Melatonin alleviated the immune response and improved the salivary gland function in primary Sjögren's syndrome

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Research article

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Abstract

Background

Excessive inflammatory reactions participate in primary Sjögren's syndrome (pSS) progression. In addition, biological clock genes have been detected in the salivary glands, which indicates that clock genes regulate the growth and development of the salivary glands as well as the quality and quantity of saliva secretion. Melatonin is an amine hormone secreted by the pineal gland that has many physiological functions, such as regulating immunity and correcting disorder in the biological clock rhythm. The purpose of this study was to clarify the correlation between pSS and the biological clock rhythm and explore the possibility of applying melatonin to treat pSS.

Methods

Melatonin (10 mg/kg/d or 15 mg/kg/d) or vehicle was administered to NOD/Ltj mice by intraperitoneal injection for 4 weeks. Clock gene expression levels in labial gland biopsy specimens from pSS patients and submandibular gland specimens from mice were measured by Western blotting (WB) and RT-PCR. The salivary flow rate of mice was measured at 12, 14, and 16 weeks. The severity of lymphocyte infiltration in the salivary glands was analysed by haematoxylin and eosin (H&E) staining. Enzyme-linked immunosorbent assay (ELISA) and immunohistochemical staining were used to detect the expression levels of related inflammatory factors in mice. The percentages of Th17, Th2, and Treg cells were analysed by flow cytometry.

Results

There was a distinct expression profile for clock genes in pSS patients compared with controls. Continuous melatonin administration improved salivary gland function in NOD/Ltj mice, with decreased lymphocyte infiltration in the submandibular glands and reduced related inflammatory factor expression in the serum and salivary glands. Melatonin treatment skewed T cells towards the Treg and Th2 subsets while suppressing Th17 responses. Additionally, melatonin administration regulated clock gene expression in NOD/Ltj mice.

Conclusion

pSS pathogenesis and progression are correlated with abnormal circadian gene expression. Melatonin improves the hypofunction of the salivary glands and inhibits the inflammatory development of pSS in NOD/Ltj mice. This study provides a theoretical basis and potential approach for the clinical prevention and treatment of pSS.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease that targets mucosal tissues and their supporting secretory glands. The eyes and the mouth are primary targets in pSS. pSS has a reported prevalence of 0.15–3.3%, depending on the diagnostic criteria used[1]. Although the specific pathogenesis of pSS is still unclear, current studies show that inflammatory cells and cytokines are important in the occurrence and development of this disease[2].

The circadian clock orchestrates the daily rhythms of many physiological, behavioural and molecular processes, providing the means to anticipate and adapt to environmental changes. Mounting research identifies the circadian system as a critical regulator of immune defence. Indeed, nearly every aspect of immunity, both innate and adaptive, displays a daily oscillatory pattern, including immune cell trafficking; circulating humoral components; inflammatory processes; cytokine, chemokine and recognition receptor expression; and signalling[3]. In the past, a series of clock genes and ion and water channel genes (Ae2a, Car2, and Aqp5) that control the main functions of the salivary glands were detected in the salivary glands of humans and experimental animals[4]. Furthermore, there is increasing evidence that the circadian rhythm affects tooth development, salivary gland and oral epithelial homeostasis, and saliva production[5]. In our previous study, we also identified the role of ROR α in the pathogenesis of pSS[6].

Melatonin is an amine hormone produced by the pineal gland in mammals, including humans, and its level varies with the time of day[7]. Melatonin coordinates the activities of organisms with 24-h cycle changes in the external environment[8]. Melatonin performs dual roles in regulating immune inflammation, promoting or suppressing the immune response in different autoimmune diseases[9]. Previous studies have shown that melatonin can be used to treat various autoimmune diseases, cancers and diseases related to circadian rhythm disorders, with most treatments including melatonin showing promising therapeutic potential. As an endogenous hormone, melatonin is safe, reliable and efficacious with almost no side effects[10, 11]. However, the regulatory role of melatonin in the progression of pSS has not been reported. Based on an analysis of existing related research, we learned that pSS is not only related to an imbalance in immune inflammatory responses but also may involve in the dysregulation of circadian gene expression, which suggests the feasibility of applying melatonin in the treatment of pSS. In this article, we will focus on this perspective. Specifically, we aimed to explore whether there are differences in the expression of clock genes in the salivary glands between pSS and normal control groups and further clarify whether melatonin can play roles in regulating circadian gene expression and alleviating inflammatory progression in pSS.

