Am J Transl Res 2026;18(3):2380-2392
www.ajtr.org /ISSN:1943-8141/AJTR0170394

*Original Article*

# Melatonin ameliorates TNFα-induced oral epithelial cell inflammation via Keap1-Nrf2 axis modulation

Shuangqing Wang[1*], Wenjing Wang[2*], Rongxia Zhang[3], Yaxuan Liu[3], Yamei Wang[4], Yang Bai[4], Yunfang Bai[5]

*[1]Department of Prosthodontics, Hebei Eye Hospital, Xingtai 054001, Hebei, China; [2]Department of Stomatology, The Fourth Hospital of Hebei Medical University, Shijiazhuang 050000 Hebei, China; [3]Department of Stomatology, Second Hospital of Shijiazhuang, Shijiazhuang 050000, Hebei, China; [4]Department of Stomatology, Qinhuangdao Haigang Hospital, Qinhuangdao 066000, Hebei, China; [5]Department of Emergency, The Fourth Hospital of Hebei Medical University, Shijiazhuang 050000, Hebei, China. [*]Equal contributors and co-first authors.*

Received November 29, 2025; Accepted February 11, 2026; Epub March 15, 2026; Published March 30, 2026

**Abstract:** Background: Oral mucosal inflammatory diseases comprise a diverse array of conditions that affect the delicate tissues lining the oral cavity, posing a multifaceted challenge in clinical practice. Further studies are needed to elucidate the pathogenesis of these conditions. Melatonin has emerged as a promising antioxidant that scavenges free radicals and attenuates oxidative stress, a key contributor to inflammatory processes. Methods: This study employed two oral epithelial cell lines, human oral epithelial cells (HOEC) and human oral keratinocytes (HOK), alongside a three-dimensional (3D) epithelial cell model, to investigate the effects of melatonin on oral epithelial inflammation. Results: The findings revealed that TNFα significantly induced inflammation in oral epithelial cells, whereas melatonin inhibited TNFα-induced inflammation. Furthermore, melatonin's action in mitigating inflammation in oral epithelial cells was mediated via its receptor, MTNR1A. Mechanistically, melatonin suppressed inflammation in oral epithelial cells through the Keap1/Nrf2 signaling pathway. Additionally, melatonin attenuated TNFα-induced inflammation in the 3D oral epithelial cell model. Conclusion: These findings offer novel insights into the potential development of therapies for treating oral epithelial inflammation.

**Keywords:** Melatonin, TNFα, inflammation, oral epithelial cells, Keap1-Nrf2 axis

## Introduction

Oral mucosal inflammatory diseases encompass a heterogeneous spectrum of disorders that disrupt the integrity of oral cavity linings, imposing substantial clinical burdens due to their chronicity, recurrent presentation, and limited therapeutic efficacy [1]. Treatment strategies are equally diverse, aiming to alleviate symptoms, address underlying causes, and prevent recurrence [2]. Management approaches range from topical corticosteroids and antifungals to immunomodulatory agents and lifestyle modifications, tailored to each patient's unique presentation and needs [2]. In addition to pharmacotherapy, patient education plays a pivotal role in empowering individuals to adopt optimal oral hygiene practices, recognize symptom triggers, and seek timely professional care [3]. Despite these interventions, a critical gap persists: the lack of targeted therapies that address the oxidative stress-driven inflammatory cascades underlying disease pathogenesis. This knowledge gap underscores the urgent need to identify novel, mechanistically validated agents that can modulate key signaling pathways involved in oral mucosal inflammation.

Melatonin, often referred to as the "hormone of darkness" due to its secretion by the pineal gland in response to darkness, is a versatile molecule with wide-ranging functions beyond its renowned role in regulating the sleep-wake cycle [4]. Its emergence as a potent anti-inflammatory agent indicates new therapeutic avenues for inflammation management [4]. In addition to modulating circadian rhythms, melatonin demonstrates remarkable antioxidant properties, scavenging free radicals and mitigating oxidative stress - a pivotal contributor to inflam-

https://doi.org/10.62347/ULDG8122

matory processes [5]. As research continues to unravel the intricacies of melatonin's immunomodulatory actions, its burgeoning utility as a supplementary agent in inflammation management holds promise for enhanced therapeutic strategies and improved patient outcomes.

Beyond its canonical role in circadian rhythm regulation, melatonin acts as a key modulator of the Keap1/Nrf2 signaling pathway-a central cellular defense axis against oxidative stress that governs the transcription of antioxidant and detoxification genes [6]. Melatonin exerts its regulatory effects by promoting Nrf2 nuclear translocation, enhancing its binding to antioxidant response elements in target gene promoters, and inducing Keap1 degradation to amplify Nrf2 activation [6]. This targeted modulation of the Keap1/Nrf2 pathway underscores melatonin's capacity to restore cellular redox balance, a function particularly relevant to oral epithelial inflammation, where oxidative stress-driven damage and pro-inflammatory cascades are core pathogenic drivers. Importantly, this mechanism positions melatonin as a potential therapeutic agent for oral mucosal inflammatory disorders, with its pathway-specific activity offering a mechanistic basis for mitigating epithelial injury [7]. While preclinical studies have hinted at melatonin's anti-inflammatory potential in oral epithelial tissues, the specific mechanistic link between melatonin, the Keap1/Nrf2 pathway, and inflammation modulation in oral mucosal cells remains unelucidated, representing a critical scientific question that has not been systematically addressed.

Against this backdrop, the present study was designed to fill this knowledge void with three core objectives: (1) to evaluate the direct anti-inflammatory effects of melatonin on two representative oral epithelial cell lines (human oral epithelial cells [HOEC] and human oral keratinocytes [HOK]) under inflammatory stimuli; (2) to elucidate the mechanistic role of the Keap1/Nrf2 signaling pathway in mediating melatonin's anti-inflammatory actions in these cells; and (3) to validate the therapeutic potential of targeting this pathway for oral mucosal inflammation management. We hypothesized that melatonin would suppress pro-inflammatory cytokine production and oxidative stress in HOEC and HOK cells by activating the Keap1/Nrf2 pathway, with Nrf2 knockdown abrogating these protective effects. The primary innovation of this work lies in its cell-type-specific mechanistic validation of melatonin's Keap1/Nrf2-dependent anti-inflammatory activity in oral epithelial cells-a critical step that bridges translational gaps between basic melatonin research and its clinical application for oral mucosal inflammatory diseases. Our findings confirm that melatonin exerts anti-inflammatory effects in oral epithelial cells via the Keap1/Nrf2 signaling pathway, providing a mechanistic foundation for developing melatonin-based targeted therapies to address unmet clinical needs in oral mucosal inflammation management.

## Materials and methods

### *Cell culture*

Two human oral epithelial cell lines, HOEC (catalog #AC34021) and HOK (catalog #FE1439), were obtained from ATCC. These cell lines were cultured in Dulbecco's modified Eagle medium (ThermoFisher, catalog #10569010) supplemented with 10% fetal bovine serum (ThermoFisher, catalog #16140071) and 1% penicillin-streptomycin (ThermoFisher, catalog #151-40148). Cultures were maintained in a humidified chamber at 37°C with 5% $CO_2$. Human oral epithelial cells (HOEC) and human oral keratinocytes (HOK) were authenticated by short tandem repeat (STR) profiling to confirm their species origin, identity, and absence of cross-contamination. STR analysis was performed by the vendors.

