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Research article

Integrating network pharmacology and experimental evaluation to explore the complementary therapeutic effect and mechanism of melatonin in periodontitis

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ABSTRACT

Objective: To explore the potential targets for melatonin in the treatment of periodontitis through network pharmacologic analysis and experimental validation via *in vivo* animal models and *in vitro* cellular experiments.

Materials and methods: In this study, we first screened melatonin targets from Pharm Mapper for putative targets, Drug Bank, and TCMSP databases for known targets. Then, disease database was searched and screened for differential expressed genes associated with periodontitis. The intersection of disease and melatonin-related genes yielded potential target genes of melatonin treatment for periodontitis. These target genes were further investigated by protein-protein interaction network and GO/KEGG enrichment analysis. In addition, the interactions between melatonin and key target genes were interrogated by molecular docking simulations. Then, we performed animal studies to validate the therapeutic effect of melatonin by injecting melatonin into the peritoneal cavity of ligation-induced periodontitis (LIP) mice. The effects of melatonin on the predicted target proteins were also analyzed using Western blot and immunofluorescence techniques. Finally, we constructed an *in vitro* cellular model and validated the direct effect of melatonin on the predicted targets by using qPCR.

Results: We identified 8 potential target genes by network pharmacology analysis. Enrichment analysis suggests that melatonin may treat periodontitis by inhibiting the expression of three potential targets (MPO, MMP8, and MMP9). Molecular docking results showed that melatonin could effectively bind to MMP8 and MMP9. Subsequently, melatonin was further validated in a mouse LIP model to inhibit the expression of MPO, MMP8, and MMP9 in the periodontal tissue. Finally, we verified the direct effect of melatonin on the mRNA expression of MPO, MMP8, and MMP9 in an *in vitro* cellular model.

Conclusions: Through a combination of network pharmacology and experimental validation, this study provides a more comprehensive understanding of the mechanism of melatonin to treat periodontitis. Our study suggests that MPO, MMP8, and MMP9 as key target genes of melatonin to

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treat periodontitis. These findings present a more comprehensive basis for further investigation into the mechanisms of pharmacological treatment of periodontitis by melatonin.

Abbreviations

GCF	gingival crevicular fluid
SMILES	simplified molecular-input line-entry specification
TCMSP	Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform
NCBI	National Center for Biotechnology Information
PPI	Protein-Protein Interaction
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
BDSV	BIOVIA Discovery Studio Visualizer
Tb.Sp	bone trabecular separation
Tb.N	number of bone trabeculae
BS/BV	bone surface area to bone volume ratio
BV/TV	bone volume over total volume
CEJ	cementoenamel junction
ABC	alveolar bone crest
qPCR	quantitative polymerase chain reaction
MPO	Myeloperoxidase
MMP	Matrix metalloproteinases
ROS	reactive oxygen species
Mel	melatonin

1. Introduction

Periodontitis is the sixth most prevalent disease in humans, and the most common chronic inflammatory disease [1–3]. More than 70 % of adults in China are affected by periodontitis, with a global prevalence of 11 % for the severe form of the disease [4,5]. Periodontitis is induced by periodontal dysbiosis due to the accumulation of biofilm, and subsequent imbalance of immunological microenvironment and persistent tissue damage, leading to bleeding gums, periodontal pocket formation, tooth mobility, and final tooth loss [6,7]. Moreover, periodontitis is widely reported to have substantial impact on systemic disorders, including diabetes, atherosclerosis, Alzheimer's disease, and chronic kidney disease [8,9].

Previous studies reported a complicated bidirectional relationship between periodontitis and systemic diseases. Pro-inflammatory cytokines, immune cells, bacteria, and their metabolites from periodontal tissues can transmit to remote organs and tissue via the circulatory system, thereby induce or worsen many systemic diseases [10,11]. Also, systemic diseases can increase the risk of periodontitis and affect the effectiveness of periodontal treatment via enhanced reactive oxygen species (ROS), maladaptive trained immunity, and potential neuro-immunological mechanisms [12]. Therefore, preventing and treating periodontal infections allows a positive feed-back loop on the patient's general health [13,14].

However, in acute infections and in patients with systemic diseases, conventional nonsurgical or surgical therapy alone may not provide satisfactory therapeutic results, thus a complementary treatment is urgently required, such as the electroacupuncture and the application of different agents (melatonin, hypochlorite, or chlorhexidine, etc.), which have different results in terms of the clinical attachment level and anti-inflammatory effect [15,16].

Melatonin is frequently used in the medical field as an anti-inflammatory and antioxidant adjunctive therapy; however, there are few studies in the dental field [17–21]. When melatonin is secreted from the pineal gland into the bloodstream, it passively diffuses into the saliva of the mouth. The amount of melatonin that enters the saliva varies from 24 % to 33 % of the total amount in the blood [22–24]. In the oral cavity, melatonin is regarded as a regulator of bone turnover and immunomodulatory agent and has been assayed in saliva, gingival crevicular fluid (GCF), and gingival tissues [25–27]. These effects of melatonin are closely related to the treatment of periodontitis disease. Indeed, a combination of recent basic studies and clinical studies suggest that melatonin may have potential therapeutic effects on periodontitis [28–32]. In animal studies, melatonin significantly reduced the infiltration of neutrophils in the mandible of rats with experimental periodontitis, eliminated oxygen free radicals, and reduced the secretion of pro-inflammatory cytokines to reduce the destruction of periodontal tissues [33–36]. In human studies, Hesam et al. found that after six months of taking 10 mg of melatonin orally each night at bedtime, patients with periodontitis had a significant reduction in periodontal probing depth and a significant improvement in probe bleeding [37–40]. Also, it was found that oral melatonin for adjunctive medication after routine periodontal scaling had a positive effect on periodontal status and glycaemic control in patients with type 2 diabetes and chronic periodontitis [41,42]. Nonetheless, the exact mechanism and potential targets of melatonin adjunctive therapy of periodontitis

is still unclear and needs to be further explored. Our null hypothesis is that melatonin has no potential therapeutic effect on periodontitis.

