褪黑激素：透過調解下視丘–腦下垂體–腎上腺軸治療壓力加劇牙周組織破壞的潛力藥物

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摘 要

環境壓力的因素對於發炎性疾病（如牙周炎）的進展與嚴重程度，扮演著關鍵的角色，並且已被指出對牙周病治療結果有負面的影響。睡眠不足是其中一個壓力因子，它會調節已經建立的牙周病病理生理學的過程，並透過激活下視丘-垂體-腎上腺（HPA）軸來加速牙周組織的降解。由於褪黑激素可以與HPA 軸相互作用，因此可以逆轉睡眠障礙等壓力因素造成的影響，成為牙周炎的潛在治療方法。以改良的多平台法（MMPM）為模型，誘導患有或不患有實驗性牙周炎的四組雄性 Wistar 大鼠，進行 7 天的睡眠剝奪，其中一組在睡眠剝奪後，給予褪黑激素治療。而 HPA 軸對於大鼠應對壓力反應（睡眠剝奪）的影響將通過評估海馬迴和其他大腦區域的皮質酮和糖皮質激素受體（GR）的水平來驗證。慢性不可預測壓力模型和穿梭箱試驗將用於決定褪黑激素給藥是否可以減輕或逆轉大鼠受到壓力引起的行為缺陷。此外，會利用微型電腦斷層掃描及組織切片測量來分析齒槽骨破壞、發炎細胞浸潤至牙周組織、及牙周附連喪失的情形。另外也會收集被絲線綑綁住的臼齒周圍的黏膜牙齦組織，進行骨髓過氧化酶 （MPO）和 RT-PCR 分析，以確定發炎的病理機制，並評估特定發炎調控介質的基因表達，如 TNF-α （腫瘤壞死因子 α）、IL-1β（介白素-1 β）、COX2（環氧化酶）和 PGE2（前列腺素 E2）。透過免疫組織化學染色（IHC）和抗酒石酸酸性磷酸酶（TRAP）染色，可以發現被固定住的大臼齒的牙周組織會表現出多種發炎和成骨的調節因子：TNF-α（腫瘤壞死因子），IL-1（白血球介素因子 1），RANKL（破骨細胞分化因子）和 OPG（破骨細胞抑制因子）。 因此，我們可以假設：利用褪黑激素調解下視丘-腦下垂體-腎上腺軸（HPA axis）具有治療和壓力因素有關的牙周疾病的潛力。

關鍵詞：褪黑激素、壓力、HPA 軸、牙周病、皮質醇

Melatonin: A Potential Therapeutic Drug for Stress-aggravated Periodontal Destruction through the Hypothalamic-pituitary-adrenal Axis

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Abstract

Environmental stress factors play a pivotal role in the development and progression of inflammatory diseases, such as periodontitis, and have been suggested to have negative influence on the outcome of periodontal treatment. Poor sleep quality is one of such stress factors, which may modulate the pathophysiological processes of the already established periodontal inflammation and accelerate the degradation of periodontal tissues through activating the hypothalamic-pituitary-adrenal (HPA) axis. As melatonin could interact with HPA axis, it may reverse the effects caused by stress factors, such as sleep disturbance, and become

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a potential treatment for periodontitis. The modified multiple platform method (MMPM) will be used as a model to induce sleep deprivation to four groups of male Wistar rats with or without experimental periodontitis for seven days, and treatment with melatonin will be given to one group following sleep deprivation. The effect of the HPA axis on the rats in response to stress (sleep-deprivation) will be validated by evaluating corticosterone and glucocorticoid receptor (GR) levels in the hippocampus and other brain regions. Chronic unpredictable stress model and shuttle-box test will be used to determine whether melatonin administration can alleviate or reverse the stress-induced behavior deficit in rats. Moreover, alveolar bone destruction, inflammatory cell infiltrate in periodontal connective tissue, and attachment loss will be analyzed by microcomputer tomography and histometric measurements. The mucogingival tissues surrounding the ligatured molars will also be collected for myeloperoxidase (MPO) and RT-PCR analysis to determine inflammatory pathologies and to evaluate the gene expression of specific inflammatory mediators, such as Tumor Necrosis Factor-α (TNF-α), interleukin-1β (IL-1β), Cyclooxygenase-2 (COX2) and Prostaglandin E2 (PGE2). Differential expression profiles of the inflammatory and osteogenic mediators of periodontal tissue surrounding ligatured molars, such as TNF-α, IL-1β, the receptor activator of nuclear factor-κB ligand (RANKL), and osteoprotegerin (OPG), will also be observed through immunohistochemical (IHC) staining and Tartrate-resistant acid phosphatase (TRAP) staining. Thus, we hypothesize that melatonin could be a potential therapeutic agent for modulating periodontal disease aggravated by stress factors through alteration of the HPA axis.

