Title: Melatonin as adjunctive therapy in the treatment of periodontitis associated with obesity. An *in-vivo* experimental study.

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The authors declare no conflict of interest.

ABSTRACT

Aims:

To study the effect of adjunctive systemic administration of melatonin to standard mechanical cause-related periodontal therapy in obese animal.

Materials and methods:

Forty-two male Wistar rats were randomly divided into six groups: 1) control group (C-Perio), 2) control group with adjunctive chlorhexidine (C-Perio-CHX), 3) Control group with adjunctive melatonin (C-Perio-Mel),4) Obese group (HFD-Perio), 5) Obese group with adjunctive chlorhexidine (HFD-Perio-CHX), 6) control group with adjunctive melatonin (HFD-Perio-Mel). The animals were subjected to a standard mechanical periodontal treatment follow by chlorhexidine or melatonin. Periodontal outcomes were evaluated by inflammatory parameters, micro-tomography, periodontal probing depth (PD), modified gingival index (MGI), plaque dental index (PLI) and bleeding on probing (BOP). Systemic effects were evaluated by measuring levels of inflammatory cytokines, insulin, leptin, osteocalcin (OC), osteopontin (OPN), plasminogen activator inhibitor-1 (PAI-1), intercellular adhesion molecule 1 (sI-CAM-1), E-selectin and lipid profile.

Results:

Clinical parameters and alveolar bone healing were ameliorating with both treatments, even with betters results to melatonin groups. Cytokines, lipid profiles, insulin, leptin, OPN, PAI-1, sI-Cam and E-selectin were significantly reduced in animals treated with melatonin, especially in HFD-Perio-mel group.

Conclusions:

Adjunctive melatonin therapy significantly reduced alveolar bone loss and exerted a protective effect against the recognized co-morbidities of periodontitis and obesity.

CLINICAL RELEVANCE

Scientific rationale for study

A demonstrated comorbidity effect of periodontitis and obesity has been described suggesting a reduction of circulating melatonin as a possible connection between these two pathologies. We investigated the effect of systemic adjunctive melatonin treatment of periodontal and metabolic biomarkers.

Principal findings

Melatonin adjunctive treatment reduced alveolar bone loss and control the systemic dysregulation, mainly in the obese animals.

Practical implications

Our findings established a new perspective to the treatment of periodontitis in obese subjects, not only improving periodontal parameters but also controlling inflammation, vascular injury and lipid metabolism, all of them implicated in a worsening of oral health.

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Introduction

The association between periodontitis and obesity has been demonstrated both in experimental(Perlstein and Bissada, 1977) and epidemiological studies(Keller et al., 2015). In fact, overweight and obese subjects are 2.3 times more likely to suffer from periodontitis when compared to normal-weight individuals (Suvan et al., 2011). Results from a systematic review evaluating 19 studies assessing these associations have reported statistically significant associations between periodontitis and obesity (OR 1.81 (1.42, 2.30)), and overweight (OR 1.27 (1.06, 1.51)) and obese and overweight combined (OR of 2.13 (1.40, 3.26) (Suvan et al., 2011).

To explain these epidemiological associations, apart from the well-established sharing of common risk factors, a co-morbidity effect has been attributed based on the chronic inflammatory nature of both diseases, what results in increased systemic inflammation, metabolic dysregulation and dyslipidemia (Virto et al., 2017). In spite of these common inflammatory-mediated mechanisms, however, the specific underlying biological pathways remain partly unknown. Some authors have also suggested the possible influence of alterations in the circadian cycle, since significant reductions in the melatonin hormone levels have been reported in experimental studies in both, obesity and periodontitis (Virto 2) (Shimizu et al.). In fact, when melatonin has been administered in obese subjects, significant reductions in body weight and adipose tissue deposits have been reported, together with diminished release of pro-inflammatory biomarkers (Favero et al., 2015, Rios-Lugo et al., 2010). Similarly, adjunctive topical application of melatonin together with standard periodontal treatments have resulted in significant reductions in gingival inflammation (Arabacı et al., 2015, Cutando et al., 2014).