With the advent of the era of bioinformatics and the age of Big Data, network pharmacology has become an important tool for innovation in the field of information science and medicine [43]. Network pharmacy is a new discipline that explains disease mechanisms and drug action mechanisms from the perspective of biological networks. Today, network pharmacy has made significant breakthroughs in theoretical foundations and algorithm development, and has been widely applied in traditional and modern medical research [44,45]. This study was designed to explore the effect and mechanism of melatonin to treat periodontitis in four aspects. First, network pharmacological analysis was used to predict potential pharmacological targets of melatonin during periodontitis treatment. Then, molecular docking methods were applied to verify the binding ability of melatonin upon selected key genes in silico. Thirdly, the therapeutic effects and mechanisms of melatonin were validated in a ligation-induced periodontitis mouse model. Finally, the direct effect of melatonin on the screened therapeutic targets were verified in a HL60 cell model (China, Procell) with LPS (*Escherichia coli*, Sigma, L2755, 10 μg/mL) stimulation.

2. Materials and methods

2.1. Ethical approval

All animal operations were performed in accordance with the guidelines of the Animal Care and Use Committee of China. Animal operations were approved by the ethics committee of Chongqing Medical University Affiliated Hospital of Stomatology (Ethical approval No.2023034).

2.2. Network pharmacology analysis

2.2.1. Screening of potential targets

The known and potential targets of melatonin were screened as previously described [46]. Briefly, we first used the PubChem (https://pubchem.ncbi.nlm.nih.gov/) database to retrieve the 3D structure and the simplified molecular-input line-entry specification (SMILES) representation of melatonin. Then, to predict putative potential targets for melatonin, the 3D structure was uploaded to the Pharm Mapper (http://lilab-ecust.cn/pharmmapper/index.html) webtool, and the SMILES was input to Swiss Target Prediction (http://swisstargetprediction.ch/). Last, we searched the Drug Bank (https://go.drugbank.com/) and TCMSP (https://old.tcmsp-e.com/tcmsp.php) database for known melatonin targets.

DisGeNET (https://www.disgenet.org/), GeneCards (https://www.genecards.org/), and NCBI (https://www.ncbi.nlm.nih.gov/) Gene databases were searched to identify protein targets associated with periodontitis. To improve the reliability of the results, periodontitis-related targets with a Gene-Disease Associations score $\geq$0.1 in DisGeNET or the relevance score $\geq$10 in GeneCards were included.

2.2.2. Protein-protein interaction (PPI) network analysis

The STRING (https://cn.string-db.org/) database was used to construct Protein-Protein Interaction (PPI) networks. The cytoscape software was applied to visualize and analyze the PPI networks. The resulting PPI networks was subjected to module analyses with the Plugin MCODE with the default parameters (degree cutoff $\geq$2, node score cutoff $\geq$0.2, K-core $\geq$2, and Max depth = 100). Furthermore, topological analysis of the PPI networks was performed and node degrees of these DEGs were analyzed.

2.2.3. Functional enrichment analysis

The GO database (http://www.geneontology.org) integrates biological modeling databases and other biological experimental research results to analyze cellular components (CC), molecular functions (MF), and biological processes (BP) items for analysis. The Kyoto Encyclopedia of Genes and Genomes database (KEGG, https://www.genome.jp/kegg/) is a knowledge base for systematic analysis of gene functions, linking genomic information with higher order functional information. Junction combined with GO and KEGG enrichment analysis can be used to obtain functional information from a large number of genes and to mine the signaling pathways associated with drugs and diseases. GO and KEGG pathway analysis were performed using the online databases DAVID (https://david.ncifcrf.gov/), KOBAS (http://bioinfo.org/kobas), and Metascape (http://metascape.org). $P < 0.05$ was considered to be statistically significant.

2.3. Molecular docking

According to the small molecule Chemical Abstracts Service number, the 3D structure of the small molecule sdf format was downloaded from the PubChem database, import into ChemBio3D Ultra 14.0 to minimize the energy, set the Minimum RMS Gradient to: 0.001, and saved the small molecule as mol2 format. The optimized small molecules were imported into AutodockTools-1.5.6 for hydrogenation, calculating charge, distributing charge, and setting rotatable bond, and saving in "pdbqt" format. We downloaded these proteins in PDB format and used BIOVIA Discovery Studio Visualizer (BDSV, https://www.3ds.com/products/biovia/discovery-studio/visualization) 2020 to remove redundant conformations and water molecules from the proteins and predict their binding sites. The protein structure was introduced into AutoDocktools (v1.5.6) for hydrogenation, charge calculation, charge distribution, and atomic type assignment, and preservation in "pdbqt" format in preparation for docking.

2.4. Animal experiment

All animal experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All treatments were performed in a gentle manner, and every effort was made to minimize animal suffering. The protocol was reviewed and approved by the Dental Ethics Committee of Chongqing Medical University.

All animals were housed in temperature and humidity rooms with free access to food and water, ambient temperature (22–24 °C) and a 12-h light/dark cycle, maintaining 6–8 animals in each cage. Forty C57BL/6 mice aged 6–8 weeks and weighing 18–22 g were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China) and divided randomly into 3 groups: CTR (control, n = 6); LIP (ligature-induced periodontitis, n = 6); LIP + Melatonin (ligature-induced periodontitis treated with Melatonin, n = 6). The LIP model was established by intraperitoneal injection of Ketamine (100 mg/kg) with xylazine (5 mg/kg) into the mice for anesthesia, followed by placement of 5–0 ligature wires around the bilateral maxillary second molars. On the day of periodontitis model establishment, mice in the LIP + Melatonin group received an intraperitoneal injection of 100 mg/kg body weight melatonin for 7 days [47–50], while the remaining two groups received physiological saline injections. At the 7th day, we used CO_2 asphyxiation to euthanize mice, to ensure that the mice receive minimal torture during execution.

2.5. Micro-computed tomography

After 24 h of fixation with 4 % paraformaldehyde, the maxilla was scanned using a high-resolution CT system (Skyscan, Belgium) with using a voxel size of 17.5 μm, a voltage of 70 kV, and a current of 800 μA. Three-dimensional reconstructions were generated using 3D Slicer software. The area of interest was defined as the alveolar bone of the maxillary second molars. The vertical bone loss was identified by the distance between the cementoenamel junction (CEJ) and the alveolar bone crest (ABC). The calculation indicators of bone morphology parameters were determined by the value of Tb.Sp (bone trabecular separation), Tb.N (number of bone trabeculae), BS/BV (bone surface area to bone volume ratio), and BV/TV (bone volume over total volume).