### *3D oral epithelial model culture*

The culture of the 3D oral epithelial model followed a previously established protocol [8]. Briefly, a scaffold-based method using Matrigel® (hereafter referred to as Matrigel®, Corning, catalog #356231) was used, and oral epithelial cell lines, HOEC (20,000 cells/well) and HOK (20,000 cells/well) were mixed (1:1) with 50% Matrigel®, followed by seeding onto the bottom of a 96-well plate (8 mL per well). The plate was incubated in a cell culture incubator for at least 10 min to allow the Matrigel to solidify, followed by the addition of 100 µL of cell culture media to each well. The entire medium was renewed every other day. The 3D models were validated by assessing 3D spheroid morphology (as shown in **Figure 6**) and were cultured for a

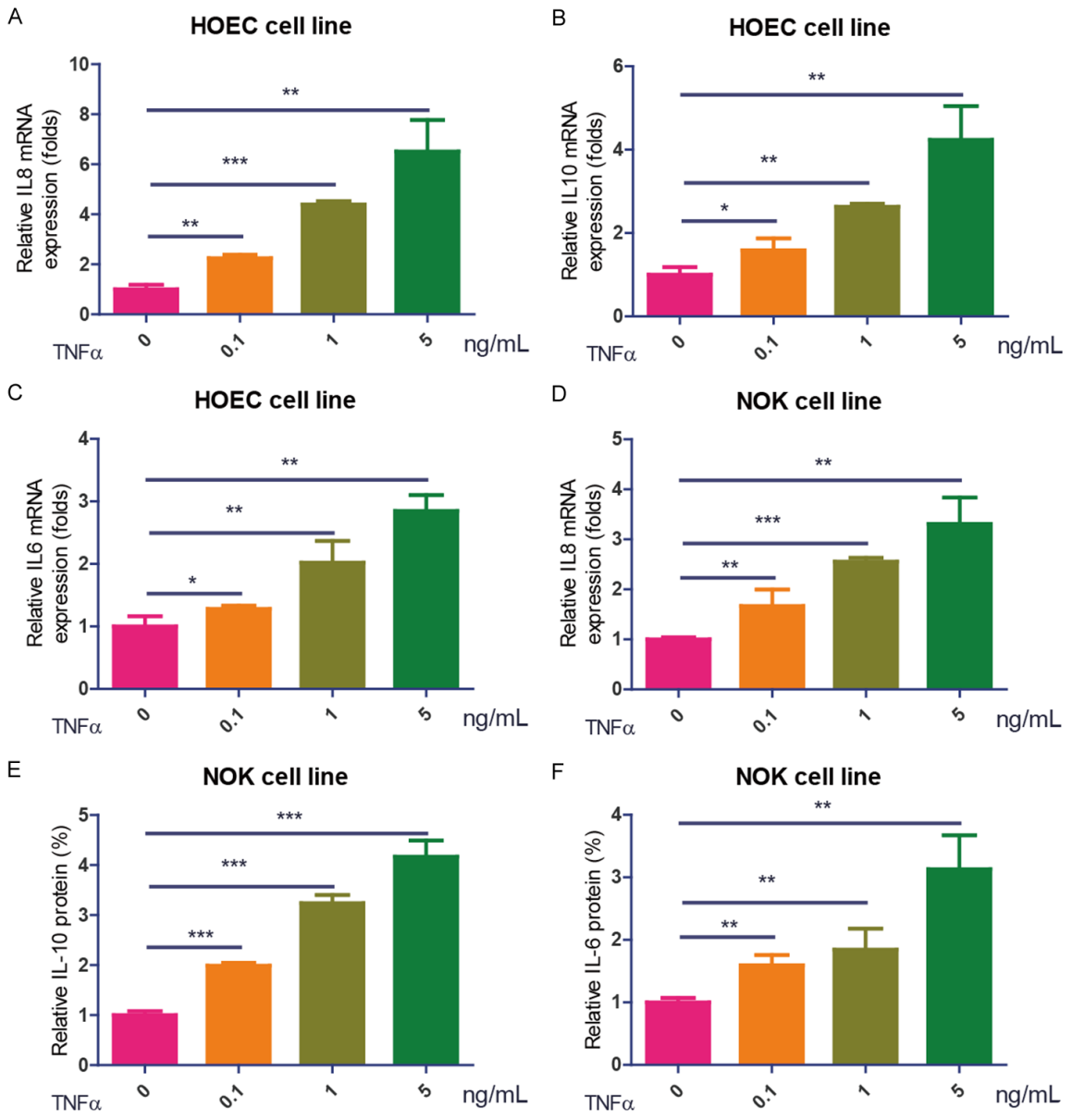



**Figure 1.** TNFα significantly induces inflammation in oral epithelial cells. A. TNFα significantly increased expression of IL8 in HOEC cells (**P<0.01, ***P<0.001). B. TNFα significantly increased expression of IL8 in HOK cells (**P<0.01, ***P<0.001). C. TNFα significantly increased expression of TNFα in HOEC cells (*P<0.05, **P<0.01). D. TNFα significantly increased expression of TNFα in HOK cells (***P<0.001). E. TNFα significantly increased expression of IL6 in HOK cells. TNFα significantly increased expression of IL6 in HOK cells (*P<0.05, **P<0.01). F. TNFα significantly increased expression of IL6 in HOK cells (**P<0.01).

4-day period before the initiation of subsequent experimental procedures.

*Enzyme-linked immunosorbent assay (ELISA) for detecting inflammatory and anti-inflammatory factors*

The ELISA assay was performed as described previously [9, 10]. Cell supernatants from HOEC and HOK cultures were harvested to quantify interleukin (IL)-6, IL-8, and IL-10 levels after treatment for 48 hours. Detection of these cytokines in cell supernatants was performed using specific ELISA kits for IL-8 (Abcam, catalog #ab214030), IL-6 (Abcam, catalog #ab178013), and IL-10 (Abcam, catalog #ab185986). The assays were conducted according to the manufacturer's instructions