Materials And Methods

Labial gland specimen collection

All samples collected in this experiment were taken from patients who provided informed consent. The experiment was reviewed and approved by the Ethics Committee of Tongji Medical College of Huazhong

University of Science and Technology. Labial gland specimens were obtained from 11 individuals who underwent diagnostic evaluation for sicca symptoms indicative of pSS and were diagnosed with the American-European Sjögren's syndrome consensus criteria. Seven of these patients were diagnosed with pSS, and all of them were hospitalized for the first time, with no history of hormone, immunosuppressant, biological agent or anticholinergic drug receipt. None of the patients had a history of head and neck radiotherapy or evidence of other desmoplasia, lymphoma, essential mixed cryoglobulinemia, AIDS, or hepatitis B or C virus infection at the time of this study. The samples in the normal control group were obtained from normal salivary glands around cysts in patients with labial mucinous gland cysts. All specimens were collected between 9:00 and 10:00 am.

Mice

Thirteen-week-old female NOD/Ltj mice were used as a model of Sjögren's syndrome, and sex- and age-matched outbred ICR mice were used as normal controls. All animals were purchased from Huafukang (Beijing) and raised in the Animal Experimental Center of Tongji Medical College of Huazhong University of Science and Technology. The room temperature was maintained at 25°C with a 12:12-h light/dark cycle (lights on at 08:00 and off at 20:00). Mice had free access to water and a standard laboratory chow diet under specific pathogen-free conditions. Mice were housed for 1 week to allow adaptation to the environment before treatment. Animals were divided into 6 groups, with an average of 6 mice per group: ICR mice served as the normal control group (NC). NOD/Ltj mice were injected intraperitoneally with vehicle or melatonin 1 h before turning off the light every day for four weeks, and the administered melatonin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a 20% ethanol and 80% normal saline (2 µg/µl) solution. The NOD/Ltj mice in the model group were not given any treatment, while other NOD/Ltj mice were injected intraperitoneally with melatonin at a dosage of 10 mg/kg/d(10M) or 15 mg/kg/d(15M) or with an equal amount of solvent(10M CN, 15M CN). We detected the function of the salivary glands (measurement of the volume of saliva secreted in 10 minutes) in mice at 12 weeks, 13 weeks and 14 weeks. After 4 weeks of drug intervention, mice were sacrificed by orbital blood collection, and samples were collected at 9:00 in the morning. The salivary glands were isolated, with samples of the salivary glands being used for haematoxylin and eosin (H&E) and immunohistochemical staining; the remainder of the glands were used for Western blotting (WB) and RT-PCR. Peripheral venous blood was collected for enzyme-linked immunosorbent assay (ELISA), and the spleens were removed for analysis by flow cytometry. All experimental protocols were performed following the Guideline for the Care and Use of Laboratory Animals of Ethics Committee of Drug Clinical Trials at Tongji Medical College of Huazhong University of Science and Technology (statement no. S803).

Measurement of stimulated salivary flow

Each animal was injected intraperitoneally with 0.1 µg/µl sodium pentobarbital (1.0 mg/kg body weight, Hangzhou Minsheng Pharmaceutical, China). Saliva collection began within four minutes of pilocarpine administration, and saliva was collected from the oral cavity into Eppendorf tubes for 10 minutes using a 100-µl micropipette. The Eppendorf tubes were weighed before and after saliva collection, and the final

amount of saliva was standardized and is reported as mg/10 minutes. After saliva collection was completed, the mice were rewarmed and left alone once w vital signs returned to normal.

Histological assessment

The submandibular glands of mice were fixed in 4% paraformaldehyde immediately after isolation. Next, the specimens were embedded in paraffin blocks and cut into paraffin sections for H&E staining. H&E-stained sections were imaged by microscopy (Olympus Corporation, Japan). The focus score (FS) of lymphocytes was calculated with ImageJ 6.0 software (Media Cybernetics) using the method proposed by Greenspan, in which focus score=1 was defined as a single focus composed of >50 mononuclear cells per 4 mm² tissue, to assess the severity of salivary gland damage.

Immunohistochemical analysis

After deparaffinization, paraffin sections were dehydrated in ethanol, followed by antigen retrieval. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide at room temperature for 20 minutes, and then the sections were incubated with 10% goat serum (ZSGB-BIO, Beijing) for 30 minutes. The tissue sections were incubated with 100 µl anti-IL-6 (1:400, Proteintech, Wuhan) and anti-IL-1β (1:200, Abcam, UK) primary antibodies overnight at 4°C, and the slides were then washed with TBST for five minutes to remove any residual primary antibody, followed by a 30-minute incubation with biotinylated secondary antibodies (ZSGB-BIO, Beijing). Then, the slides were washed with TBST for fifteen minutes to remove any residual secondary antibody and stained with an avidin-biotinylated enzyme complex for 20 minutes. The staining was visualized by using 3,3-diaminobenzidine (ZSGB-BIO, Beijing), and counterstaining was performed with haematoxylin for 1 minute. Immunohistochemically stained sections were imaged with a photomicroscope (Olympus, Japan).