2.6. Western blot analysis

MPO, MMP8, and MMP9 levels in mouse gingival tissue were measured by Western blot. Mouse gingival tissue was collected into EP tubes and quickly placed on ice, and RIPA buffer containing protease inhibitors was added to the EP tubes. The EP tubes were ground in a grinder and the supernatant was centrifuged at 4 °C at 12,000 g for 15 min before the protein content of the supernatant was determined by BCA protein assay. Equal amounts of protein from each sample were separated on a 10 % gel and electroblotted on a PVDF membrane, then incubated with primary antibody (rabbit polyclonal anti-MPO, 1:1000, Abcam, ab208670) (rabbit polyclonal anti-MMP8, 1:1000, Abcam, ab81286) (rabbit polyclonal anti-MMP9, 1:1000, Abcam, ab76003) and overnight at 4 °C, followed by the appropriate hrp-coupled secondary antibody (1: 6000, goat anti-rabbit IgG (H + L)-HRP couples. Proteintech B900210). The target protein bands were visualized with chemiluminescent reagents (Merck Millipore, USA) on a ChemiDoc MP Imaging System (Bio-Rad, USA).

2.7. Immunofluorescence

The gingiva was placed in a low concentration of alcohol for 2 h and then gradually moved into a high concentration of alcohol to remove the water from the tissue. The tissue block was then placed in xylene, a clearing agent dissolved in both alcohol and paraffin, to replace the alcohol in the tissue block. The transparent tissue block was placed in the melted paraffin wax and kept in a wax melting chamber. After the paraffin wax was completely immersed in the tissue block for embedding, the embedded gingival tissue block was placed on the slicer to prepare paraffin sections. Paraffin sections were dewaxed and rehydrated, followed by antigen repair in citric acid. The sections were incubated in hydrogen peroxide for 25 min at room temperature and protected from light, and washed three times with PBS. The sections were then closed with 10 % rabbit serum for 30 min, and the closure solution was removed and primary antibody was added incubated overnight at 4° (MPO GB11224 RAB 1:5000) (MMP8 GB11867 RAB 1:2000) (MMP9 GB11132 RAB 1:2000). The sections were washed three times, then the secondary antibody corresponding to the primary antibody was added and incubated for 50 min protected from light. The cell nucleus was stained with DAPI (1:1000) for 5 min. Then the sections were washed three times, blocked and images were acquired (330–380 nm excitation wavelength and 420 nm emission wavelength for DAPI, 465–495 nm excitation wavelength and 515–555 nm emission wavelength for MPO, 510–560 nm excitation wavelength and 590 nm emission wavelength for MMP8, 608–648 nm excitation wavelength and 672–712 nm emission wavelength for MMP9).

2.8. Cell culture

HL60 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin at a density of $2*10^6$/ml, and then the cells were spread into 12-well plates. Next, 13 μl of DMSO was added to each well to give a final concentration of 1.3 % DMSO. Finally, the well plates were placed in a cell culture incubator for 72 h of induction [51].

2.9. Cell experiment

Cells were spread into 12-well plates at a density of two million per milliliter, and first the LPS + Mel group was pretreated by adding 25 μmol melatonin for half an hour, followed by the addition of LPS (*E.coli*, Sigma, L2755, 10 μg/ml) in the LPS and LPS + Mel groups for 2 h.

2.10. Quantitative real-time PCR

Cellular RNA was extracted from HL60 cells after induced differentiation using a rotary column (Beyotime, R0032). Quantitative polymerase chain reaction (qPCR) was performed as described in the Supplementary Information. This was followed by reverse transcription with 5 × PrimeScript RT Master Mix (Takara, Japan) and PCR amplification with TB Green™ Premix Ex Taq™ (Takara, Japan). The reactions and assays were performed on a CFX96TM system (Bio-Rad, USA). GAPDH was used as a standard internal reference gene. The relative mRNA expression of each target gene was calculated using the 2ˆ-ΔΔCT method. The list of primers is given in Table 1.

2.11. Statistical analysis

All data were statistically analyzed using Prism 9 (Graphpad, USA). Multiple group comparisons were performed using one-way ANOVA or Kruskal-wall test, and Tukey's or Bonferroni post-hoc tests were used to determine the differences between the two groups. Data were expressed as mean ± standard deviation. In all statistical analyses, differences with a $p <$ value of 0.05 were considered significant. Differences were marked with an asterisk to indicate statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns not statistically significant.

3. Results

3.1. Network pharmacology analysis

3.1.1. PPI networks analysis and screening for the hub gene

Initially, potential and confirmed targets of melatonin were identified using a combination of database searches. Melatonin's 3D structure was retrieved from PubChem, and PharmMapper was utilized to predict potential targets. Known melatonin targets were obtained from DrugBank and TCMSP databases (Fig. 1a). We then searched the DisGeNET, GeneCards, and NCBI gene databases to identify 454 targets that were highly associated with periodontitis (Fig. 1b). After that, the 381 selected melatonin-related targets (blue circles) were combined with the 454 periodontitis-related targets (red circles) screened in the disease database to obtain 8 potential targets of melatonin related to periodontitis, as shown in the Venn diagram (Fig. 1c). The above 8 overlapping targets were imported into STRING to construct the PPI network, and the results were shown in Fig. 1d. In short, we found that Ptgs2, Esr1, MPO, and MMP9 had high connectivity.