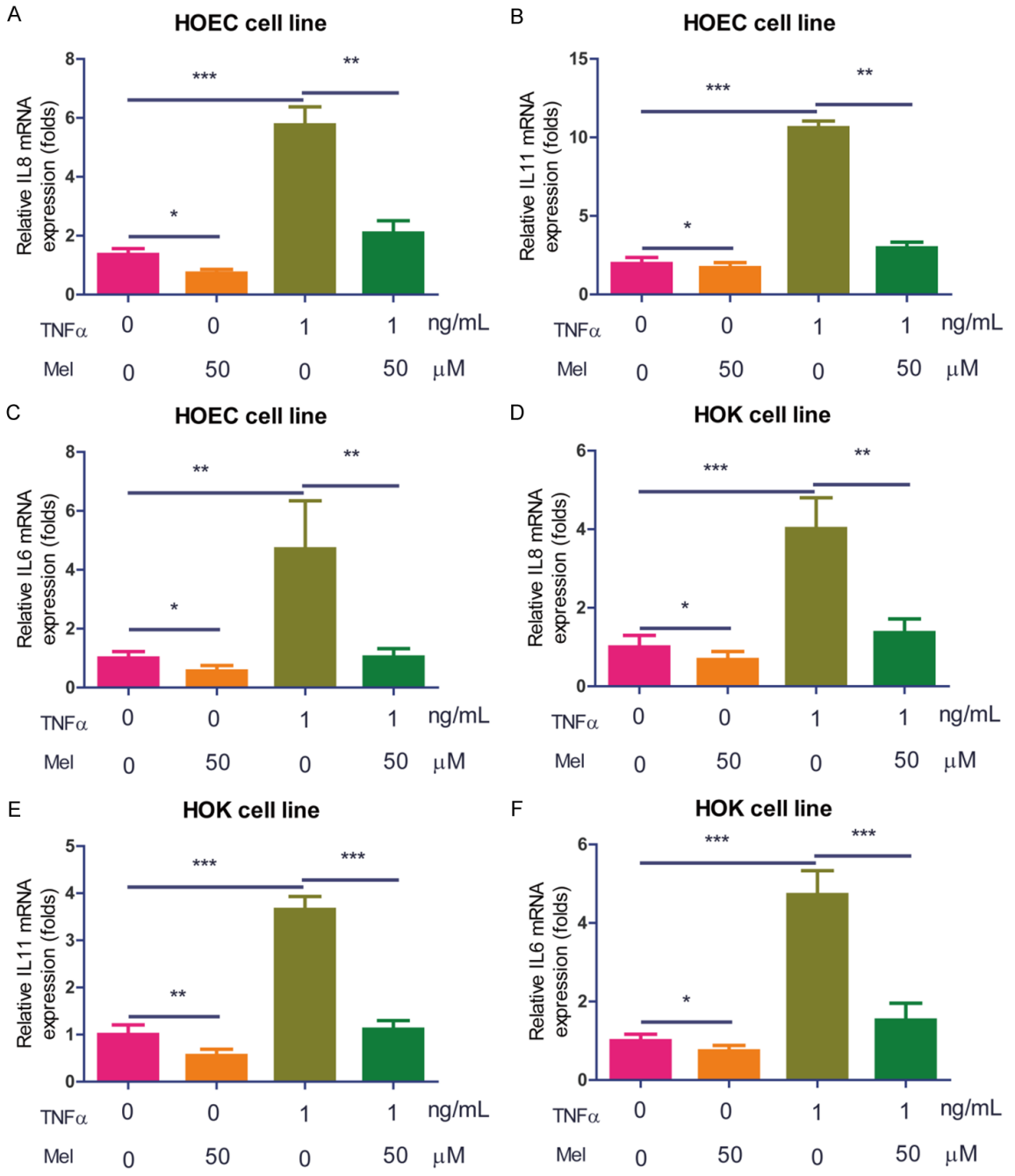



**Figure 2.** Melatonin inhibits TNFα induced inflammation. A. Melatonin (50 μM) inhibited expression level of IL-8 at basal levels and TNFα stimulated conditions in HOEC cells (*P<0.05, ***P<0.001). B. Melatonin (50 μM) inhibited expression level of IL-11 at basal levels and TNFα stimulated conditions in HOEC cells (*P<0.05, ***P<0.001). C. Melatonin (50 μM) inhibited expression level of IL-6 at basal levels and TNFα stimulated conditions in HOEC cells (*P<0.05, **P<0.01, ***P<0.001). D. Melatonin (50 μM) inhibited expression level of IL8 at basal levels and TNFα stimulated conditions in HOK cells (**P<0.01, ***P<0.001). E. Melatonin (50 μM) inhibited expression level of IL-11 at basal levels and TNFα stimulated conditions in HOK cells (**P<0.01, ***P<0.001). F. Melatonin (50 μM) inhibited expression level of IL-6 at basal levels and TNFα stimulated conditions in HOK cells (*P<0.05, **P<0.01, ***P<0.001).

(Shanghai ExCell Biotechnology) and read at a wavelength of 600 nm. These assays enabled precise measurement of IL-8, IL-6, and IL-10 concentrations, facilitating assessment of inflammatory responses in HOEC and HOK cells.

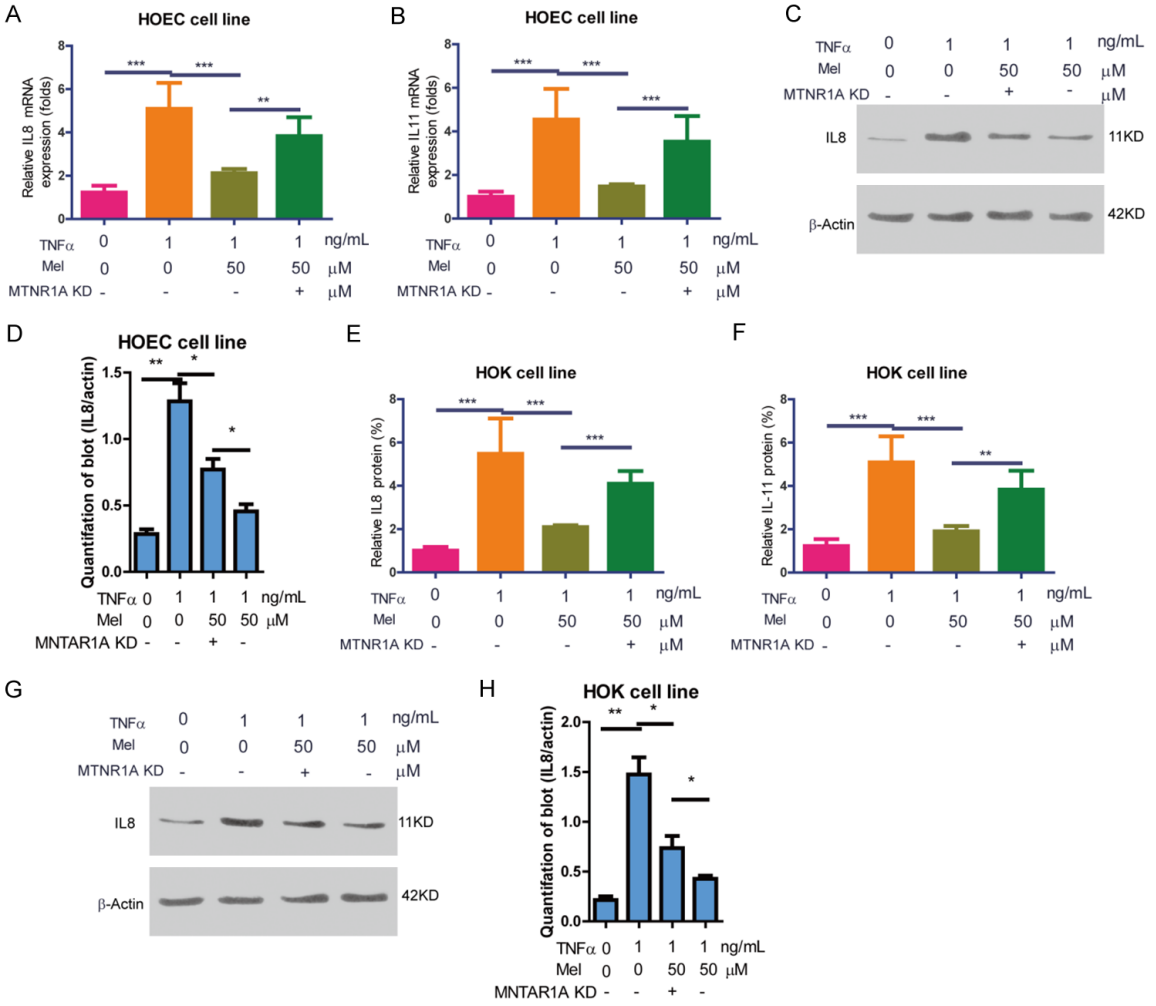



**Figure 3.** Melatonin inhibits inflammation of oral epithelial cells via its receptor, MTNR1A. (A) MTNR1A knockdown (KD) significantly compromised effects of melatonin on expression levels of IL-8 in HOEC cells (**P<0.01, ***P<0.001). (B) MTNR1A knockdown (KD) significantly compromised effects of melatonin on expression levels of IL-11 in HOEC cells (***P<0.001). (C) MTNR1A knockdown (KD) significantly compromised effects of melatonin on protein levels of IL8 in HOEC cells. (D) Quantification of IL-8 protein of (C). (E) MTNR1A knockdown (KD) significantly compromised effects of melatonin on expression levels of IL-8 in HOK cells (***P<0.001). (F) MTNR1A knockdown (KD) significantly compromised effects of melatonin on expression levels of IL11 in HOK cells (**P<0.01, ***P<0.001). (G) MTNR1A knockdown (KD) significantly compromised effects of melatonin on protein levels of IL8 in HOK cells. (H) Quantification of IL-8 protein of (G).

*RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

TRIzol reagent (Beyotime Institute of Biotechnology) was used for total RNA extraction from HOEC and HOK cells (seeded in 96-well plates at a density of 6,000 cells per well), following the manufacturer's instructions. The BeyoRT First Strand cDNA Synthesis kit (catalog #D7166; Beyotime Institute of Biotechnology) was used for cDNA synthesis from the isolated RNA. RT-qPCR was performed to measure expression levels of genes including IL-8, IL-10, and IL-6 in both HOEC and HOK cells using BeyoFast SYBR Green qPCR Mix (2×; catalog #D7260-25 ml; Beyotime Institute of Biotechnology) on a 7500 Fast Real-time PCR System (Applied Biosystems, ThermoFisher Scientific, Inc.). The qPCR program comprised initial denaturation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. GAPDH served as an internal reference

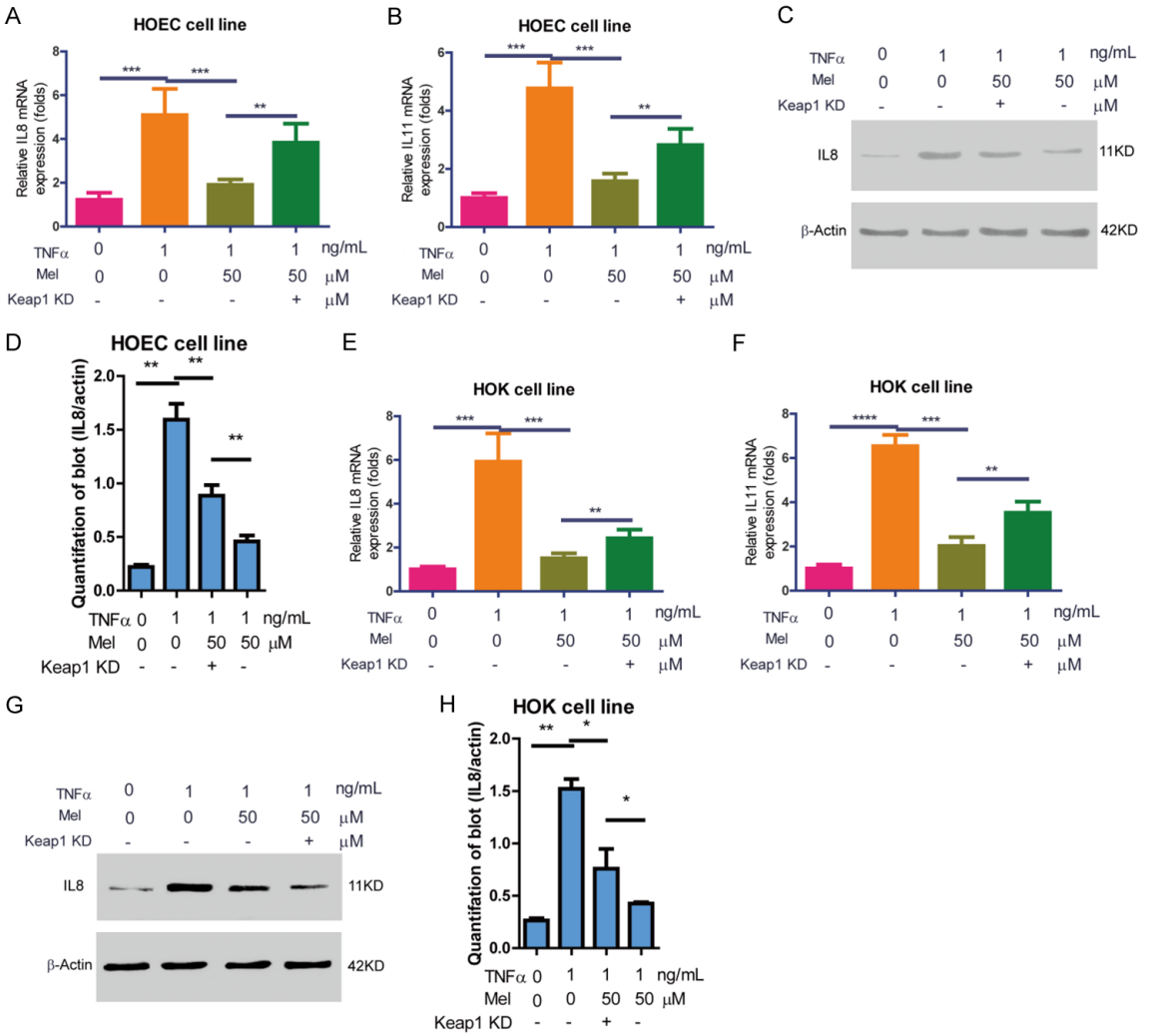



**Figure 4.** Melatonin inhibits inflammation of oral epithelial cells via Keap1. (A) Keap1 KD significantly compromised effects of melatonin on expression levels of IL-8 in HOEC cells (**P<0.01, ***P<0.001). (B) Keap1 KD significantly compromised effects of melatonin on expression levels of IL-11 in HOEC cells (**P<0.01, ***P<0.001). (C) Keap1 KD significantly compromised effects of melatonin on protein levels of IL-8 in HOEC cells. (D) Quantification of IL-8 protein of (C). (E) Keap1 KD significantly compromised effects of melatonin on expression levels of IL-8 in HOK cells (***P<0.001). (F) Keap1 KD significantly compromised effects of melatonin on expression levels of IL-11 in HOK cells (**P<0.01 ***P<0.001). (G) Keap1 KD significantly compromised effects of melatonin on protein levels of IL-8 in HOK cells. (H) Quantification of IL-8 protein of (G).

gene. Relative gene expression levels were determined using the $2^{-\Delta\Delta Cq}$ method [11]. The primers for IL-8, IL-10, IL-6, and GAPDH used in this study are listed in **Table 1**. Each experiment was performed in triplicate.

*Western blotting analysis*

Cell supernatants from HOEC and HOK cultures were harvested to perform western blotting after treatment for 48 hours. Cell lysates were prepared using RIPA buffer (50 mM Tris, pH 7.6; 150 mM NaCl; 1% Triton X-100; 1 mM EDTA; 0.5% sodium deoxycholate; and 0.1% SDS) containing a protease inhibitor (Roche, Indianapolis, IN). Protein concentrations were measured using the BCA method (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific, Lenexa, KS). Equal amounts of protein (35 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Blots were probed overnight at 4°C using polyclonal antibodies specific for IL-8 (Anti-IL-8 antibody, Abcam, catalog number: ab7747) or β-actin (Abcam, catalog number: ab8226). Membranes