RNA extraction and real-time quantification PCR

Total RNA was extracted from frozen human labial gland specimens and Submandibular gland specimens in mice using Trizol(vazyme, NanJing, China) reagent according to the instructions of the manufacturer. 1ug DNA(Cdna) of each sample was synthesized from RNA using Prime Script™ RT Master Mix (TaKaRa BioTechnology, Japan). Real-time polymerase chain reaction (PCR) amplification of cDNA aliquots was performed by the SYBR® Premix Ex Taq kit (TaKaRa, BioTechnology, Japan) on StepOne Real-Time(Thermo Fisher, USA), Operating procedures were in accordance with the instructions. The levels of mRNA were normalized in relevance to Gapdh. The data analysis of genetic expression was used the method of $2^{-\Delta\Delta C_t}$. All the primer sequences were provided in Supplementary Table 1.

Western blot analysis

Proteins were isolated from the salivary glands of patients and mice, and Western blotting was performed. Total protein was extracted from samples using RIPA buffer (Beyotime, Shanghai, China) containing phosphatase and protease inhibitors. The supernatant was collected and separated, and its

protein concentration was measured using a BCA protein assay kit (Beyotime, Shanghai). Aliquots (30 µg) of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF nitrocellulose membranes. The membranes were blocked with 5% (w/v) skim milk in TBST for 1 h and then incubated with a primary antibody overnight at 4°C. The primary antibodies used in this experiment included a rabbit anti-BMAL1 antibody (1:1000, ab3350, Abcam, USA), rabbit anti-CLOCK antibody (1:1000, D45B10, [Cell Signaling Technology](#), USA), rabbit anti-PER1 antibody (1:500, ab136451, Abcam, USA), rabbit anti-PER2 antibody (1:1000, ab179813, Abcam, USA), rabbit anti-CRY1 antibody (1:800, 13474-1-AP, Proteintech, China), rabbit anti-CRY2 antibody (1:500, ab38872, Abcam, USA), rabbit anti-NR1D1 antibody (1:500, 14506-1-AP, Proteintech, China), and mouse anti-GAPDH antibody (1:500, ab38872, Abcam, USA). The secondary antibodies included a [goat anti-rabbit IgG H&L \(HRP\) antibody \(1:2000, ab275018, Abcam, USA\)](#) and [goat anti-mouse IgG H&L \(HRP\) antibody \(1:2000, ab205719, Abcam, USA\)](#). Protein bands were visualized with an enhanced chemiluminescence (ECL) Western blotting substrate (Thermo Scientific Pierce, P180196).

ELISA

Peripheral blood was collected from the retro-orbital plexus of mice. The samples were allowed to stand at room temperature for 2 h, and the serum was obtained by centrifugation. The concentrations of TGF-β1, IL-10, IL-17, IFN-γ, melatonin, and anti-SSA/Ro and anti-SSB/La autoantibodies were detected by ELISA in accordance with the instructions of the manufacturer. TGF-β1, IL-10, IL-17, and IFN-γ ELISA kits were purchased from MULTI SCIENCES Biotechnology (Wuhan, China), a melatonin ELISA kit was purchased from *Elabscience* Biotechnology (Wuhan, China), and anti-SSA/Ro and anti-SSB/La antibody ELISA kits were purchased from MyBioSource (San Diego, California, USA).

Flow cytometry

Fresh spleens were collected from mice and processed in a plastic dish with sterile PBS to obtain a single-cell suspension. Red blood cells (RBCs) were lysed using RBC lysis buffer (BD Biosciences, USA) with reference to the manufacturer's instructions. The splenocytes were then suspended in PBS and counted before staining.

For regulatory T (Treg) cell staining, mononuclear cells from the spleen of mice (1×10^6 cells/sample) were stained with a FITC-conjugated anti-CD4 antibody (BD Biosciences, USA) and an APC-conjugated anti-CD25 antibody (BD Biosciences, USA) for 30 minutes at 4°C in the dark. Then, staining buffer was added, the cells were centrifuged for 5 minutes at 500 g/min, and the supernatant was aspirated. The cells were fixed and permeabilized with a fixation/permeabilization solution, which was obtained from BD Biosciences, followed by resuspension in 100 µL BD Perm/Wash buffer containing a PE-conjugated anti-Foxp3 antibody (BD Biosciences, USA) and staining for 1 h at 4°C in the dark.