3.1.2. Bioinformatic annotation

Based on the number of hit genes and *P*-values, we performed GO enrichment analysis in three aspects: biological processes, cellular components, and molecular functions, and the five pathways with the highest enrichment in each aspect are presented (Fig. 2a). We found that these genes were enriched in oxidative respiratory burst response and inflammatory response, which may be closely related to MPO [52,53]. Then, to verify the relationship between the above enriched pathways and the potential targets previously screened for melatonin in the treatment of periodontitis, we used Chord diagram to investigate their correlation (Fig. 2b). We could see that MPO and PTGS2 were highly correlated in terms of oxidative respiratory burst response and stimulatory response to LPS, while MMP9 and MMP8 were also highly correlated with extracellular matrix disassembly. The results of KEGG analysis showed that the TNF signaling pathway and Estrogen signaling pathway were highly enriched (Fig. 2c). Similarly, we used Sankey diagram to show the correlation of KEGG enriched pathways with the previously screened relevant targets (Fig. 2d). It found that the TNF signaling pathway, which was closely related to inflammation, had a high correlation with MMP9. Previous studies have demonstrated that neutrophils were also closely associated with MPO, MMPs [54,55]. This may suggest that MPO, MMP8, and MMP9 may be potential

Table 1
List of primers.

Gene	Direction	Primer (5′-3′)
GAPDH	F (forward)	CACTCCTCCACCTTTGACGC
	R (reverse)	CTGTTGCTGTAGCCAAATTCGT
MPO	F	TGATCGGTTTTGGTGGGAG
	R	TCTTAGACACGGTGGTGATGC
MMP8	F	TGGGAACGCACTAACTTGACC
	R	TGGTGAAGATGAGAGGTGATGC
MMP9	F	GCACTGGGCTTAGATCATTCC
	R	CTGGATGCCGTCTATGTCGTC

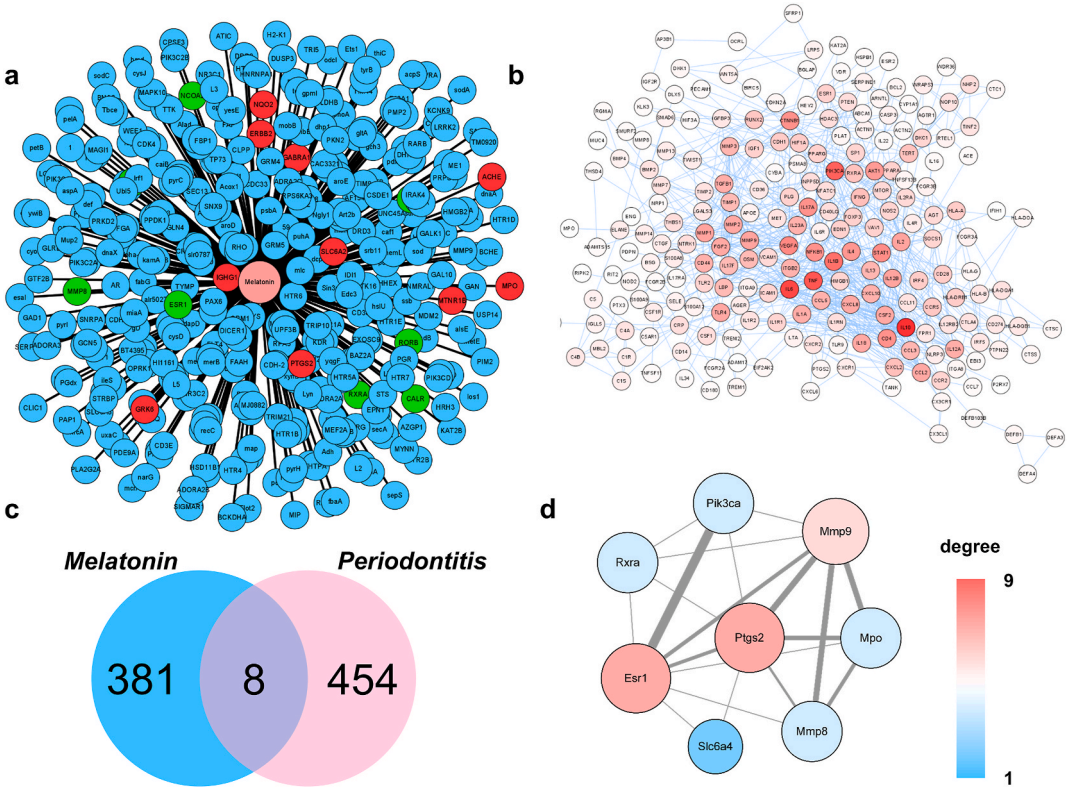

Fig. 1. Screening for potential melatonin targets in periodontitis. (a) Network of known and potential protein targets of melatonin. Pink: melatonin. Green: known targets. Blue: potential targets predicted by the model. Red: predicted and known targets. (b) Periodontitis-related targets in the disease database (c) Venn diagram for screening melatonin targets in periodontitis. (d) PPI network construction for 8 crossover targets.

key targets for melatonin in the treatment of periodontitis among the several targets we screened.

3.2. Molecular docking of potential key targets with melatonin

To investigate whether the above eight targets could bind directly to melatonin, we performed molecular docking experiments. As shown in Fig. 3 a-h and Table 2, the experimental results indicated that both MMP8 and MMP9 can bind melatonin stably with binding energies of −8.1 and −8.5 kcal/mol. At the same time COX2 and RXRA can also bind melatonin stably with binding energies of −8.2 and −8.1 kcal/mol. In contrast, MPO, ESR1, PI3K, and SLC6A4 had a slightly weaker binding capacity. These results suggest that melatonin may treat periodontitis by inhibiting the expression of MMP8 and MMP9.

3.3. Melatonin ameliorated bone loss in ligation-induced experimental periodontitis

Next, we conducted animal experiments to verify our hypothesis. The experimental design is shown in Fig. 4a–b. To assess whether melatonin could alleviate experimental periodontitis in mice, we ligated the maxillary second molar of mice for LIP model establishment, and after successful establishment of LIP. Melatonin was injected intraperitoneally on the same day for treatment concurrently with the LIP modeling. Then the alveolar bone loss in the mesial and distal regions of the maxillary second molars of three groups of mice was measured by micro-CT. Alveolar bone loss was determined at the mesial and distal region of the second molar at the maxilla (Fig. 4c). Data on mesial and distal alveolar bone loss in the studied groups were shown in Fig. 4d. Compared to the CTR group, the CEJ-ABC distance in the LIP group was significantly increased in the mesial (0.137 ± 0.020 mm vs. 0.316 ± 0.045 mm) and distal (0.169 ± 0.020 mm vs. 0.324 ± 0.045 mm) sites, indicating that the filament ligation led to alveolar bone loss. Compared to the LIP group, the mesial (0.19 ± 0.020 mm) and distal (0.2 ± 0.020 mm) CEJ-ABC distance was significantly lower in the LIP + Mel group, but still higher than that in the CTR group. (Fig. 4d). Bone volume fraction (BV/TV) and bone trabeculae number (Tb.N) were significantly reduced in the LIP group and were alleviated by melatonin treatment. While bone trabecular separation showed a significant increase in the LIP group while under melatonin treatment, this increase was effectively suppressed under melatonin treatment. These results confirmed that melatonin indeed played a therapeutic role in the development of experimental periodontitis (Fig. 4e–f).