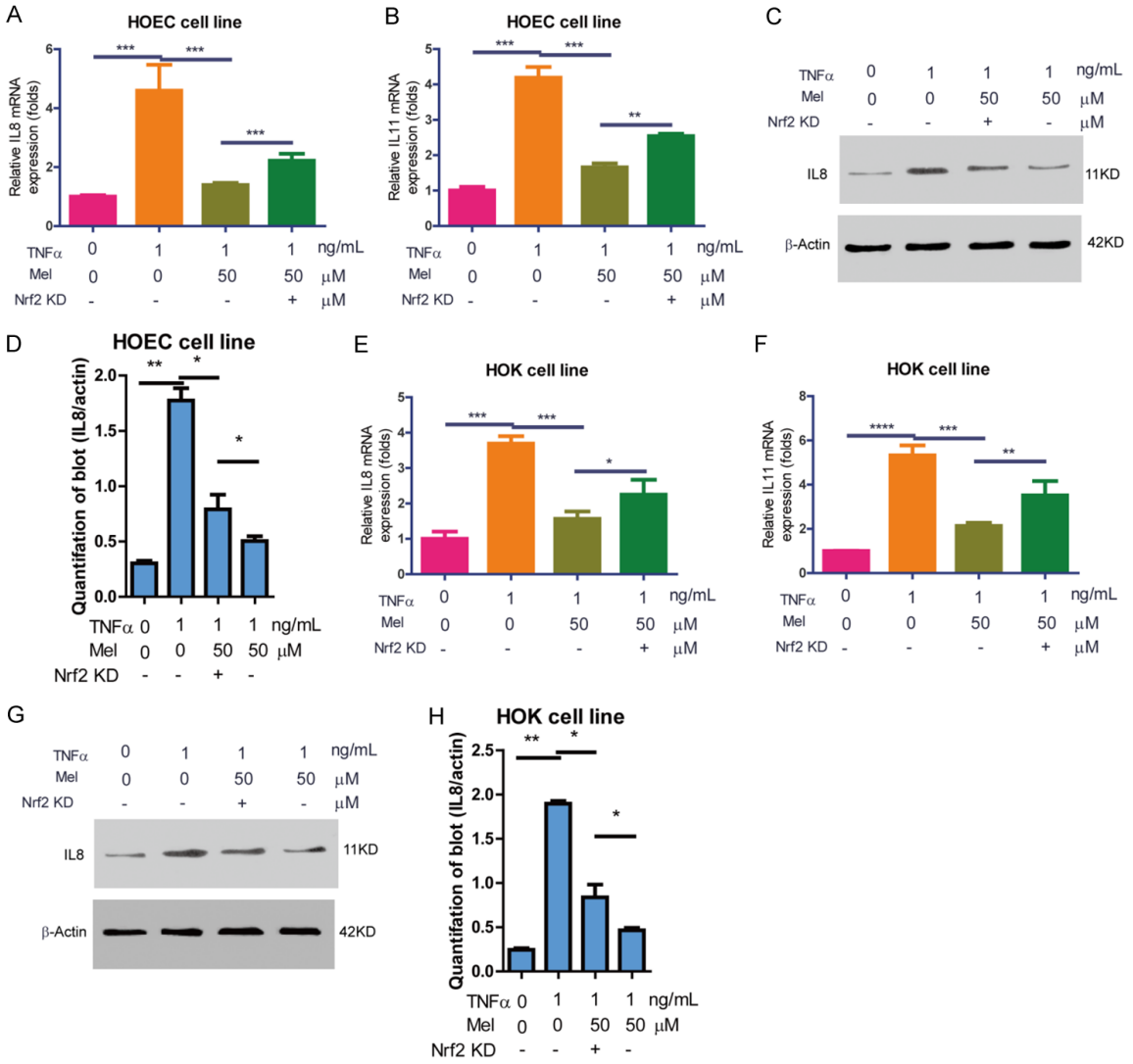



**Figure 5.** Melatonin inhibits inflammation of oral epithelial cells via Nrf2. (A) Nrf2 KD significantly compromised effects of melatonin on expression levels of IL-8 in HOEC cells (***P<0.001). (B) Nrf2 KD significantly compromised effects of melatonin on expression levels of IL-11 in HOEC cells (**P<0.01, ***P<0.001). (C) Nrf2 KD significantly compromised effects of melatonin on protein levels of IL-8 in HOEC cells. (D) Quantification of IL-8 protein of (C). (E) Nrf2 KD significantly compromised effects of melatonin on expression levels of IL-8 in HOK cells (*P<0.05, ***P<0.001). (F) Nrf2 KD significantly compromised effects of melatonin on expression levels of IL-11 in HOK cells (**P<0.01, ***P<0.001). (G) Nrf2 KD significantly compromised effects of melatonin on protein levels of IL-8 in HOK cells. (H) Quantification of IL-8 protein of (G).

were then washed and probed with secondary antibodies (Beyotime, catalog number: A0208) for 2 h at room temperature. Protein bands were visualized using Odyssey CLx. Band intensities were quantified via ImageJ and normalized to the internal reference protein (e.g., β-actin).

*Small interfering (si)RNA-based knockdown assay*

Gene knockdown (KD) was achieved using siRNA, following a previously described method [12]. Sequences targeting MT1, Keap1, and Nrf2 were designed using the siRNA-Target-Finder (GeneScript) and subsequently synthesized and procured from Synbio Technologies. The sequence for the negative control (NC) siRNA within the empty vector was 5'-UUCUCCGAACGUGUCACGU-3', siRNA-MTNR1A was 5'-AAGAAGCTCAGGAACGCAGGA-3', while that for siRNA-Keap1 was 5'-AAGTGCGAGATCCTGCAGTCC-3', and for siRNA-Nrf2 was 5'-AAGTAGGTAACTGTAGTCCAC-3'.

The siRNAs, including both non-targeting control siRNA and target-specific siRNA, were transiently transfected into HOEC and HOK cells