Keywords: Melatonin, Stress, HPA Axis, Periodontal Disease, Cortisol

I. Introduction

The hallmark of periodontal disease is the progressive destruction of gingival soft tissue and alveolar bone, which is initiated by colonization of dental plaque biofilm [1]. The progression of periodontitis mainly depends on the host immune responses to the bacterial pathogens and their virulence factors (e.g. lipopolysaccharide and proteases), this may in turn disrupt connective tissue and lead to osteoclastogenesis and bone resorption [1]. Moreover, apparition and evolution of periodontal diseases are influenced by many local or systemic risk factors as well as other acquired environmental factors such as stress [1–4]. Dysregulation of immune response and stress factors play pivotal roles in the development and progression of inflammatory periodontal disease, and have been suggested to negatively influence the outcome of periodontal treatment [1].

Sleep is essential to emotional and physical health; inadequate sleep over a period of time has been defined as a sleep trouble, in which the patient has difficulties in falling and/or in staying asleep, and this is responsible for depressive or irritable mood, loss in concentration, along with decreased learning and memory capacities [5]. People who have had sleep troubles complain of reduced ability involving memory, learning, logical reasoning, which may foreshadow psychiatric problems [5]. With the increasing trend in poor sleep quality in the modern societies worldwide, the correlation between sleep depletion and up-regulation of hormones and inflammatory markers in the body have been observed and suggested to increase the risk of several systemic disorders [5, 6], such as obesity [5], cardiovascular diseases [7], diabetes [8], arthritis [9]. In terms of periodontal disease, the response pattern to various stress factors have been examined in experimentally induced periodontitis in rodents and in patients with periodontitis [1–4]. As one of the psychological stress factors, poor sleep quality may not cause periodontal destruction by itself, but may modulate the pathophysiological processes of the already present periodontal inflammation thus leading to accelerated degradation of periodontal tissues [10].

Stressful conditions elicit activation of the hypothalamus–pituitary–adrenal (HPA) axis, a major route of communication between the central nervous system (CNS) and immune responses. The HPA system functions to restore the homeostasis once activated by inflammatory, physical, chemical, and l stresses through the release of

hypothalamic corticotrophin releasing hormone (CRH), which stimulate pituitary adrenocorticotrophin hormone (ACTH) secretion, and ACTH in turn stimulates glucocorticoids (cortisol; corticosterone) to be secreted from the adrenal glands [11–13]. Physiologically, the circadian rhythm hinders the secretion of ACTH or cortisol as the HPA axis is inhibited during sleep time; on the contrary, wakefulness or sleep disturbance would cause an increase in cortisol levels due to continued activity of the HPA axis [11, 13]. An activated HPA axis leads to a series of hormonal-releasing cascades and is regulated by a feedback mechanism, such as sustained tonus of corticosterone and should increase the nocturnal melatonin surge [11].

Melatonin is a hormone synthesized and secreted mainly in the pineal gland, with secretions reaching its peak during darkness but is inhibited during daytime. Secretion of melatonin drives enduring changes in many physiological systems, including the autonomic nervous system, the brain–gut axis, and the HPA axis [11, 13]. Thus melatonin can also interact with the HPA axis, which has been implicated in governing a person's circadian rhythm, as well as the modulation of certain behaviors, immune system, and responses to stress [11, 13]. Notably, decreased sleep-associated ACTH and cortisol nadir values were found in a study where totally blind individuals were administrated pharmacological doses of melatonin orally, which signifies that melatonin has the potential to improve sleep function by synchronizing in time the inhibition of HPA activity with central nervous sleep processes [14].