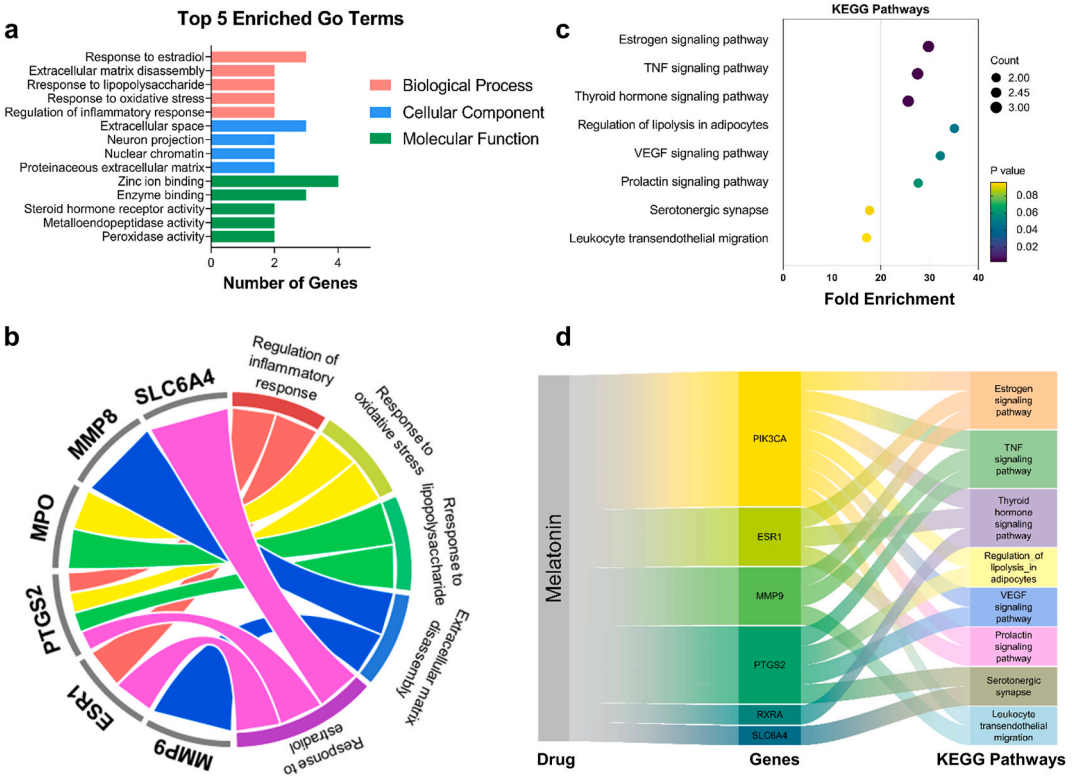

Fig. 2. The degree of enrichment of GO and KEGG for selected targets. (a) Bar graph showing the top 5 biological processes (BP), cellular components (CC) and molecular functions (MF) terms enriched by three groups of DEGs. (b) Correlation analysis of eight cross-targets with GO enrichment pathways. (c) Analysis of the top 8 enriched KEGG pathways. (d) Correlation analysis of the eight cross-targets with KEGG-enriched pathways.

3.4. MPO, MMP8, and MMP9 are key targets for melatonin treatment of periodontitis

Next, to verify whether the therapeutic effect was related to the potential targets, we chose MPO, MMP8, and MMP9 since they possessed high connectivity in network analysis (MPO, MMP9), or high binding affinity in molecular docking (MMP8, MMP9), and all closely related to neutrophils, one of the most important innate immune cells participating in periodontitis. We verified the protein expression levels of these potential targets using Western Blot (Fig. 5a–d). In the LIP group, the expression levels of both MPO, MMP8, and MMP9 were significantly increased, while their expression decreased under Melatonin treatment. To summary, these results suggest that upregulation of the expression levels of MPO/MMP8/MMP9 is closely associated with the establishment of LIP, while melatonin treatment alleviates this effect.

Then, to verify our conjecture at the histological level, we did immunofluorescence staining of periodontal tissues of mice (Fig. 5e). The results showed that the expression of both MPO and MMP8 were significantly higher in the gingival tissues of the LIP group compared to the CTR group, whereas the expression of MPO, MMP8, and MMP9 were significantly downregulated under melatonin treatment, confirming the therapeutic effect of melatonin.

To further identify whether melatonin could act directly on these three targets, we selected the HL60 line to verify the direct effect of melatonin on the expression of these targets. First, we induced the differentiation of immature HL60 cells into mature differentiated neutrophils (Fig. 5f). We added LPS to the medium to simulate the effect of periodontitis *in vitro*, and melatonin was also added for therapeutic effect. Afterwards, we used qPCR to observe the transcriptional levels of these three key targets, and the primer sequences are shown in Table 1. We can see that MPO transcript levels increased 1.5-fold, MMP8 increased 3-fold, and MMP9 increased significantly 6-fold in the LPS stimulation compared to the CTR group. Melatonin treatment suppressed this effect and restored it to the level of the CTR group (Fig. 5g). At the same time, we also examined the mRNA levels of three other genes with high PPI connectivity and stable binding to melatonin molecules (Fig. S1). We found that although LPS significantly up-regulated the mRNA levels of COX-2 and ESR1, melatonin treatment did not reverse this effect. And the mRNA levels of RXRA in the LPS and LPS + Mel groups were significantly down-regulated compared to the CTR group. This suggests that COX2, ESR1, and RXRA may not be potential targets for melatonin treatment of periodontitis.