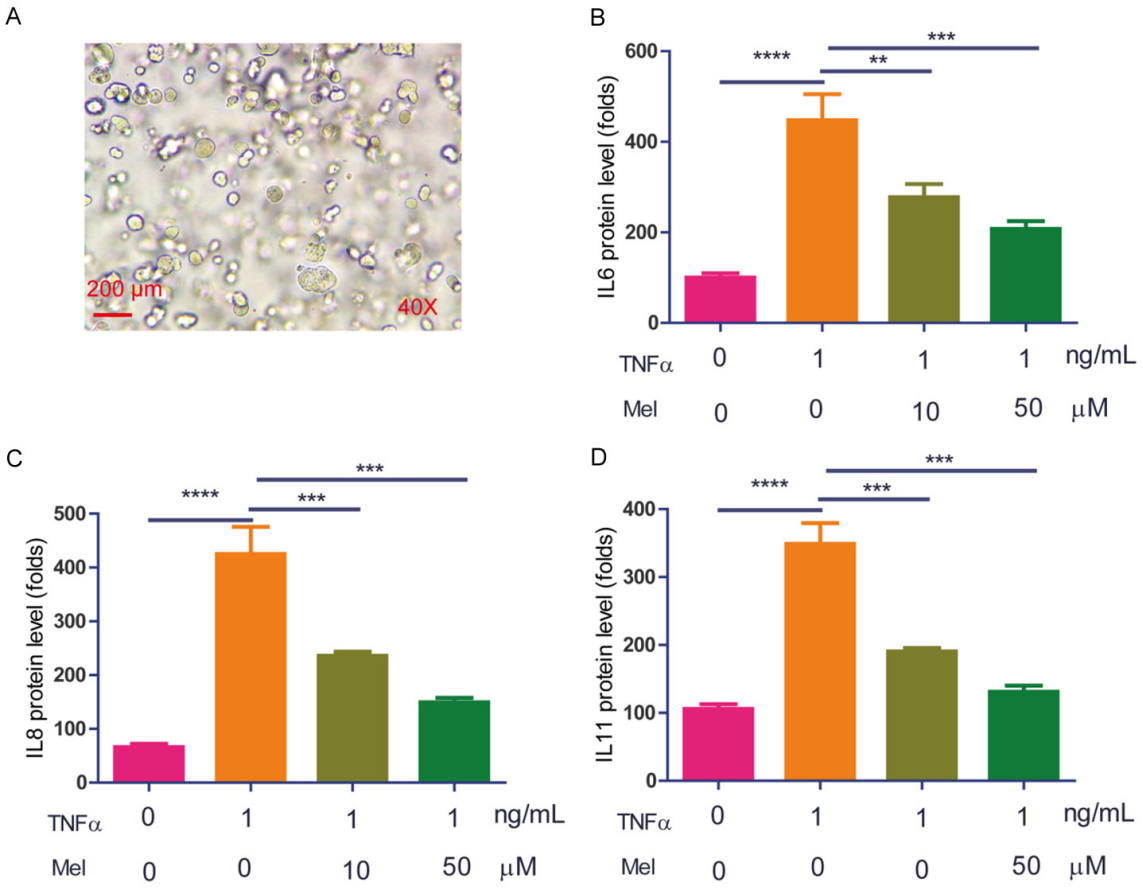



**Figure 6.** Melatonin inhibits TNFα induced inflammation on 3D oral epithelial cell model. A. A 3D oral epithelial cell model was developed and used for investigating effects of melatonin on inflammation in oral epithelial cells (magnification: 40×). B. Melatonin significantly reduced TNFα induced increase of protein levels of IL6 in a 3D oral epithelial cell model (**P<0.01, ***P<0.001). C. Melatonin significantly reduced TNFα induced increase of protein levels of IL8 in a 3D oral epithelial cell model (***P<0.001, ****P<0.0001). D. Melatonin significantly reduced TNFα induced increase of protein levels of IL11 in a 3D oral epithelial cell model (***P<0.001, ****P<0.0001).

**Table 1.** Primers used in the present study

| Gene | Primer | Sequence |
|---|---|---|
| MT1 | Sense | GACCATGCAGGGCAACGG |
| | Anti-sense | CCACAAAGATGTTTCCTGCGTT |
| Keap1 | Sense | ATGGCCACATCTATGCCGTC |
| | Anti-sense | GCTCTGGCTCATACCTCTCC |
| Nrf2 | Sense | AGGTTGCCCACATTCCCAAA |
| | Anti-sense | ACGTAGCCGAAGAAACCTCA |
| GAPDH | Sense | AATGGGCAGCCGTTAGGAAA |
| | Anti-sense | GCCCAATACGACCAAATCAGAG |

using FuGENE HD Transfection Reagent (catalog no. E2311; Promega Corporation) as per the manufacturer's guidelines, in a cell culture incubator (maintained at 37°C with 5% $CO_2$). Transfection with siRNA was performed 24 h before subsequent experiments. Knockdown efficiency was assessed using RT-qPCR and western blot assays, following the protocols described in this study.

*Statistical analysis*

Data are expressed as mean ± standard error of the mean. Statistical analysis of continuous variables was conducted using one-way ANOVA followed by Tukey's post-hoc test, while categorical variables were analyzed using Fisher's exact test. Correlation analysis was performed using Pearson's correlation coefficient. Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc.). A *p*-value <0.05 was considered statistically significant.

## Results

*TNFα significantly induces inflammation in oral epithelial cells*

To study the effects of TNFα on oral epithelial cell inflammation, HOEC and HOK cells were treated with different doses (0, 0.1, 1, and 5 ng/mL) of TNFα for 48 h. TNFα significantly increased expression of inflammation markers, including IL-8 in both HOEC (**Figure 1A**) and HOK (**Figure 1B**) cells, TNFα in both HOEC (**Figure 1C**) and HOK (**Figure 1D**) cells, and IL-6 in both HOEC (**Figure 1E**) and HOK (**Figure 1F**) cells. Overall, these findings demonstrated that TNFα significantly induces inflammation in oral epithelial cells.

*Melatonin inhibits TNFα-induced inflammation*

Accumulating evidence suggests that melatonin plays an important role in anti-inflammatory effects [5]. Therefore, it is important to explore the impact of melatonin on inflammation in oral epithelial cells. We found that melatonin (50 μM) could inhibit expression of inflammation markers, including IL-8 in both HOEC (**Figure 2A**) and HOK (**Figure 2D**) cells, IL-11 in both HOEC (**Figure 2B**) and HOK (**Figure 2E**) cells, and IL-6 in both HOEC (**Figure 2C**) and HOK (**Figure 2F**) cells with either non-TNFα treatment or TNFα treatment conditions. Taken together, our data demonstrate that melatonin inhibits TNFα-induced inflammation in oral epithelial cells.

*Melatonin inhibits inflammation of oral epithelial cells via its receptor, MTNR1A*

Since melatonin exerts its biological effects primarily through binding to specific G protein-coupled receptors (GPCRs), among which the melatonin receptor family (MTNRs) plays an indispensable role in mediating downstream signaling cascades that regulate cellular activities - including inflammation, proliferation, and stress responses - we aimed to dissect the molecular mechanism underlying melatonin's anti-inflammatory actions in oral epithelial cells. Notably, among the major melatonin receptor subtypes (MTNR1A/MT1 and MTNR1B/MT2), MTNR1A has been widely characterized as a key mediator of melatonin's anti-inflammatory effects across multiple tissues and cell types. Accumulating evidence demonstrates that MTNR1A is highly expressed in epithelial tissues, including the oral mucosa, and its activation has been shown to inhibit pro-inflammatory cytokine production (e.g., TNF-α, IL-6, and IL-1β) and suppress NF-κB signaling - pathways critically involved in oral epithelial inflammation. In contrast, MTNR1B exhibits a more restricted tissue distribution and has been less frequently implicated in epithelial inflammatory regulation compared to MTNR1A. Given these observations, combined with the lack of clarity regarding which melatonin receptor subtype mediates anti-inflammatory responses in oral epithelial cells, we specifically focused on MTNR1A to test the hypothesis that melatonin alleviates oral epithelium inflammation through the activation of this receptor subtype. Oral epithelial cells were treated with siRNA-MTNR1A. MTNR1A KD significantly compromised the effects of melatonin on the expression of inflammatory markers, including IL-8 (**Figure 3A**) and IL-11 (**Figure 3B**), in HOEC cells. Moreover, MTNR1A KD reduced the effects of melatonin on IL-8 protein levels (**Figure 3C** and **3D**) in HOEC cells. Similarly, MTNR1A KD significantly compromised the effects of melatonin on the expression of inflammatory markers, including IL-8 (**Figure 3E**) and IL-11 (**Figure 3F**), in HOK cells. Moreover, MTNR1A KD compromised the effects of melatonin on IL-8 protein levels (**Figure 3G** and **3H**) in HOK cells. Taken together, these results demonstrate that melatonin inhibits inflammation in oral epithelial cells via its receptor, MTNR1A.