Despite these reported beneficial effects, there is no evidence of the potential benefit of melatonin when is used as adjunctive therapy in the treatment of periodontitis associated with obesity.

It was, therefore, the main objective of this experimental *in-vivo* investigation to study the efficacy of adjunctive systemic administration of melatonin to standard mechanical cause-related periodontal therapy compared with the same mechanical debridement plus the gold standard antimicrobial agent, chlorhexidine (CHX).

Materials and methods

Animals and Experimental Design

This investigation was carried out in the Experimental Animal Center at the University Complutense of Madrid (UCM), Madrid, Spain, once the Institutional UCM Animal Care Committee and the Regional Authorities approved the study protocol and the proposed animal care and welfare measures.

This pre-clinical *in vivo* investigation used 42 two-month old Wistar rats with an initial body weight of 180 g. and was conducted according to the Spanish and European Union regulations (European Communities Council Directive 86/609/EEC) (Assessment et al., 1996) and following the ARRIVE guidelines (Kilkenny et al., 2010).

The experimental animals were maintained during the study under standard housing conditions, with controlled light (12-hour light/12-hour dark schedule; lights on at 08:00 hours) and temperature (22°C ± 2°C). They were weighed once a week and any procedures involving any potential pain were made under anesthesia using a combination of ketamine (0.08 mL/100 g) and xylacine (0.04 mL/100 g) under appropriate concentrations (Brandelero et al., 2012).

Induction of obesity and periodontitis

Obesity was induced by feeding the experimental animals *ad libitum* with a high-fat diet (HFD) (TD03307, Envigo, Castellar del Vallès, Barcelona, Spain) containing 35.2% fat, 35.5% carbohydrates, 20.4% protein, and 8.9% vitamins and minerals in 100 g of chow. Obesity was diagnosed when the rats using this HFD weighted 25% more than the rats fed with the standard diet (Envigo, Castellar del Vallès, Barcelona, Spain) containing 3% fat, 60% carbohydrates, 16% protein, and 21% vitamins and minerals in 100 g of chow. Obesity induction was usually achieved after 2 months with this HFD.

Experimental periodontitis was induced using a modification of the method described by Polak et al (Polak et al., 2009). In brief, two bacterial pathogens, *Porphyromonas gingivalis* strain ATCC W83K1 and *Fusobacterium nucleatum* strain DMSZ 20482 were grown in brain heart infusion broth (Becton, Dickinson and Company, Franklin Lakes, NJ) under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37°C. When achieving a pre-established density (10^9 cells/mL, adjusted by spectrophotometry at 550 nm) bacteria were separated from the culture media by centrifugation (10 minutes at 4,000 rpm), were re-suspended in sterile 2% carboxymethyl cellulose (Sigma, St. Louis, MO) and vortexed (200 to 300 rpm in continuous mode for 1 to 2 minutes). One milliliter of this bacterial suspension was inserted in a sterile syringe and administered to the experimental animals without anesthesia through oral gavages for 4 consecutive days during 12 weeks (Virto et al., 2017). The bacterial culture growth phase, suspension media, infection doses, and induction procedures were all standardized throughout the study.

Experimental design

This investigation was designed as a parallel-arm in-vivo experimental study using a randomized block, examiner blind design. The sample consisted on 21 non-obese rats

with induced periodontitis (control group (C-Perio)) and 21 obese rats with induced periodontitis (experimental group (HFD-Perio)).

These groups were each randomly assigned to the following interventions:

a) no treatment (n=7)

b) experimental treatment consisting on standard mechanical debridement plus adjunctive melatonin (n=7)

c) control treatment consisting on standard mechanical debridement plus adjunctive chlorhexidine (n=7)

This design resulted in 6 groups (C-Perio, C-Perio-CHX, C-Perio-Mel, HFD-Perio, HFD-Perio-CHX and HFD-Perio-Mel)

The standard mechanical periodontal treatment consisted in scaling the roots of the molars under the gingival margin using a mini-five curette (Micro Mini Five® Gracey Curette. Huffredy) by an experienced periodontist under direct lighting and magnification (JG).