The relationships between countless types of stress and periodontitis have long been investigated [1, 4, 15]. A series of studies using rodent models have revealed that the inappropriate HPA axis regulation and a subsequent inability to mount a suitable glucocorticoid response to gingival inflammation may influence the susceptibility to periodontal disease [16–20]. However, the information regarding the effect of poor sleep quality and HPA axis on the progression of periodontitis is still limited. Moreover, there are no reports to date describing the therapeutic effects of melatonin which may act to regulate the HPA axis and thus decrease susceptibility and progression of periodontal disease.

II. Evidence and Possible Mechanisms

1. Effect of Poor Sleep Quality on Progression of Periodontitis

Inadequate sleep over a period of time not only directly but also indirectly affects a person's immuno-inflammatory systems by causing systemic alterations to the host defense system [21]. As mentioned previously, the HPA axis reacts to stress triggers such as sleep deprivation (SD) in experimental animals, and causes elevation of CRH, ACTH and corticosterone levels in an attempt to regulate the body's host defenses [11, 13]. Although glucocorticoids should suppress inflammation, prolonged release of glucocorticoids may instead induce glucocorticoid resistance, resulting in increasing counts of leukocytes and monocytes as well as production of inflammatory cytokines [12]. Such an increase is not without cost and is considered a two-edged sword in that over-activity of the host defense is responsible for the tissue damage and extent of inflammatory conditions. Therefore, it is reasonable to believe that periodontitis as an inflammatory disease would also be affected when sleep quality is poor [22].

It has been shown that enhanced HPA axis can significantly increase the susceptibility and progression of periodontitis [16–18], which may be attributable to the well-documented ability of the sympathetic nervous system (SNS) to regulate immune system function primarily via the adrenergic neurotransmitter noradrenaline released at sympathetic nerve terminals [18, 23]. On the contrary, substances can modulate immune and central nervous system (CNS) responses, such as glycine, which has beneficial effect on destructive periodontal bone loss [24]. Therefore, the responsiveness of the HPA axis may play a major role in immune regulation and the resultant outcome of inflammatory periodontal diseases. These findings may implicate the concept of a

bidirectional immune-brain-immune regulatory network with importance for periodontal health and disease [1, 4, 16, 25].

2. Effect of Melatonin on Periodontal disease

Given the properties of melatonin and its presence in the oral cavity [26–33], application of melatonin displayed a variety of beneficial biological activities in oral diseases, such as caries [27], viral infection [28], candidiasis [34], xerostomia [29], oral ulcer [30], and oral cancer [31].

In terms of periodontal disease, melatonin possess several beneficial actions through restoration of antioxidants [35], modulation of immuno-inflammatory response [35], protection and recovery of the integrity of gingival tissues[36], and metabolism of bone [37–38]. Clinically, patients with periodontitis are assessed to have a diminished level of salivary melatonin, which can recover after periodontal therapy, thus can be correlated with a decrease of local periodontal inflammation [39]. Moreover, topical treatment of inflamed gingival tissue with melatonin can be associated with an improvement in the gingival index and pocket depth [38]. During the process, a reduction in salivary levels of receptor activator of nuclear factor kappa-B ligand (RANKL), acid phosphatase, alkaline phosphatase, osteopontin and osteocalcin, but increased salivary concentrations of OPG can be observed [38]. Melatonin may act at the area of the osteoclast lacuna, where it inhibits bone resorption because of its antioxidant properties and ability to neutralize reactive species [40]. Furthermore, melatonin stimulates the synthesis of type I collagen fibers, increases the gene expression level of bone sialoprotein and other protein markers of bone, including alkaline phosphatase, osteopontine, and osteocalcine in preosteoblasts, thus significantly shortens the time needed for their differentiation into mature osteoblasts from 21 to 12 days in human osteoblasts *in vitro* [41]. These results indicate that melatonin has a favorable effect of retarding osteoclast differentiation, promoting osteoblast proliferation, differentiation and activity, improving the quality of alveolar bone, and impeding the progression of periodontal disease [41, 42].