*Melatonin inhibits inflammation in oral epithelial cells via Keap1*

Melatonin has been reported to be involved in Keap1/Nrf2 signaling [13, 14], prompting us to explore whether its effects on oral epithelial inflammation are mediated through Keap1/Nrf2. Oral epithelial cells were treated with siRNA-Keap1. Keap1 KD significantly compromised the effects of melatonin on the expression levels of inflammatory markers, including IL-8 (**Figure 4A**) and IL-11 (**Figure 4B**), in HOEC cells. Keap1 KD also compromised the effects of melatonin on IL-8 protein levels (**Figure 4C** and **4D**) in HOEC cells. Similarly, Keap1 KD significantly compromised the effects of melatonin on expression of inflammatory markers, including IL-8 (**Figure 4E**) and IL-11 (**Figure 4F**), in HOK cells. Keap1 KD also diminished the

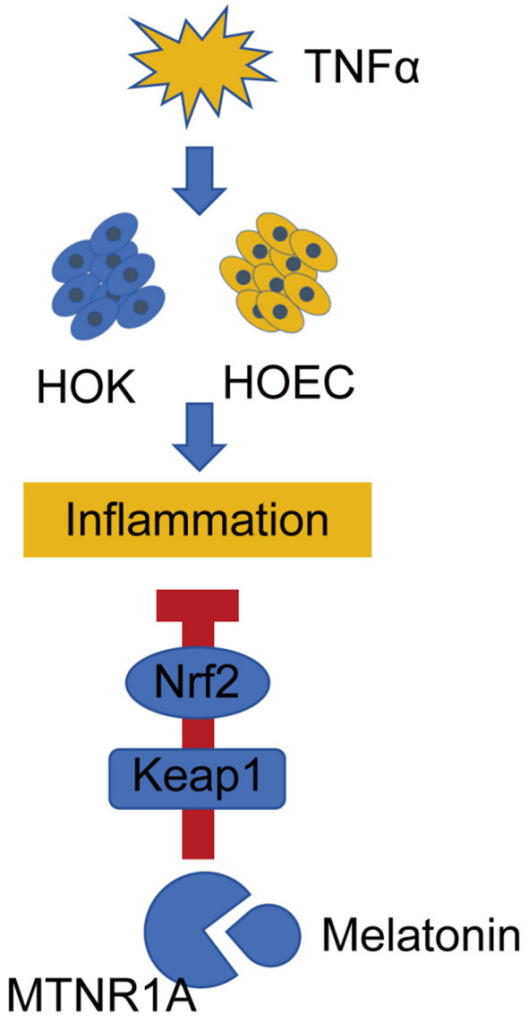



**Figure 7.** Schematic of melatonin's anti-inflammatory mechanism in TNFα-induced oral epithelial cells. TNFα induces inflammation by promoting pro-inflammatory cytokines (IL-6/IL-8/IL-11). Melatonin binds to MTNR1A to further attenuate oral epithelial cell inflammation via the Keap2-Nrf2 signaling pathway.

effects of melatonin on IL-8 protein levels (**Figure 4G** and **4H**) in HOK cells. Taken together, these results demonstrate that melatonin inhibits inflammation in oral epithelial cells via Keap1.

*Melatonin inhibits inflammation in oral epithelial cells via Nrf2*

We further explored whether the effects of melatonin on oral epithelial inflammation were mediated through Keap1/Nrf2. Oral epithelial cells were treated with siRNA-Nrf2. Nrf2 KD significantly impaired the effects of melatonin on expression of inflammatory markers, including IL-8 (**Figure 5A**) and IL-11 (**Figure 5B**), in HOEC cells. Moreover, Nrf2 KD compromised the effects of melatonin on IL-8 protein levels (**Figure 5C** and **5D**) in HOEC cells. Nrf2 KD also significantly compromised the effects of melatonin on expression of inflammatory markers, including IL-8 (**Figure 5E**) and IL-11 (**Figure 5F**), in HOK cells. Nrf2 KD also compromised the effects of melatonin on IL-8 protein levels (**Figure 5G** and **5H**) in HOK cells. Furthermore, influence of Nrf2 on the downstream gene, HO-1, was explored, which indicated that knockdown of Nrf2 reduced expression of HO-1 in both types of cells (Supplementary Figure 1). Taken together, these results demonstrate that melatonin inhibits inflammation in oral epithelial cells via Nrf2.

*Melatonin inhibits TNFα-induced inflammation in a 3D oral epithelial cell model*

We further explored the effects of melatonin on inflammation in oral epithelial cells. A 3D oral epithelial cell model was developed and used to investigate the effects of melatonin on oral epithelial cell inflammation (**Figure 6A**). ELISA was used to detect effects of melatonin on TNFα-induced inflammation in our 3D oral epithelial cell model. Melatonin significantly reduced TNFα-induced increases in the protein levels of inflammatory markers, including IL-6 (**Figure 6B**), IL-8 (**Figure 6C**), and IL-11 (**Figure 6D**), in a dose-dependent manner. Taken together, these experiments demonstrated that melatonin inhibits TNFα-induced inflammation in a 3D oral epithelial cell model.

## Discussion

In this study, we investigated the potential anti-inflammatory effects of melatonin on oral epithelial cells in the context of TNFα-induced inflammation, focusing on the involvement of the Keap1-Nrf2 axis (**Figure 7**). Our findings reveal that melatonin administration effectively attenuates TNFα-induced inflammation in oral epithelial cells through modulation of the Keap1-Nrf2 pathway. These findings represent a significant advancement in our understanding of the role of melatonin in combating inflammatory processes within the oral cavity, suggesting its therapeutic potential for managing oral inflammatory diseases. By elucidating the underlying mechanisms involving Keap1-Nrf2 signaling, our study sheds light on the intricate

molecular pathways through which melatonin exerts its anti-inflammatory effects and provides valuable insights for the development of novel therapeutic strategies targeting oral inflammatory conditions.

The induction of inflammation in oral epithelial cells by TNFα stands as a pivotal aspect in understanding oral health and disease [1]. Research consistently demonstrates that TNFα significantly activates inflammatory pathways in oral epithelial cells, leading to the release of various inflammatory mediators and recruitment of immune cells [15]. This inflammatory cascade contributes to the pathogenesis of several oral diseases including periodontitis, oral mucositis, and oral lichen planus [15]. Understanding the mechanisms by which TNFα induces inflammation in oral epithelial cells is paramount for developing targeted therapies to mitigate oral inflammatory conditions and ultimately improve oral health outcomes. In this study, we demonstrated that TNFα induced increases in inflammation markers in two oral epithelial cell lines in a dose-dependent manner (**Figure 1**), consistent with previous studies. For instance, Hosokawa et al. found that TNFα could induce inflammation in oral epithelial cells [16].

The inhibitory effect of melatonin on TNFα-induced inflammation presents a fascinating avenue for potential therapeutic intervention in various inflammatory conditions [5]. Melatonin exerts potent anti-inflammatory effects on various cell types, including oral epithelial cells, by inhibiting the production of pro-inflammatory cytokines, reducing oxidative stress, and enhancing antioxidant defenses [5]. Melatonin's ability to regulate immune responses and promote tissue repair further underscores its potential as a therapeutic agent for mitigating TNFα-induced inflammation in oral tissues and beyond [17]. In the present study, melatonin inhibited TNFα-induced inflammation in a 3D oral epithelial cell model (**Figure 2**). Thus, melatonin may be an effective agent for inhibiting inflammation. Our results are consistent with those of previous studies. For example, melatonin inhibited lipopolysaccharide-induced inflammation in cultured mouse mammary tissue [18]. A previous study also demonstrated that postoperative melatonin administration reduces oxidative stress and inflammation, while significantly improving cardiac function in patients undergoing coronary artery bypass grafting (CABG) surgery [19]. To the best of our knowledge, our study is the first to demonstrate that melatonin alleviates inflammation in oral epithelial cells.

Inhibition of inflammation in oral epithelial cells by melatonin through its receptor, MTNR1A, elucidates a fascinating mechanism underlying the anti-inflammatory properties of this hormone [14]. MTNR1A, a high-affinity melatonin receptor, is abundantly expressed in various tissues, including oral epithelial cells [14]. Research indicates that melatonin exerts its anti-inflammatory effects by binding to MTNR1A and subsequently modulating downstream signaling pathways involved in inflammation [15]. Activation of MTNR1A by melatonin inhibits pro-inflammatory cytokine production, suppresses NF-κB activation, and regulates inflammatory gene expression in oral epithelial cells [14]. This interaction highlights the intricate interplay between melatonin and its receptors in orchestrating immune responses and maintaining tissue homeostasis. Understanding the role of MTNR1A-mediated signaling in mediating the anti-inflammatory actions of melatonin provides valuable insights into potential therapeutic strategies for managing oral inflammatory conditions and promoting oral health [15]. In the present study, melatonin inhibited inflammation in oral epithelial cells via MTNR1A (**Figure 3**). Further exploration of the molecular mechanisms underlying this interaction may uncover novel therapeutic targets for developing targeted interventions for oral inflammation and related diseases.

