illustrating the morphometric findings of the DM+PLAC (B: ligated and C: unligated teeth), DM+INS (D: ligated and E: unligated teeth), DM+RSV (F: ligated and G unligated teeth), DM+RSV+INS (H: ligated and I: unligated teeth), NDM (J: ligated and K: unligated teeth).



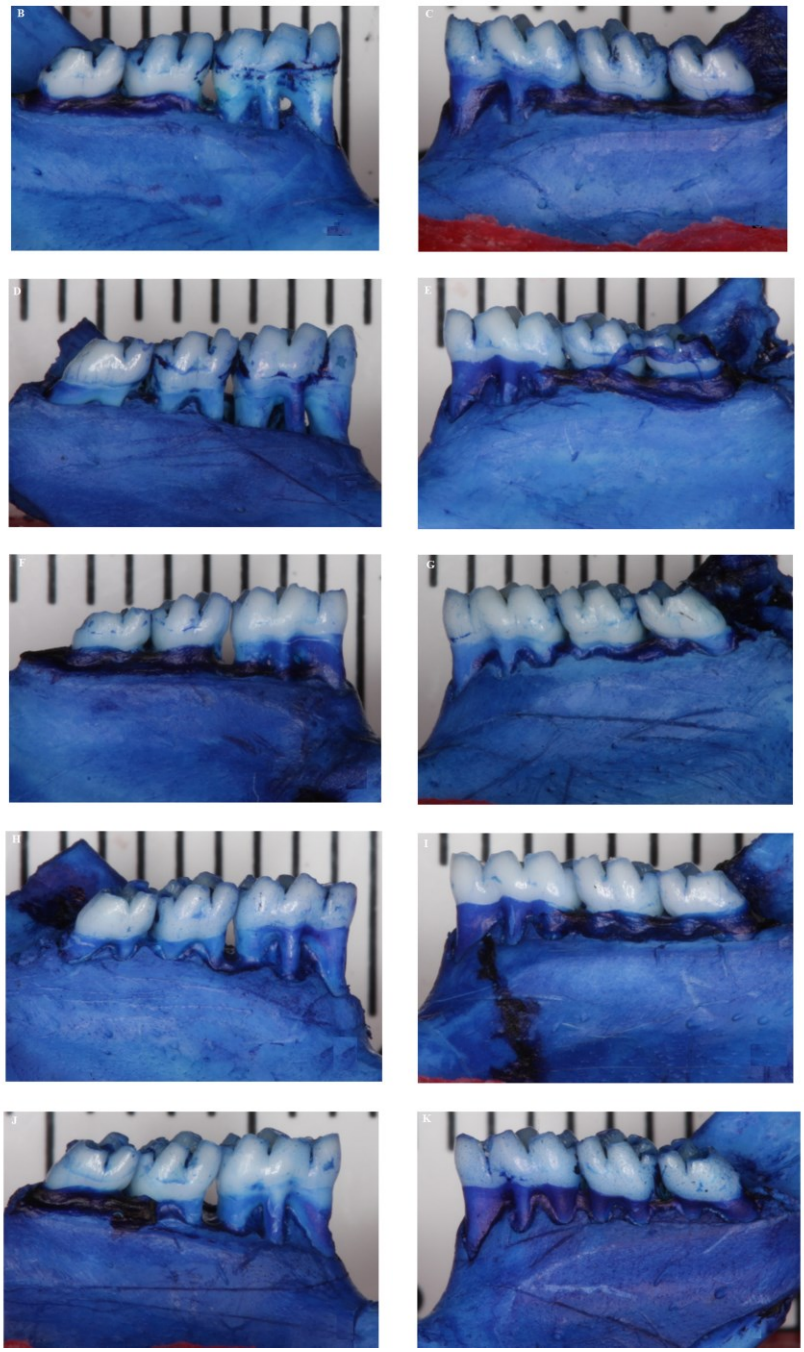


FIGURE 2. Means and standard deviation of immune-inflammatory mediators concentrations (pg/ml) measured by Luminex/MAGpix assay.

A – IL4

No significant inter and intra group differences were observed from Kruskal-Wallis/Dunn, $p > 0.05$.

B - IL-1β

* Represents statistical intra-group difference (Paired Student's t test, $p<0.05$).

† Represents inter group difference when compared to DM+PLAC, DM+INS, DM+RSV for ligated sites (Kruskal-Wallis/Dunn; $p<0.05$).