The experimental adjunctive treatment consisted on the administration of 25µg/mL of melatonin in ethanol dissolved in the drinking water for four weeks. The resulting daily melatonin dosage was approximately 2.3 mg/kg (equivalent to 25-30 mg/day for a 75-kg human adult). To discard the possible therapeutic effect of the alcohol, 0.015% of ethanol was also added to the water drinking solution in the rest of the treatment groups (Cano Barquilla et al., 2014).

The control adjuntive treatment consisted on topical application of 0.5 ml of a solution containing chlorhexidine (0.12%) and cetyl-peridinium-chloride (CPC) (0.05%), throught a disponsable syringe twice daily, during 8 days (Ribeiro et al., 2004).

Figure 1 depicts the flow chart of this experimental study, depicting the two induction periods (24 weeks) and the treatment period (8 days or 4 weeks).

Clinical outcomes were recorded at week 11 (start of the experimental periodontitis induction), at week 24 (end of the experimental periodontitis induction) and at week 26 or 29, depending of the treatment. In this study, baseline is considered week 24 and post-therapy the end of the respective therapies.

The following parameters were used to evaluate the clinical status of the periodontal tissues:

- Plaque accumulation

Mandibular molars were smeared with 2% basic fuchsine for 30 seconds and then washed. Stained purple surfaces were photographed (Liu et al., 2012) and then measured using a dedicated image software (Image Tool3) (Cavagni et al., 2013).

- Gingival index (MGI)

The Modified Gingival Index (MGI) by Lobene et al. (1986) (Lobene et al., 1986) was used using the following visual criteria under magnification and appropriate lighting, ((0) absence of any inflammation, presence of mild (1,2), moderate (3) and severe inflammation (4)) (Lobene et al., 1986).

- Bleeding on probing (BOP)

Ten seconds after light periodontal probing of the first molar. “1” was assigned in presence of bleeding a “0” in absence (Lang et al., 1986).

- Probing pocket depth (PD)

PD was measured from the gingival margin to the bottom of the pocket using a round-ended periodontal probe with a tip of 0.4 mm in diameter (Hu- Friedy Mfg. o, LLC, Chicago) under light pressure. Previous studies in the same experimental animals have

defined probing depths of ≤ 0.30 mm as gingival health (Liu et al., 2012) and periodontitis when probing depths were ≥ 0.5mm (Björnsson et al., 2003, Simch et al., 2008).

At week 29 the animals were sacrificed by decapitation in the morning (at 09:00h) under conditions of minimal stress. Trunk blood was collected in polystyrene EDTA tubes (VacutainerTM, Becton Dickinson, San Agustín de Guadalix, Madrid, Spain) and once centrifuged at 1500g for 15 min, plasma samples were stored at -80ºC. The biochemical analysis consisted on the evaluation of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, and MCP-1), osteocalcin (OC), osteopontin (OPN), leptin, insulin, plasminogen activator inhibitor-1 (PAI-1), intercellular adhesion molecule 1 (sI-CAM-1) and E-selectin by means of high-sensitivity multiplex map rat immunoassays (Millipore corporation, Billerica, MA, USA) using a platform flow cytometry analyzer (Luminex-200 System, Luminex Corporation, Oosterhout, the Netherlands). Results were measured using the dedicated software (xPonent software, Luminex Corporation, Oosterbout, The Netherlands) and were expressed as picograms or nanograms per milliliter. Plasma free fatty acids (FFA), high density lipoprotein (HDL), low density lipoprotein (LDL) and total cholesterol (TC) were also determined using colorimetric kits (BioVision Incorporated, Milpitas, USA). Triglycerides were analyzed in a drop of blood using a hand-held device, just after the animal sacrifice (Accutrend® Plus system, Roche Diagnostics, Mannheim, Germany).

Gingival specimens were dissected from the upper jaws and immediately frozen at -80ºC. These gingival specimens (10 mg) were homogenized using a high-speed benchtop homogenizer (FastPrep, 24^{TM} 5G, MP Biomedicals, USA) in 1 mL of phosphate buffer

with protease inhibitor and < 2% triton. Pro-inflammatory cytokines IL-1β, IL-6, TNF-α, and MCP-1 were measured using a flow cytometry analyzer as are described below.