For helper T (Th) cell staining, single-cell suspensions of thymocytes were prepared

in cold RPMI-1640 medium and then seeded in 12-well flat-bottomed culture plates; each sample contained more than 10^6 cells. The cells were stimulated with Leukocyte Activation Cocktail in the presence of a protein transport inhibitor (BD Biosciences, USA) for 6 h at 37°C in 5% CO₂. Then, the samples were stained with a FITC-conjugated anti-CD4 antibody (BD Biosciences, USA) for 25 minutes at 4°C in the dark. The steps for fixation and permeabilization were performed in accordance with the protocols of the manufacturer. Next, 100 µl Perm/Wash buffer was added to the samples, and the samples were stained with a BV421-conjugated anti-IL-17 antibody (BD Biosciences, USA) and PE-conjugated anti-IL-4 antibody (BD Biosciences, USA) according to an intracellular staining protocol at 4°C in the dark for 35 minutes. Finally, the cells were washed with Perm/Wash buffer and resuspended in 150 µL PBS.

The percentages of Treg cells and Th cells were analysed on a FACSCalibur flow cytometer (BD Biosciences, USA). All the data were analysed with FlowJo version 10 software (Tree Star, Ashland, Oregon, USA).

Statistical analysis

The statistical significance of inter-group differences was determined using a two-tailed Student's t-test or one-way analysis of variance (ANOVA) in GraphPad Prism (San Diego, CA, USA). Data are presented as the mean $\pm$ SEM or mean $\pm$ SD. P values are denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Results

Expression of circadian clock genes in the salivary glands of pSS patients and pSS-like model animals

To investigate whether there are correlations between circadian clock genes and pSS, we used RT-PCR and Western blotting to detect the expression of clock genes in the salivary glands of pSS patients and pSS-like model animals (Fig. 1). We found that compared with that of normal people, the expression of BMAL1, CRY1 and CRY2 in the labial salivary glands (LSGs) of pSS patients was decreased, while the expression of PER1, PER2, CLOCK, ROR α , and NR1D1 was increased (Fig. 1a-c). The same trend was observed for the expression of these clock genes in the submandibular glands of pSS-like model animals (Fig. 1d-f). Compared with that in those of 13-week ICR mice (the normal control group), the expression of BMAL1, CRY1 and CRY2 in the submandibular glands of pSS-like model animals was decreased, while the expression of PER1, PER2, CLOCK, ROR α , and NR1D1 was increased (Fig. 1d-f). Thus, our experiments confirmed the abnormal expression of clock genes in the submandibular glands of pSS patients, which suggests that the progression of pSS may be related to the abnormal expression of clock genes.

Melatonin alleviates experimental Sjögren's syndrome features in NOD/Ltj Mice

Based on the abilities of melatonin to regulate the immune response and circadian rhythm, this study applied melatonin to treat pSS-like model animals. To investigate whether melatonin can alleviate the

progression of pSS in NOD/Ltj mice, we measured the salivary flow rate and analysed the morphological structure of the submandibular glands in 13-week NOD/Ltj mice before the mice were injected intraperitoneally with melatonin. We found the formation of infiltrating lymphocyte foci in the submandibular glands of 13-week NOD/Ltj mice (Fig. 2a), and the salivary flow rate of 13-week NOD/Ltj mice was lower than that of normal control ICR mice (Fig. 2d). The salivary flow rate was measured after 2 weeks of different treatments (Fig. 2d). After 4 weeks of continuous treatment, the salivary flow rate was measured again, and the submandibular glands were taken for histological evaluation (Fig. 2a, d). We found that after 4 weeks of different treatments, the salivary flow of untreated mice (model, 10 M CN, or 15 M CN) had decreased rapidly, while that of mice in the melatonin treatment group (10M, 15M) had increased significantly (Fig. 2d). We also quantified the severity of submandibular gland lymphocyte infiltration and calculated the focus score and lymphocyte infiltration area percentage (Fig. 2b, c). All the above parameters confirmed that melatonin treatment controlled and attenuated the progression of salivary gland inflammation and improved salivary gland function in NOD mice, but there were no significant differences between the two melatonin treatment groups (Fig. 2b-d).