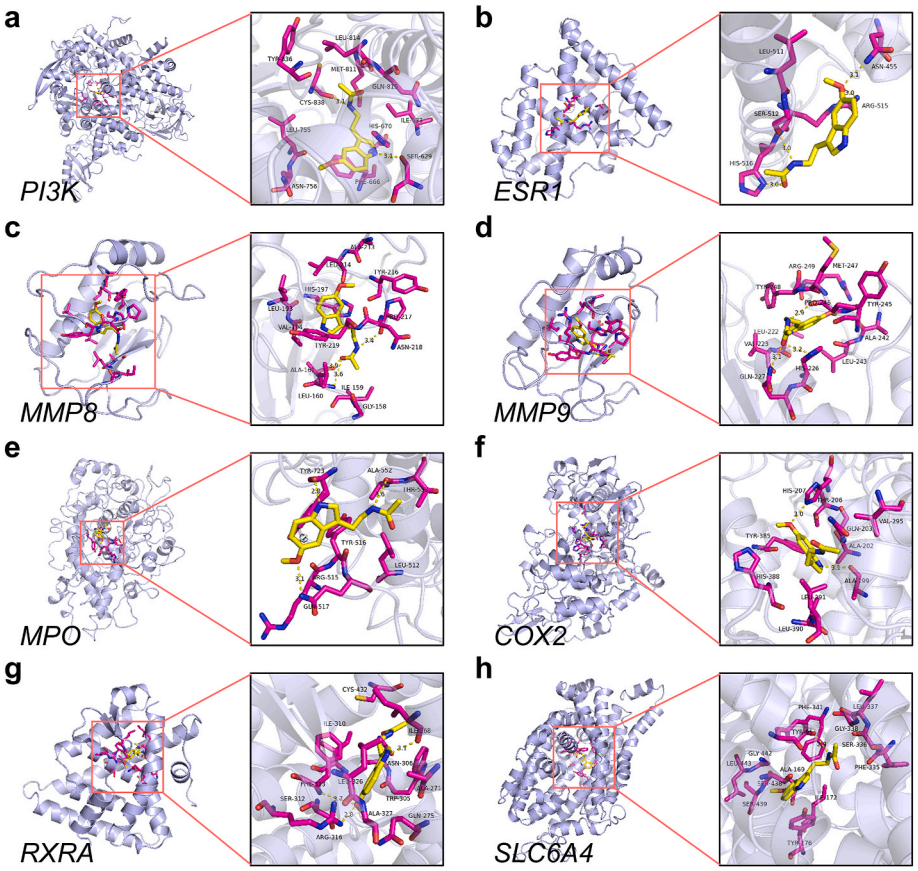

Fig. 3. Molecular docking of eight cross-targets in the Wayne diagram with melatonin. (a) PI3K. (b) ESR1. (c) MMP8. (d) MMP9. (e) MPO. (f) COX2. (g) RXRA. (h) SLC6A4.

Table 2
Molecular docking affinity of eight potential targets.

Targets	PDB ID	Box_center (x, y, z)	Box_size (x × y × z)/Å	Affinity (Kcal/mol)
PI3K	4JPS	(-2.750, −10.938, 16.869)	18.75 × 18.75 × 18.75	−7.6
ESR1	6SBO	(44.174, 4.061, 16.508)	18.75 × 18.75 × 18.75	−7.1
MMP8	1I76	(30.489, 58.392, 55.723)	18.75 × 18.75 × 18.75	**−8.1**
MMP9	6ESM	(2.760, 47.803, 22.820)	18.75 × 18.75 × 18.75	**−8.5**
MPO	5MFA	(-26.881, 0.345, 1.529)	47.25 × 47.25 × 47.25	−6.2
COX2	5F19	(28.948, 28.208, 66.918)	18.75 × 18.75 × 18.75	**−8.2**
RXRA	1MV9	(54.712, 45.030, 30.215)	18.75 × 18.75 × 18.75	**−8.1**
SLC6A4	5I6X	(-32.635, −18.694, 0.583)	18.75 × 18.75 × 18.75	−7.7

4. Discussion

In this study, we used a combination of network pharmacology and experimental validation to investigate the potential mechanism of melatonin in treating periodontitis. Our analysis revealed three key findings: First, through network pharmacology analysis, we identified MPO, MMP8, and MMP9 as potential key targets of melatonin for the treatment of periodontitis. Second, we found that in an experimental periodontitis mouse model, peritoneal melatonin treatment was effective in alleviating periodontitis induced alveolar bone loss, and this treatment effect was highly correlated with the inhibition of MPO, MMP8, and MMP9 expression. Finally, we demonstrated that melatonin directly suppressed the high transcriptional levels of MPO, MMP8, and MMP9 induced by LPS in an *in vitro* model.

The results of previous studies had shown that MMP8 and MMP9 are significantly increased in the saliva of patients with periodontitis [56–58]. Also, both MMP8 and MMP9 were extremely valuable diagnostic markers for the treatment of periodontitis [59,60]. MMP8 is currently one of the most promising biomarkers of periodontitis in oral fluids and MMP9 has been shown to be a more sensitive marker of periodontal inflammation during orthodontic treatment [61,62]. Meanwhile, the MPO, MMP8, and MMP9 levels