Dysregulation of immuno-inflammatory and stress responses play a significant role in the development and progression of periodontal disease. However, further studies are still needed to elucidate the pathological mechanisms explaining the possible relationship between stress, HPA axis activation, and susceptibility/progression of periodontal disease, as well as the therapeutic strategy.

Based on the above, we hypothesize that orally administrated melatonin may act to regulate the immune and HPA axis responses, and thus ameliorate the stress-enhanced inflammatory periodontal destruction and inhibit the production of inflammatory cytokines/mediators.

III. Experimental Testing

We suggest the following approaches to test the hypothesis:

1. Ligature-induced experimental periodontitis model will be established following the method recently published in our previous studies [43–44]. Male Wistar rats (5–6 weeks old; 250–300 g) will be divided into four groups: (1) the control (C) group, would be healthy rats to be compared with variables; (2) the ligature (L) group; (3) the ligature plus sleep deprivation (L-SD) group will undergo both experimentally induced periodontitis and sleep deprivation, which deprived of sleep for 18h a day; (4) the L-SD plus melatonin group (L-SDM) will be similar to the L-SD group except they will be given melatonin (50 mg/kg) orally once a day.
2. Sleep disturbance will be induced using the modified multiple platform technique [45] following to the chronic-partial sleep deprivation protocol by Machado et al [46]. Groups L-SD and L-SDM will be submitted to sleep deprivation for 18h per day (beginning at 16:00) for seven days beginning on Day 7. After each 18h sleep deprivation period, the rats will be allowed to sleep for 6h (beginning 10:00 – 16:00). On

Day 14, Group L-SDM rats will be treated with melatonin but continued to be sleep deprived until Day 21.

3. Chronic unpredictable stress model, shuttle-box test, will be used to determine whether melatonin administration can alleviate or reverse the stress-induced behavior deficit in rats [47]. On Day 14, and 21, before and following the drug or vehicle treatment, rats will be subjected to shuttle box testing, and this test was always initiated 24 h after the last stressful event [47]. The escape–avoidance test will be carried out in a two-way shuttle box (60 × 20 × 20 cm) with a floor divided into two equal sized chambers separated by a wood partition (1.5 cm above the grid floor) as described previously. The number of escape failures, defined as the absence of a crossing response before or during shock delivery, will be recorded.
4. Following behavioral testing, the effect of the HPA axis on the rat in response to stress (sleep-deprivation) can be observed by evaluating corticosterone and glucocorticoid receptor (GR) levels in the hippocampus and other brain regions [48]. Blood samples (20–30 μl) from the lateral tail vein will be collected into heparinized capillary tubes on Day 0, 7, 14, and 21. The corticosterone and melatonin concentration will be measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's recommended protocol. In addition, the total RNA from rat hippocampus will be isolated and subjected to reverse transcription polymerase chain reaction (RT-PCR) to analyze GR expression.
5. On Day 14 and 21, the whole skull of sacrificed animals will be processed for morphological and histological preparation. Alveolar bone destruction (distance from cementoenamel junction to alveolar bone crest, CEJ-ABC), inflammatory cell infiltrate in connective tissue, and attachment loss will be analyzed by micro computer tomography and histometric measurements as described in previous studies [43–44]. Differential expression profiles of the inflammatory and osteogenic mediators of periodontal tissue surrounding ligatured molars, such as TNF-α, IL-1β, RANKL, and OPG will be observed through immunohistochemical (IHC) staining and tartrate-resistant acid phosphatase (TRAP) staining [43].
6. The mucogingival tissues surrounding the ligatured molars will be collected for myeloperoxidase (MPO) and RT-PCR analysis to determine inflammatory pathologies, and to evaluate the gene expression of specific inflammatory mediators, such as TNF-α, IL-1β, COX2 and PGE2 following methods described previously [43].