Melatonin regulates the expression of inflammatory factors in the submandibular glands of NOD/Ltj mice

T cell proliferation depends on many lymphocyte-activating factors. IL-1 β and IL-6 are early markers of the inflammatory response and play key roles in promoting T cell activation and proliferation[12]. We detected the expression of IL-1 β and IL-6 in the submandibular glands of NOD/Ltj mice after different treatments by immunohistochemistry (cells stained with each antibody are shown in brown). As shown in the staining results, compared with that in those of the mice not given melatonin treatment, the expression of IL-1 β and IL-6 in the submandibular glands of the mice treated with melatonin was decreased (Fig. 3a, b). The decreased expression levels of IL-1 β and IL-6 were consistent with the trend in HE staining shown in Fig. 2, and the degree of lymphocyte infiltration in the submandibular glands of NOD/Ltj mice was reduced after 4 weeks of continuous treatment with melatonin.

Melatonin regulates the production of serum cytokines and autoantibodies in NOD/Ltj mice

To further clarify the role of melatonin in pSS, we examined the concentration of melatonin in the salivary glands and compared the results between NOD mice and ICR mice. We found that the concentration of melatonin in the salivary glands of the normal control mice was higher than that in those of the diseased animals (Fig. 4a). The level of melatonin in salivary gland homogenates from NOD mice was detected again after different treatments were administered for 4 weeks, and the results showed that the concentration of melatonin in the submandibular glands of the treated mice was higher than that in those of the untreated mice (Fig. 4b). Subsequently, we also measured the expression levels of other inflammatory factors in the serum of NOD/Ltj mice in different treatment groups (Fig. 4c-f). During the progression of pSS, IL-10 and TGF-1 β play important roles in the Treg-mediated inhibition of inflammation, which is contrary to the effects of IL-17A and IFN- γ on immunity. In our experiment, the concentrations of IL-10 and TGF-1 β in the serum of diseased mice were significantly lower than those in

the serum of ICR mice, while the concentrations of IL-17A and IFN- γ in the serum of diseased mice were significantly higher than those in the serum of ICR mice. The serum level of IL-10 in the treated groups was higher than that in the untreated groups (Fig. 4c). The concentrations of IL-17a and IFN- γ in the serum of NOD/Ltj mice treated with melatonin were lower than those in the serum of NOD/Ltj mice not given melatonin (Fig. 4e, f). Finally, we measured the concentrations of anti-SSA and anti-SSB autoantibodies in the serum of NOD/Ltj mice in different treatment groups. The results showed that the concentrations of anti-SSA and anti-SSB autoantibodies in the serum of NOD/Ltj mice were higher than those in the serum of ICR mice, and the concentrations of anti-SSA and anti-SSB autoantibodies in the serum of NOD/Ltj mice treated with melatonin were lower than those in the serum of mice not given melatonin treatment (Fig. 4g, h). All the above parameters were not significantly different between the 10 M group and 15 M group (Fig. 4b-h).

Melatonin promotes the differentiation of T cells into Treg cells and Th2 cells and inhibits the proliferation and differentiation of Th17 cells

We next investigated how melatonin directs CD4⁺ T cell responses in diseased animals. The percentage of defined Th17 cells, which play an important role in the progression of pSS, in the spleen of NOD/Ltj mice was examined via flow cytometry (Fig. 5a). We found that the percentage of Th17 cells in the spleen of diseased animals was significantly increased, indicating that the systemic inflammatory response was enhanced in NOD/Ltj mice. However, melatonin treatment reduced the frequency of Th17 cells (Fig. 5d). We also detected the percentages of Th2 cells (Fig. 5b) and Treg cells (Fig. 5c), which are related to the inhibition of the inflammatory response, in the spleen of mice in different experimental groups via flow cytometry. We observed that the frequencies of Treg cells and Th2 cells in the spleen of NOD/Ltj mice were far lower than those in the spleen of ICR control mice by flow cytometry and that the 4 weeks of daily treatment with melatonin significantly restored the numbers of Treg cells and Th2 cells in NOD/Ltj mice (Fig. 5e, f). In general, although statistical analyses of the above results showed no differences between the two melatonin treatment groups, our data still show that melatonin injection can support T cell differentiation into Treg cells and Th2 cells and inhibit Th17 cell proliferation and differentiation, thus inhibiting the inflammatory response and alleviating Sjögren's syndrome.

Melatonin regulates clock gene expression in the salivary glands of pSS-like model animals

To explore the molecular mechanism by which melatonin alleviates experimental Sjögren's syndrome features, given the regulatory effect of melatonin on the circadian rhythm and existence of circadian rhythm disorder in pSS, we assessed changes in clock gene expression after melatonin treatment by RT-PCR and Western blotting. We observed that the expression of BMAL1, CRY1 and CRY2 was increased and that of CLOCK, PER1, PER2, ROR-ERB α and ROR α was decreased in the submandibular glands of NOD/Ltj mice treated with melatonin for 4 weeks compared with those of untreated mice (Fig. 6). Our study showed that melatonin was indeed able to regulate the expression of clock genes in NOD mice and that the expression levels of clock genes in treated mice tended to be normal in the control group.