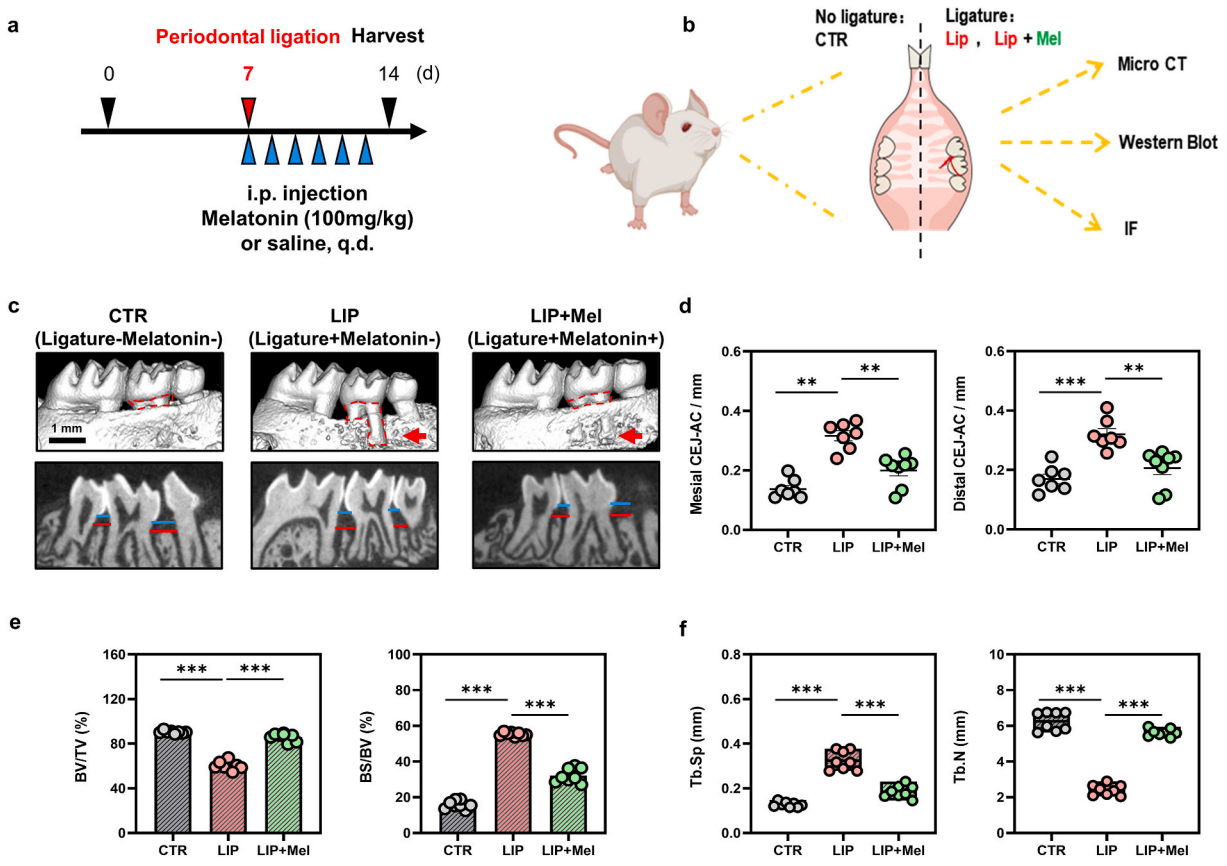

Fig. 4. (a) Timeline of an experiment into ligature-induced periodontitis and Mel treatment. (b) Schematic diagram of ligature induced periodontitis and Mel treatment in mice. (c) 3D reconstruction of Micro-CT scanned images of the alveolar bone. Scale bar = 1 mm (d)The quantitative statistics of mesial (left) and distal (right) CEJ-ABC distances of alveolar bone loss. (e) Comparison of the bone volume fraction (BV/TV) and bone surface-volume ratio (BS/BV). (f) Comparison of the Bone trabecular separation (Tb.Sp) and Number of bone trabeculae (Tb.N).

within the saliva of patients with periodontitis also decrease more significantly after three months of undergoing periodontal treatment [63–65].

In the present study, our network pharmacology results showed that periodontitis and melatonin share 8 common targets. Among them, MMP8 and MMP9, which are associated with matrix hydrolysis, are two key gene targets. In which MMP8 and MMP9, which are associated with matrix hydrolysis, is a key gene target. GO and KEGG enrichment analysis further showed that the oxidative respiratory burst effect was highly relevant in the development of periodontitis and also was a relevant target for melatonin to exert its antioxidant effect, which may suggest that MPO plays an important role in development of periodontitis [66–69]. And these are also consistent with the results of previous studies that MPO expression was significantly increased in the setting of periodontitis.

However, there are no studies demonstrating a correlation between the therapeutic effects of melatonin in periodontitis on MPO, MMP8, and MMP9. Therefore, in our study, we speculate that melatonin treats periodontitis by inhibiting the expression of MPO, MMP8, and MMP9. So next we did molecular docking of melatonin with MPO, MMP8, and MMP9, to verify whether melatonin could bind directly to these key targets. The results showed that melatonin bind to MMP9 and MMP8 in a more stable and direct way, which suggests that melatonin might be able to directly inhibit the expression of MMP9 and MMP8, but the binding to MPO was weaker.

The next animal experiments also confirmed our hypothesis. We performed animal experiments to evaluate the effect of melatonin on neutrophil-related protein targets. We found that the expression of MPO, MMP8, and MMP9 were significantly higher in the LIP group and melatonin could inhibit this high expression, while paraffin sections of gingival tissue showed the same results. The fluorescence intensity of MPO and MMP8 was significantly increased in the LIP group, while the fluorescence intensity of MPO, MMP8, and MMP9 were significantly decreased under Melatonin treatment. This verified our conjecture that melatonin can treat periodontitis by inhibiting the expression and function of MPO, MMP8, and MMP9 in periodontal tissues under periodontitis environment. Finally, in an *in vitro* model, our experimental results also show that melatonin directly suppresses the high transcript levels of MPO, MMP8, and MMP9 induced by LPS. Because both MPO and MMPs are closely related to neutrophils, the above experimental results also suggest that neutrophils may be important target cells for melatonin treatment of periodontitis.