IV. Preliminary study

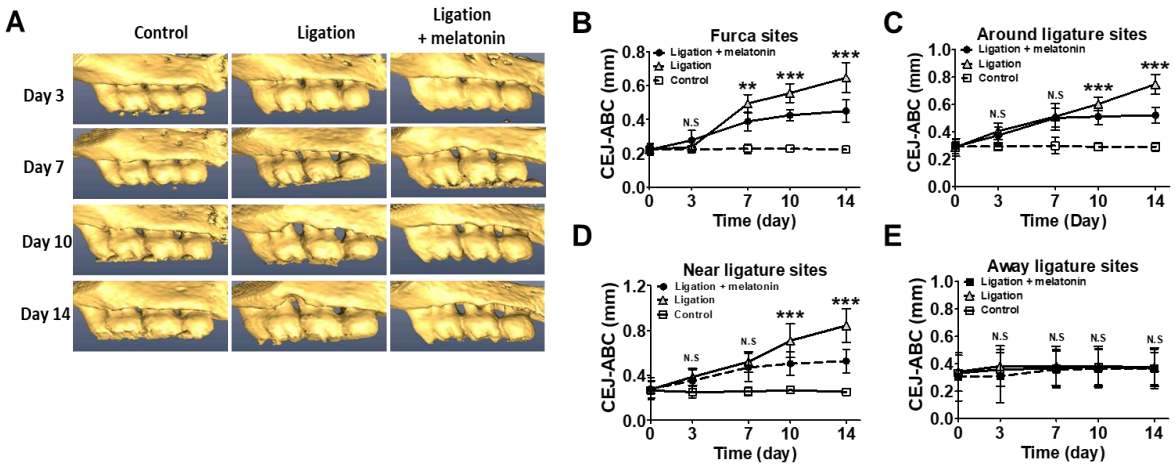
To test the hypothesis, we conducted a polit study to evaluate in vivo and in vitro effects of melatonin on periodontitis by examining alveolar bone destruction in rat-model of experimental periodontitis along with its influence on osteoblastic activities. For the materials and methods used in the pilot study, Sprague-Dawley rats were separated into control (C), ligature (L), and ligature-melatonin (L-Mel) groups (n=5 per group). Ligature-induced experimental periodontitis in rats were established by having 3-O silk wrapped bilaterally around the cervical margins, pushed into the gingival sulcus and knotted at the mesiobuccal side of the maxillary second molars. Rats in Ligature-melatonin group were treated with 50 mg/kg through oral gavage for 14 days. After 14 days, the rats were sacrificed for Micro-CT and histological analysis. In in vitro study, MC3T3-E1 cells were treated with Melatonin at concentrations of 12.5–400 nM, and mineralization effects were examined through alkaline phosphatase (ALP) activity and Alizarin Red Staining (ARS).

The preliminary results revealed that increased alveolar bone destruction in ligation-induced experimental periodontitis during a 14-day course, while melatonin treatment significantly decreased alveolar bone loss in rat with melatonin administration compared to ligation group (Figure 1). The body weight of rats in each group was recorded and data showed no difference among different groups (Figure 2). In in vitro study, cell viability test showed that Melatonin treatment had no toxicity to MC3T3-E1 cells for concentrations up to 400 μM (Fifure 3).

In addition, the preliminary data indicated that Melatonin increased mineralization activity of MC3T3-E1 cells after differentiation in vitro at 200μM (Figure 4).

Figure 1

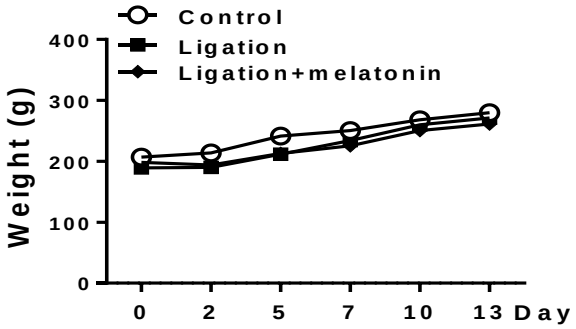
Systemic administration of Melatonin reduced alveolar bone destruction in experimental periodontitis



(A) Micro-CT scans of control, ligature, and ligature-melatonin rats after systemic administration of Melatonin (50 mg/kg) for 14 days; (B-E) Bone destruction at various sites.