Discussion

In our study, we found that the expression of BMAL1, CRY1 and CRY2 was decreased in salivary gland samples from patients with pSS and diseased animals compared to corresponding normal controls, while the expression of CLOCK, PER1, PER2, REV-ERB α , and ROR α was increased. Melatonin administration significantly improved salivary gland secretory function and relieved sialadenitis in NOD/Ltj mice.

Circadian rhythms are endogenous processes with an oscillatory pattern that follows a daily cycle, mostly controlled by the circadian system. The molecular clocks that control circadian rhythms are being revealed to be important regulators of physiology and disease. Disruption of 24-h rhythms is closely related to many diseases, including cancers and cardiovascular disease[3, 13]. Previous studies have confirmed that clock proteins play important roles in the regulation of immune function and the inflammatory response. The proliferation of lymphocytes and production of cytokines show circadian rhythm changes[14, 15]. The circadian rhythm regulates the degree of inflammation by acting on inflammatory response signalling pathways. Inflammatory signalling pathways are closely related to the pathological changes of various chronic diseases in vivo[16, 17]. The proinflammatory cytokines produced by the activation of signalling pathways promote the activation of T cells, which plays an important role in the progression of various autoimmune diseases, including pSS[18, 19]. Conversely, inflammatory mediators also interfere with the expression of clock genes[20], but the relevant mechanisms involved are complex and have not been fully elucidated yet.

A curative agent for pSS is lacking, and current treatments are often employed based on results in other autoimmune diseases[21]. Suppression of an excessive abnormal immune response is critical to attenuate the symptoms of patients with pSS, and glucocorticoids or immunosuppressive therapy is therefore frequently utilized[22]. The commonly used immunosuppressive drugs include hydroxychloroquine (HCQ), rituximab, methotrexate and TNF inhibitors, but these agents inevitably induce a series of side effects, such as osteoporosis, infection, gastric ulcer, myelosuppression, hepatorenal toxicity, and immunosuppression[23]. It is not only the expectation of doctors but also the demand of patients that a new, safe and effective drug that can be quickly promoted and effectively used in the clinical treatment of Sjögren's syndrome be found.

Melatonin is an amine hormone produced by the pineal gland of mammals, including humans. In addition to its receptor-mediated physiological effects, melatonin also has many receptor-independent effects. Melatonin has a wide range of functions, including circadian rhythm regulation, sleep quality improvement, cancer inhibition, antioxidative activity, anti-ageing activity and immunoregulation[24, 25]. Because of the great potential shown by melatonin in regulating the immune response, melatonin has been used in many clinical studies of autoimmune diseases. Melatonin has dual roles in regulating immunity, and it was found to aggravate the progression of rheumatoid arthritis. In contrast, melatonin alleviates the severity of some autoimmune diseases, such as experimental reflux oesophagitis, systemic lupus erythaematosus, multiple sclerosis, inflammatory bowel disease and scleroderma[16, 26, 27]. In the

past, only a small step has been taken in the exploration of melatonin function, and its pleiotropic value makes melatonin worthy of being applied in clinical studies of other diseases.

Based on the functions of melatonin related to regulating the immune response and circadian rhythm, this study applied melatonin to treat NOD/Ltj mice, which were used as a model of pSS. It was found that in NOD/Ltj mice treated with melatonin for 4 weeks, the function of the salivary glands was obviously improved, the infiltration of lymphocytes into the submandibular glands was reduced, the expression of related inflammatory factors in the serum and salivary glands was decreased, the differentiation of Th17 cells that promote inflammatory reactions in the spleen was attenuated, and the differentiation of Th2 and Treg cells that restrain inflammatory reactions was heightened. The above results were not significantly different between the 10 M group and 15 M group, suggesting that perhaps in our pSS-like model, the concentration of melatonin in the 10 M group was high enough to achieve great drug efficacy.