Left hemi-mandibles were carefully removed and fixed in 4% formalin solution (Applichem Panreac; Barcelona, Spain). These specimens were studied by micro-computed tomography (micro-CT) using a high-resolution micro-CT system (Skyscan 1172, Bruker microCT, Kontich, Belgium) with a 4.96 µm voxel resolution and a source voltage of 100 kV and 100 µA plus an aluminum and copper filter of 0.5mm to optimize the contrast. The resulting teeth were rotated 360° around their long axes and four absorption images were recorded every 0.400° of rotation. These raw images were then reconstructed through 3D cone beam reconstruction algorithms using the standard SkyScan® reconstruction software (NRecon 1.6.10, Bruker microCT, Kontich, Belgium) to coronally-oriented serial tomograms. For these reconstructions, beam hardening was set to 25 % and ring artefact reduction to 15. After the scanning, the teeth were aligned and measured in 3D with DataViewer (Bruker microCT, Kontich, Belgium). For evaluating the alveolar bone levels, the distance between the cemento-enamel junction and the alveolar bone crest [CEJ-AC] was measured five times at six different points (mesio-buccal (MB), buccal (B), disto-buccal (DB), disto-lingual (DL), lingual (L), and mesio-lingual (ML) by an independent blinded experienced investigator.

Statistical analysis

Data were expressed as means (S.E.M) and 95% confidence intervals (95% CI). For all evaluated parameters, the normality was tested by Shapiro–Wilk test and the appropriate statistical test was selected according to this assumption. A univariate analysis of variance and independent samples t- test or ANOVA and a post-hoc Tukey's multiple comparison

tests were employed to determine the differences between groups. All analyses were performed in BM SPSS Statistics 22.0; IBM Corporation, Armonk, NY, USA. The animal was the unit of analysis and the alpha level was set at 0.05.

Results

At week 24 the obesity and periodontitis induction periods were finalised with the HFD-Perio group resulting in a 58.68% increase in weight compared with the C-Perio group.

Table 1 depicts the baseline clinical periodontal parameters at week 24. In both groups the gingival margin presented color alterations, with evidence of edema and ulceration, but there were no significant differences between the HFD-Perio and the C-Perio groups in regards to plaque accummulation and bleeding on probing. Stadistically significant differences, however, were found in probing depths (0.93 mm (0.03) vs 0.70 mm (0.06)) and gingival inflammation (3.33(0.21) vs 2.83(0.16)) being the HFD-Perio group more periodontally affected.

Table 2 represents the effect of the tested interventions (melatonin and CHX treatments, respectively) on the clinical parameters between baseline and week-26 or week-29. Both intervention resulted in resolution of gingival inflammation and reduction of BOP. Moreover, stadistically significant reductions in PLI and PD were found when compared to the non-treated group. When comparing the tested interventions in the HFD-Perio group, adjunctive systemic melatonin always achieved better clinical outcomes than the postive control group (adjunctive CHX), although significant differences were only encountered for BOP reductions

Table 3 depicts the effect of both interventions on alveolar bone healing, measured by Micro-ct as the changes in the distance between the cemento-enamel-junction and the

alveolar crest (CEJ-AC). At baseline, the mean distance CEJ-AC was similar between the groups (C-Perio vs HFD-Perio). Enhanced bone healing (reduced CEJ-AC distance) occurred in the melatonin treatment groups (C-Perio-Mel and HFD-Perio-Mel), although these reductions were higher in the HFD-Perio-Mel (15 vs 3.19%). In the CHX treatment groups (C-Perio-CHX and HFD-Perio-CHX) only the HFD-Perio-CHX group showed increased bone healing. The comparisons between the treatment groups in the HFD-Perio rats did not demonstrate significant differences, although the magnitude of the change was higher in the melatonin treated group than in the CHX group (15% vs 11.36%, respectively)

In spite of the high fat diet, the use of systemic melatonin resulted in a decrease in body weight, what resulted in 12% less weight gain in this group (HFD-Perio-Mel).