Figure 2

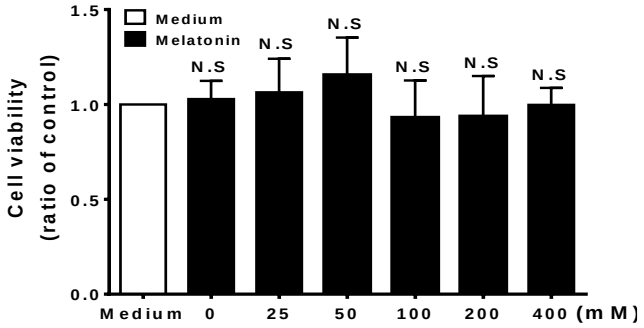
Record of the body weight of rats.



The body weight of rats in three groups was recorded during the 14-days course.

Figure 3

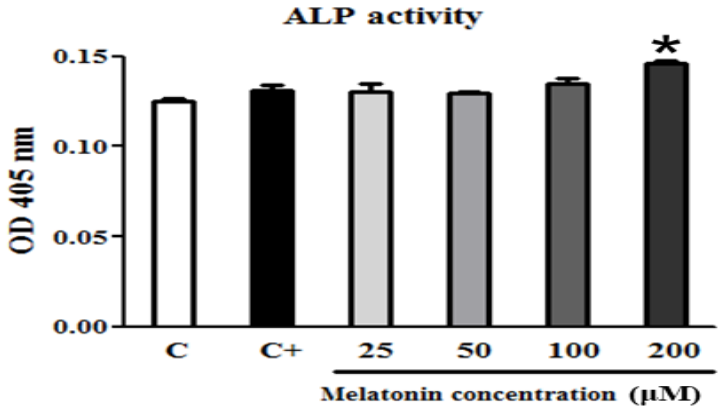
Viability test of MC3T3-E1 cells treated with Melatonin.



MC3T3-E1 were seeded at $1x10^4$/well in 96 well plate in 10%FBS/αMEM medium overnight and then treated with Melatonin at concentrations of 25-400 μM for 24 hours, and the cell viability test was performed by using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-dipheyltetrazolium bromide) assay.

Figure 4

. *Melatonin increased mineralization activity of MC3T3-E1 cells after differentiation in vitro.*



Cells were seeded at 3x104 in 24-well plate ALP activity assay was performed after 8 days of treating with melatonin at various concentrations colorimetrically using an Alkaline Phosphatase Colorimetric Assay Kit which uses p-nitrophenyl phosphate (pNPP) as a phosphatase substrate

These preliminary data provided evidence that systemic administration of Melatonin reduced alveolar bone loss in ligature-induced experimental periodontitis in rats, and melatonin may increase mineralization activity of osteoblasts. Based on these results, we will continue to combine above mentioned in vivo analysis with in vitro mechanistic studies to define the cellular and molecular basis for the potential therapeutic strategy of melatonin in periodontitis.

V. Conclusion

These results would provide evidence with an emerging literature showing that psychological stress, such as sleep disturbance, may dysregulate regulatory mechanisms within the brain involved in immune regulation, and thereby alter immune responses and influence the susceptibility/resistance to inflammatory periodontal destruction. Melatonin could contribute to reverse behavior deficits in chronically stressed animals, modulate the stress-induced HPA axis activation, and ameliorate the susceptibility and pathological development of periodontal disease under stressed conditions by means of decreasing inflammatory cytokine profiles and osteoclastogenic factors expression. Thus, melatonin may be a potential biological therapeutic drug to modulate the susceptibility and progression of periodontal destruction.

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