‡ Represents statistical inter-group difference when compared to all groups for non-ligated sites (Kruskal Wallis test, $p<0.05$).

C - IL-6

* Represents statistical intra-group difference (Paired Student's t test, $p<0.05$).

† Represents inter group difference when compared to DM+PLAC, DM+INS, DM+RSV for ligated sites (Kruskal-Wallis/Dunn; $p<0.05$).

‡ Represents inter group difference when compared to DM+PLAC, DM+INS, DM+RSV for non-ligated sites (Kruskal-Wallis/Dunn; $p<0.05$).

D - IFN-γ

† Represents inter group difference when compared to DM+PLAC for ligated sites (Kruskal-Wallis/Dunn; $p<0.05$).

‡ Represents inter group difference when compared to DM+PLAC for non-ligated sites (Kruskal-Wallis/Dunn; $p<0.05$).

E - IL-17

† Represents inter group difference when compared to DM+PLAC for ligated sites (Kruskal-Wallis/Dunn; $p<0.05$).

F - No significant inter and intra group differences were observed from Kruskal-Wallis/Dunn, $p > 0.05$.

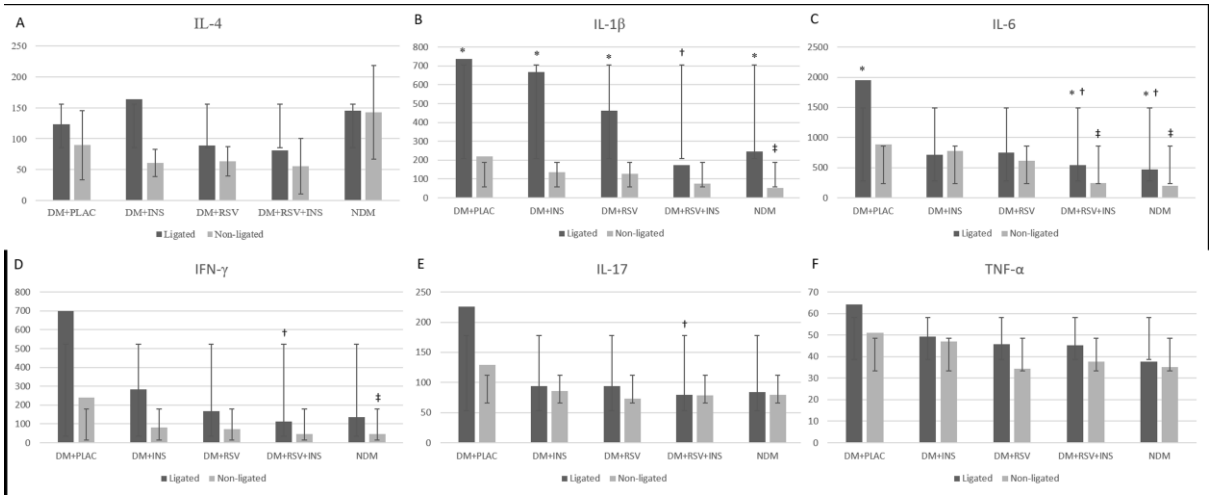


FIGURE 3. Means ± SD of superoxide dismutase one (SOD1 - A) gingival levels and nicotinamide adenine dinucleotide phosphatase oxidase (NADPH oxidase - B) (U/ml) measured by ELISA assay.

A

* Represents statistical intra-group difference (Paired Student's t test, $p<0.05$).

† Represents statistical inter-group difference when compared to the other groups, at unligated side (Kruskal Wallis test, $p<0.05$).

‡ Represents statistical inter-group difference when compared to DM+RSV, DM+RSV+INS and NDM, at ligated side (Kruskal Wallis test, $p<0.05$).

§ Represents statistical inter-group difference when compared to DM+RSV, DM+RSV+INS and NDM, at ligated side (Kruskal Wallis test, $p<0.05$).

B

* Represents statistical intra-group difference (Paired Student's t test, $p<0.05$).

† Represents statistical inter-group difference when compared to the DM+INS and DM+RSV+INS, at ligated side (Kruskal Wallis test, $p<0.05$).