Our results confirm that the use of melatonin in the treatment of pSS in an experimental animal model can indeed play roles in limiting inflammation and alleviating disease progression. However, the molecular mechanism underlying melatonin therapy for pSS has not yet been fully elucidated because of the intricate mechanism of action of melatonin in organ tissues and the complex interaction between inflammation and the circadian rhythm. As indicated in our study, the disordered expression of clock genes observed in pSS patients and animals may be the cause of pSS, or excessive inflammatory responses may disturb the normal expression of biological clock genes. Melatonin can inhibit a variety of important inflammation-related pathways and molecules and may reduce inflammation by blocking these inflammatory signalling pathways, which are closely related to the progression of pSS[9, 19, 28–30]. Moreover, as a broad antioxidant, melatonin can effectively reduce tissue damage during chronic inflammation[8, 9, 31]. It has also been proven that melatonin can inhibit the differentiation of Th17 cells in a melatonin receptor-dependent manner[24, 32]. In addition, melatonin further alleviated inflammatory responses by directly regulating the expression of the circadian clock gene. In our study, we confirmed that melatonin administration could regulate the expression levels of related circadian clock genes to restore the balance between anti-inflammatory genes and circadian clock genes in pSS-like animals. The expression of BMAL1, CRY1 and CRY2, which are related to abating inflammation in the submandibular glands, was increased, and the expression of CLOCK, PER1, PER2, Rev-Erba and ROR α , which are related to enhancing inflammation, was decreased. Therefore, melatonin alleviated inflammatory reactions in pSS-like animals by regulating the expression of circadian clock genes or directly acting on the immune system. In this process, the specific contributions of clock proteins and melatonin receptors need to be further elucidated by constructing gene-knockout animal models, and the changing expression of circadian clock genes after melatonin treatment may benefit from the alleviation of inflammation. Our study further clarifies the roles of melatonin and the circadian rhythm in the occurrence and progression of pSS and provides new strategies for pSS treatment.

Conclusion

In conclusion, its beneficial role in animal models of pSS, non-toxicity, and lack of side effects across a wide range of pharmacological concentrations make melatonin a potential drug to treat and mitigate pSS. Melatonin shows great potential in clinical medical research, but more clinical experiments are needed to evaluate its efficacy in patients; more in-depth and diversified exploration of melatonin is required before this agent can be popularized.

Abbreviations

RORα

orphan nuclear receptors retinoic acid-related receptors

pSS

primary Sjögren's syndrome

LSG

labial salivary gland

Th17

T helper 17

FS

focus score

IL-17RA

interleukin-17 receptor A

NOD

non-obese diabetic

ANA

anti-nuclear antibody

RF

rheumatoid factor

ELISA

Enzyme-linked immunosorbent assay

IgG

immunoglobulin G

SSA

Anti-Sjögren's-syndrome-related antigen antibody A

SSB

Anti-Sjögren's-syndrome-related antigen antibody B

MSG

minor salivary gland

IL-17

interleukin 17

ESR

erythrocyte sedimentation rate

H&E

hematoxylin and eosin

PBS

Phosphate-buffered saline

PCR

polymerase chain reaction

Treg

regulatory T

NC

normal control

DAPI

Diamidino-phenyl-indole

GAPDH

Glyceraldehyde-3-phosphate dehydrogenase

DMSO

Dimethyl Sulphoxide

BSA

Bovine Serum Albumin

RIPA

Radio-immunoprecipitation assay

TBST

Tris-buffered-saline Tween

Declarations

Availability of data and materials

All of the data supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate

All LSGs specimens obtaining was conducted with the approval of the ethics committee of Tongji Medical College, Huazhong University of Science and Technology, and informed consent was obtained from all participants. All animal experiments were conducted with the approval of the animal ethics committee of Huazhong University of Science and Technology(IACUC Number: S803).

Consent for publication

All authors have read and approved the manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

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Contributions

BC and YD conceived the project, BC provided the research fund. BC, YD, YL, XHW, SLY designed the experiments. YL and XHW collected samples and conducted the experiments. All authors participated in drafting the manuscript. YL and XHW performed the statistical analyses. BC and YD revised the manuscript. All authors reviewed the manuscript. All authors read and approved the final manuscript.

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Everyone who contributed significantly to the work has been listed.

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Figures

Figure 1

Expression of clock genes in the salivary glands of pSS patients and pSS-like model animals. (a) The mRNA expression of clock genes in the LSGs of normal control and pSS patients was assessed by real-time quantitative PCR. Relative mRNA levels were normalized to the β -actin level. (b) Western blotting was used to detect the protein expression of PER1, PER2, CLOCK, BMAL1, CRY1, ROR α , NR1D1 and CRY2 in the LSGs of pSS patients (n=7) compared to those of normal participants (n=3). (c) A greyscale analysis is shown, and the data were normalized to the GAPDH results. The results are presented as the mean $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (d) The mRNA expression of clock genes in the submandibular glands of control mice and NOD mice was determined by real-time quantitative PCR. Relative mRNA levels were normalized to the β -actin level. (e) The expression of clock genes in NOD mice and ICR mice was detected by Western blotting. (f) A greyscale analysis is shown, and the data were normalized to the GAPDH results. The results are presented as the mean $\pm$ SD (n=3 per group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Figure 2