Table 4 depicts the effect of the two adjunctive therapies on the levels of pro-inflammatory cytokines measured in gingival tissue. The animals treated with melatonin resulted in statistically significant reductions in all cytokines (IL-1β, IL-6, MCP-1 and TNF-α), while in those treated with CHX only MCP-1 and TNF-α where significantly reduced.

Table 5 reports the same effect on pro-inflammatory cytokines measured in plasma. Similar trends were found, but significantly greater reductions for IL-1β occurred in the melatonin treated group when compared with the CHX treated group.

The effect of both treatments on plasma biochemical biomarkers are depicted in Table 6. A relevant increase in osteocalcin plasma levels occurred in both treatment groups when compared with the baseline levels, although differences were not statistically significant. Moreover, both treatments reduced OPN, insulin and leptin plasma concentrations when

compared to non-treated animals, being the use of melatonin the most effective. In regards to the possible effect on wound healing, the administration of melatonin in HFD animals resulted in a significant ($p<0.05$) reduction of PAI-1, E-Selectin and sI-CAM when compared with the baseline levels. However, no significant effects in these biomarkers occurred in the animals treated with CHX (Con-Perio-CHX and HFD-Perio-CHX). Similarly, statistically significant ($p<0.05$) reductions occurred in TG, FFA and a significant increase ($p<0.05$) of HDL levels in the groups treated with melatonin when compared to non-treated animals. In contrast, the groups treated with CHX did not have a significant effect on these parameters. No significant changes in the levels of total cholesterol were noted with both treatments.

Discussion

The results from this investigation clearly demonstrate the efficacy of adjunctive melatonin administration in the treatment of obese rats with periodontitis. This intervention resulted in reduced gingival inflammation and BOP, with significant reductions in PD. When comparing with the control interventions significant differences were encountered for BOP reductions. These improved clinical outcomes were coupled with the enhanced bone healing demonstrated by Micro-CT mainly in obese rats with periodontitis treated with adjunctive melatonin (15%).

These reported significant improvements in clinical periodontal outcomes with the adjunctive use of melatonin have been previously reported in experimental studies using the same animal model (Virto et al., 2018). Arabaci, T et al (Arabacı et al., 2015) and Kara, A et al (Kara et al., 2013) used the ligature experimental model to induce periodontitis and after removing the ligatures, the animals were treated with an intraperitoneal dose of melatonin during 15 days resulting in reduced alveolar bone

resorption. In humans, Cutando et al (Cutando et al., 2014) reported significant reductions in clinical periodontal parameters (PD and MGI) in diabetic patients with chronic periodontitis treated with adjunctive melatonin. Similarly, Montero, J et al (Montero et al., 2017) applied melatonin topically (1% orabase cream formula) for 20 days in patients with diabetes and periodontitis resulting in significant improvements of clinical parameters (PD and MGI). In contrast, Balci Yuce H, et al (Balci Yuce et al., 2016) did not find a significant effect of using melatonin on periodontal outcome measurements, but they used melatonin as a preventive measure rather than as therapeutic.

This investigation has also reported positive results on the biochemical biomarkers studied in both plasma and gingival samples with the use of adjunctive melatonin in the obese rats with periodontitis. Significant reductions for IL-1β, IL-6, MCP-1 and TNF-α were found in the melatonin treated group in the gingival samples and significant reductions in IL-1βin the plasma samples. These findings are in a good agreement with Renn T Y, et al. (Renn et al., 2018) who reported a significant effect of melatonin in the reducing pro-inflammatory cytokines. Liu et al. (Liu et al., 2014) also demonstrated that melatonin compensated the elevated pro-inflammatory cytokines levels usually encountered in hypoxic rats.

In previous investigations from our research group we have reported that obese rats with periodontitis demonstrate a significant increase in inflammatory biomarkers (Virto et al., 2018) and a significant reduction of melatonin levels (new reference) what may explain the positive effect of the adjunctive administration of melatonin. Furthermore, obesity is no only considered a co-morbidity factor for periodontitis, but also the adipose tissue is an important source of the release of pro-inflammatory cytokines characterized in systemic inflammation ((Cavagni et al., 2013). In fact, this study has shown that the adjunctive melatonin treatment, resulted in higher reductions in the inflammatory

biomarkers in the obese rats with periodontitis, hence demonstrating a direct effect of this hormone on obesity, which was also demonstrated by the tendency to body weight reduction in the rats treated with melatonin in spite of the high fat diet.