Notably, in our investigation, we revealed a new line of thought on melatonin for the treatment of periodontitis by combining network pharmacology with experimental validation. Our experiments verified our hypothesis that melatonin dose have a potential

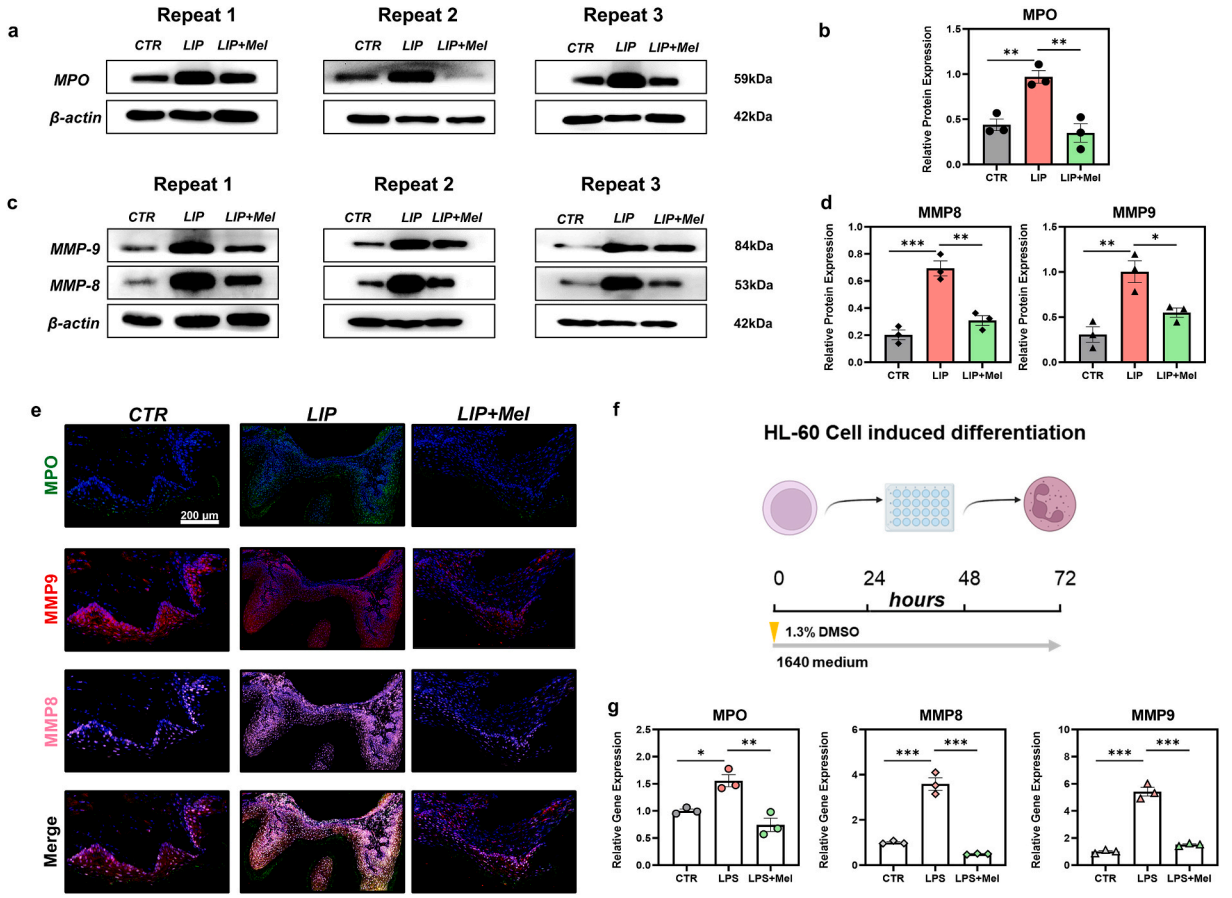

Fig. 5. (a) Western blot analysis of MPO expression in gingival tissue from CTR, LIP and LIP + Mel. (b) Quantification of MPO expression, normalized to β-actin expression. n = 3. (c) Western blot analysis of MMP8 and MMP9 expression in gingival tissue from CTR, LIP and LIP + Mel. (d) Quantification of MMP8 and MMP9 expression, normalized to β-actin expression. n = 3. (e) Immunofluorescence images of the gingival tissue. Scale bar, 200 μm. Magnification (5.0×). (f) Schematic diagram of HL60 cell induced differentiation. (g) mRNA expression levels of three key targets, MPO, MMP8 and MMP9.

therapeutic role in periodontitis. At the same time, our results answered the question that MPO, MMP8, and MMP9 are a promising target for the treatment of periodontitis. This experiment provides a more complete mechanism for the treatment of periodontitis with melatonin by combining network pharmacology with experimental validation. This may provide new insights into the molecular mechanisms of melatonin treatment of periodontitis, and it may also help to further explore the role of melatonin in the regulation of its potential targets.

Nonetheless, there are some limitations to this study. We only demonstrated that melatonin reduces the expression of MPO, MMP8, and MMP9 in the periodontitis setting, but we failed to explore the underlying causes. The mechanisms involved in the reduction of their expression by melatonin are not yet known, which need to be explored in further experiments.

5. Conclusions

In conclusion, this study shows that melatonin can effectively treat ligation-induced periodontitis as a complementary agent and by reducing the function and expression of MPO, MMP8, and MMP9. However, the exact mechanism of melatonin for periodontitis is not clear and this needs to be further investigated. Our experiments provide some insights into melatonin treatment of other local inflammatory diseases.

Ethics approval and consent to participate

All animal experiments were performed in accordance with the Declaration of Helsinki and the NIH Guide for the Care and Use of Laboratory Animals. All treatments were performed gently, and every effort was made to minimize animal suffering. The protocol was reviewed and approved by the Dental Ethics Committee of Chongqing Medical University (Ethics No. 2023034).

Consent for publication

Not applicable.

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Data availability statement

Bioinformatic analysis used data from public database and can be easily accessed according to the method. All data generated during this study are included in this article and supplementary materials. Raw data is available upon reasonable request.

CRediT authorship contribution statement

Kamoran Tuerhong: Writing – original draft, Validation. **Kehao Liu:** Methodology, Data curation. **Danfeng Shen:** Methodology, Investigation. **Qianyu Zhang:** Methodology, Formal analysis. **Qi Huang:** Formal analysis. **Mingcong Yang:** Formal analysis. **Ziyu Huang:** Formal analysis. **Lu Wang:** Supervision, Methodology, Funding acquisition, Data curation. **Sheng Yang:** Supervision, Software, Investigation, Funding acquisition, Data curation, Conceptualization. **Yuzhou Li:** Validation, Supervision, Resources, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32494.

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