Melatonin alleviates experimental Sjögren's syndrome features in NOD/Ltj mice. (a) H&E staining was used to evaluate the degree of damage to the submandibular glands of NOD/Ltj mice in different treatment groups ($n = 6$). Scale bar=200 μm ; the area marked by the red frame is enlarged 5 times in the black frame. (b) The lymphocyte focus score (number of lymphocytic foci (>50 lymphocytes) per 4-mm² tissue) is represented in the graph. The results are presented as the mean $\pm$ SD ($n = 6$ per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (c) The graph shows the lymphocyte infiltration area percentage in the submandibular glands of NOD/Ltj mice that were treated with melatonin or vehicle. The results are presented as the mean $\pm$ SD ($n=6$ per group; **** $p < 0.0001$). (d) The salivary flow of mice was assessed at the 12th week, the 14th week and the 16th week. The results are presented as the mean $\pm$ SD ($n=6$ per group; #### $p < 0.0001$; ** $p < 0.01$, **** $p < 0.0001$).

Figure 3

Expression of IL-1 β and IL-6 in the submandibular glands of NOD/Ltj mice in different treatment groups. (a) The expression of IL-1 β in the submandibular glands of NOD/Ltj mice treated with or without 10 M or 15 M melatonin was detected by immunohistochemistry. NOD/Ltj mice receiving continuous melatonin treatment for 4 weeks had lower expression of IL-1 β in the submandibular glands than those not treated with melatonin (n=3, a representative image is displayed for each group, scale bar=20 μ m). (b) The expression of IL-6 in the submandibular glands of NOD/Ltj mice with different treatments was evaluated (n=3, a representative image is displayed for each group, scale bar=20 μ m).

Figure 4

Melatonin regulates serum cytokines and autoantibodies in pSS-like animals. (a) Melatonin concentrations in the serum of 13-week NOD/Ltj mice and 13-week ICR mice were examined using ELISA kits. The results are presented as the mean $\pm$ SD ($n=6$ per group; **** $p < 0.0001$). (b) Melatonin concentrations in the serum of normal control ICR mice and NOD/Ltj mice receiving different treatments for 4 weeks were measured. The results are presented as the mean $\pm$ SD ($n = 6$ per group; *** $p < 0.001$, **** $p < 0.0001$). (c-f) The levels of cytokines associated with inflammation including IL-10 (c), TGF-1 β (d), IL-17A (e) and IFN- γ (f) were measured. The results are presented as the mean $\pm$ SD ($n=6$ per group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (g, h) The levels of the pSS-related autoantibodies anti-SSA (g) and anti-SSB (h) in the serum were examined using ELISA kits ($n=6$ per group; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Figure 5

Melatonin inhibits the Th17 cell response and promotes the differentiation of Treg and Th2 cells. (a) Flow cytometry was used to assess IL-17 (Th17) expression in CD4⁺ splenocytes. Th17 cells (CD4⁺/IL-17A⁺) are presented in Q2. (b) The percentage of IL-4-producing CD4⁺ T cells is shown in Q2. (c) CD25 and FoxP3 expression in CD4⁺ T cells from the spleen of ICR mice and NOD/Ltj mice given different treatments was evaluated. (d) Th17 levels were significantly higher in untreated NOD/Ltj mice than in ICR mice. The results are presented as the mean $\pm$ SEM (n=6 per group; *** p < 0.001; ** p < 0.01). (e) Th2 responses in the spleen of untreated or treated NOD/Ltj mice and control ICR mice were evaluated. The results are presented as the mean $\pm$ SEM (n=6 per group; ### p < 0.001; ** p < 0.01). (f) The graph illustrates Treg cell levels in the spleen of treated or untreated NOD/Ltj mice and control ICR mice. The results are presented as the mean $\pm$ SEM (n=6 per group; *** p < 0.001; **** p < 0.01).

Figure 6

Melatonin regulates circadian clock gene expression in the salivary glands of pSS-like model animals. (a-i) RT-PCR and Western blotting were used to detect the expression of PER1, PER2, CLOCK, BMAL1, CRY1, ROR α , NR1D1 and CRY2 in mice given different treatments for 4 weeks. (j-q) A greyscale analysis is shown, and the data were normalized to the GAPDH results. The results are presented as the mean $\pm$ SEM (n=3; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

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