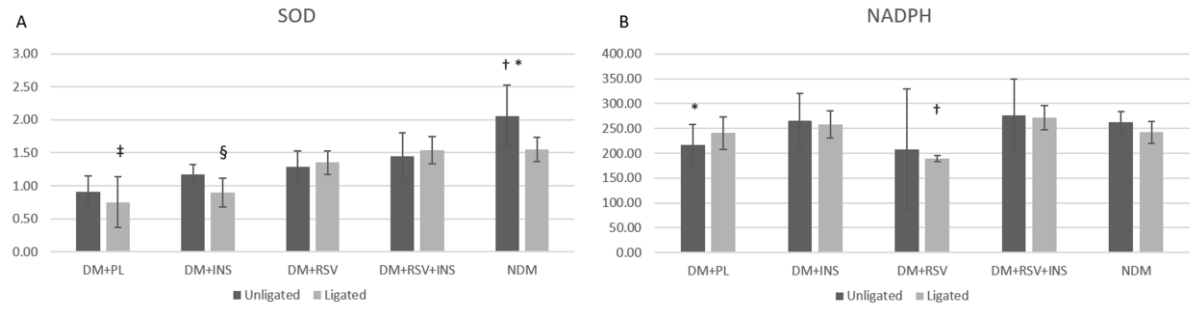


Table 1. Primer sequences for each gene, amplification profiles and the estimated length of qPCR product for each gene.

Gene	Sequence (5´–3´)	Length of qPCR product (bp)	Amplification profile [temperature (^{o}C)/time (s)]
RANKL	AGCGCTTCTCAGGAGTT	156	95/5, 55/4, 72/6
OPG	GCAGAGAAGCACCTAGC	168	95/10, 56/8, 72/7
SIRT	GGTCAGGCTGGTTTACAACG	68	95/10, 61/8, 72/10
GAPDH	TGAGTATGTCGTGGAGTCTACTG	159	95/10, 56/8, 72/7

Receptor activator of the NF-KB ligand (RANKL), osteoprotegerin (OPG), Dickkopf 1 (Dkk1), Runt-Related Transcription Factor 2 (Runx2), Sclerostin (Sost), Sirtuin (SIRT) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Table 2. Glucose levels (mg/dL) measured before the start of the treatments (Day 0) and after 30 days (Euthanasia) of the experiment in all experimental groups.

Day	DM+PLAC	DM+INS	DM+RSV	DM+RSV+INS	Non-DM
Day 0	410.27±6 7.78	404.47±5 0.58	426.93±7 2.05	442.27±7 6.22	143.20± 33.43†
Day 30	445.92±7 6.39*†	286.77±5 9.33*‡	282.07±7 7.92*‡	220.57±1 09.25*	146.80± 35.84

* Intra-group differences statistically significant when compared to the baseline ($p < 0.05$; ANOVA two way /Tukey).

† Inter-group difference statistically significant when compared to the other groups ($p < 0.05$; ANOVA two way/Tukey).

‡ Inter-group difference statistically significant when compared to Non-DM group ($p < 0.05$; ANOVA two way /Tukey).

Table 3. Mean ± (SD) of relative levels of mRNA for all genes (mRNA gene/mRNA GAPDH).

Group	*Gene/GAPDH*		
	RANKL (x10^3)	**OPG**	**SIRT**
DM+PLAC			
Ligated	49.616±28.662 □	1.098±1.646 †	4.590±5.712 ‡□
Non-ligated	14.127±18.556	5.752±10.513	14.173±9.115
DM+INS			
Ligated	43.079±29.556	5.953±5.128	15.617±14.405
Non-ligated	28.445±23.910	4.224±2.955	22.769±18.292
DM+RSV			
Ligated	42.653±24.813	6.143±4.552	28.127±27.094
Non-ligated	23.607±41.496	5.264±5.994	28.839±26.652
DM+RSV+INS			
Ligated	25.759±24.813	7.366±5.287	23.178±20.301
Non-ligated	25.809±22.212	6.159±4.678	28.274±24.652
NDM			
Ligated	17.613±26.101 □	3.124±4.640	31.142±26.488
Non-ligated	3.501± 2.641	6.369±7.879	39.159±37.824

* Intra-group differences statistically significant (Test T de Student; $p<0.05$).

† Inter-group difference statistically significant when compared to DM+INS, DM+RESV and DM+RESV+INS (Kruskal-Wallis/Dunn; $p<0.05$).

‡ Inter-group difference statistically significant when compared to DM+RESV, DM+RESV+INS and NDM (Kruskal-Wallis/Dunn; $p<0.05$).