The positive effects of the adjunctive administration of melatonin on wound healing and bone metabolism has been reported in previous investigations. Tachibana R, et al. (Tachibana et al., 2014) reported an increment of mRNA expression of OC and OPN on human dental papilla cells after 14 days of melatonin treatment and Zhang L, et al. (Zhang et al., 2010) described the same effect on human mesenchymal stem cells after 12 days of treatment. In contrast, Cutando A, et al. (Cutando et al., 2013) studying diabetic patients with periodontitis treated with melatonin demonstrated reduced levels of ostepontin, although the levels of osteocalcin rose after the melatonin therapy. The role of melatonin as a mediator in bone formation has been described in numerous studies, mainly since bone marrow cells of mice are capable of synthesizing melatonin (Conti et al., 2000). In very low concentrations, melatonin stimulates the proliferation human's osteoblasts *in vitro* (Nakade et al., 1999) and results in increased bone mineral density and trabecular structure in mice (Koyama et al., 2002).

In this investigation we also have measured the effect of adjunctive melatonin application on biomarkers related with carbohydrate and lipid metabolism, reporting reduced levels of FFA, TG, LDL, insulin and leptin and high levels of HDL when compared with baseline levels. Similar results have been described previous investigations. Bibak et al. (Bibak et al., 2014) reported improvements of hyperglycemia and triglyceridemia after melatonin administration and similarly, Rios-Lugo et al. (Rios-Lugo et al., 2010) reported a significant reduction in circulating insulin, leptin, triglycerides and cholesterol levels after the use of melatonin. Worthwhile mentioning that leptin is importance for osteoblastic cell growth and bone mineralization ?. (REF: Reseland, J. E., Syversen, U. ,

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Similarly, biomarkers associated with cardiovascular diseases (CVD) (PAI-1, E-Selectin and si-CAM plasma levels) (Chistiakov et al., 2016, Greco et al., 2015), were significantly reduced in the obese-periodontitis rats treated with melatonin (HFD-Perio-Mel). The effect of intensive periodontal treatments has also shown significant reductions in serum levels of E-selectin in periodontitis patients (Tonetti et al, 2007(Tonetti et al., 2007). Similarly, PAI-1, a highly pro-thrombotic marker of fibrinolysis(Akman et al., 2012), showed reduced levels in the animals treated with melatonin.

Among the possible regulatory effects justifying these effects, melatonin modulates the TLR4/NF-kB system with a demonstrated protective role in the development of the atherosclerotic lesion (Hu et al., 2013). During periodontitis and obesity a significant reduction of circulating melatonin levels has been demonstrated (virto 2), what is not yet clear whether it is the lack of melatonin what favors the development of obesity (Szewczyk-Golec et al., 2015) and periodontitis (Almughrabi et al., 2013, Gomez-Moreno et al., 2007) or the co-morbidity resulting from the co-existence of both diseases results in a reduction in the secretion of melatonin (Virto 2).

The results from this investigation, however, should not be directly translated to humans, since this experimental design has important shortcomings, such as the induction methods, the differences in the evaluation of clinical and biochemical outcome measurements and the differences in the administration of the treatments and dosages. In this investigation we have administered melatonin trough the drinking water with the aim of exerting the effect of the drug on multiple organs (Gulle et al., 2014). In this study we

used 2.3 mg/Kg of melatonin, but different doses have been described in the literature for the treatment of periodontitis and even large doses have been reported in humans without significant severe adverse effects (Andersen et al., 2016).

In conclusion and within the limits of this *in vivo* experimental study, adjunctive melatonin therapy significantly reduced alveolar bone loss and exerted a protective effect against the proven co-morbidities of periodontitis and obesity, such as inflammation, vascular injury and lipid metabolism. The mechanisms associated with this effect of melatonin are still not fully understood and further experimental and clinical studies are needed.

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