Inhibition of inflammation in oral epithelial cells by melatonin through the Keap1/Nrf2 signaling pathway reveals a sophisticated mechanism underlying the anti-inflammatory properties of this hormone [6]. Keap1/Nrf2 signaling is a critical cellular pathway involved in the regulation of oxidative stress and inflammation [6]. A previous study has demonstrated that melatonin activates the Keap1/Nrf2 pathway in oral epithelial cells, suppressing inflammation [16]. Upon stimulation by melatonin, Nrf2 is liberated from its inhibitor Keap1, allowing it to translocate into the nucleus and induce the expression of antioxidant and cytoprotective genes [16]. Consistently, our study confirmed that

melatonin inhibits inflammation in oral epithelial cells via the Keap1/Nrf2 pathway (**Figures 4** and **5**). This interaction highlights the specificity of the mechanism by which melatonin exerts its anti-inflammatory effects, offering potential therapeutic avenues for managing oral inflammatory conditions and promoting oral health. Further exploration of this signaling pathway may uncover novel targets for developing targeted interventions against oral inflammation and associated diseases.

The inhibition of TNFα-induced inflammation in a 3D oral epithelial cell model by melatonin represents a significant advancement in understanding the therapeutic potential of this hormone in oral health. Utilizing 3D cell models provides a more physiologically relevant platform to study complex cellular interactions and responses [17]. Our research demonstrated that melatonin effectively mitigated TNFα-induced inflammation in this model system, indicating its promising anti-inflammatory properties in oral epithelial tissues (**Figure 6**). By modulating the signaling pathways involved in inflammation, melatonin can suppress the production of pro-inflammatory mediators and attenuate tissue damage, thereby contributing to the maintenance of oral health. These findings underscore the importance of exploring melatonin as a potential therapeutic agent for managing oral inflammatory conditions and offer new avenues for the development of targeted interventions to improve oral health outcomes. Further investigations using 3D cell models will provide valuable insights into the molecular mechanisms underlying the anti-inflammatory effects of melatonin and its potential applications in clinical settings.

In conclusion, this study demonstrates that melatonin exhibits anti-inflammatory effects on TNFα-induced inflammation, mediated primarily through the melatonin receptor, MTNR1A. Specifically, we elucidated that the effect of melatonin on oral epithelial inflammation occurred via the Keap1/Nrf2 signaling pathway. This study also provides additional validation of the anti-inflammatory properties of melatonin using a 3D oral epithelial model. These findings provide valuable insights for the development of novel therapies targeting oral epithelial inflammation.

## Limitations and prospects

This study has certain limitations that warrant consideration. First, the anti-inflammatory effects of melatonin were primarily validated in two oral epithelial cell lines (HOEC, HOK) and a 3D cell model; in vivo verification using animal models of oral mucosal inflammation (e.g., periodontitis or oral mucositis models) is lacking to confirm translational potential. Second, while the Keap1-Nrf2 axis and MTNR1A receptor were identified as core mediators, crosstalk with other inflammatory pathways (e.g., NF-κB) or potential synergistic effects with clinical anti-inflammatory agents remain unexamined. For future research, we plan to: (1) establish animal models to evaluate melatonin's therapeutic efficacy in vivo and explore its pharmacokinetic profiles in oral tissues; (2) investigate the interplay between the Keap1-Nrf2 pathway and other signaling cascades to clarify the comprehensive anti-inflammatory mechanism of melatonin; and (3) optimize melatonin's delivery system (e.g., topical formulations) to enhance its bioavailability in oral mucosal lesions, laying a foundation for clinical application.

## Acknowledgements

The present study was supported by the Medical Science Research Project of Hebei (20241083).

## Disclosure of conflict of interest

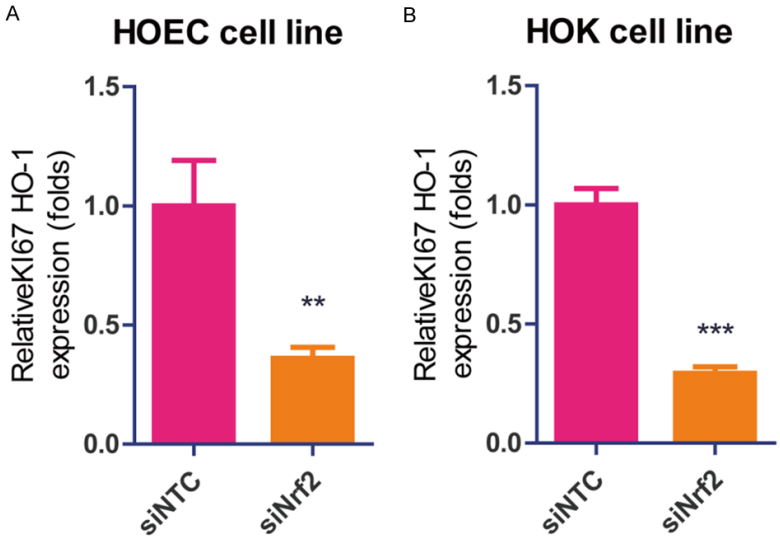
None.

**Address correspondence to:** Yunfang Bai, The Fourth Hospital of Hebei Medical University, 12 Health Road, Shijiazhuang 050000, Hebei, China. E-mail: 13191856680@163.com

## References

[1] Groeger S and Meyle J. Oral mucosal epithelial cells. Front Immunol 2019; 10: 208.
[2] Schifter M, Yeoh SC, Coleman H and Georgiou A. Oral mucosal diseases: the inflammatory dermatoses. Aust Dent J 2010; 55 Suppl 1: 23-38.
[3] Meghil MM and Cutler CW. Oral microbes and mucosal dendritic cells, "Spark and Flame" of local and distant inflammatory diseases. Int J Mol Sci 2020; 21: 1643.
[4] Vasey C, McBride J and Penta K. Circadian rhythm dysregulation and restoration: the role of melatonin. Nutrients 2021; 13: 3480.

[5] Hardeland R. Melatonin and inflammation-story of a double-edged blade. J Pineal Res 2018; 65: e12525.
[6] Vriend J and Reiter RJ. The Keap1-Nrf2-antioxidant response element pathway: a review of its regulation by melatonin and the proteasome. Mol Cell Endocrinol 2015; 401: 213-220.
[7] Diao X, Duan C, Yao L, Qin J, He L and Zhang W. Melatonin promotes the development of secondary hair follicles in adult cashmere goats by activating the Keap1-Nrf2 signaling pathway and inhibiting the inflammatory transcription factors NFkappaB and AP-1. Int J Mol Sci 2023; 24: 3403.
[8] Chitturi Suryaprakash RT, Shearston K, Farah CS, Fox SA, Iqbal MM, Kadolsky U, Zhong X, Saxena A and Kujan O. A novel preclinical in vitro 3D model of oral carcinogenesis for biomarker discovery and drug testing. Int J Mol Sci 2023; 24: 4096.
[9] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001; 25: 402-408.
[10] Michalickova D, Hrncir T, Canova NK and Slanar O. Targeting Keap1/Nrf2/ARE signaling pathway in multiple sclerosis. Eur J Pharmacol 2020; 873: 172973.
[11] Bona S, Fernandes SA, Moreira ACJ, Rodrigues G, Schemitt EG, Di Naso FC, Marroni CA and Marroni NP. Melatonin restores zinc levels, activates the Keap1/Nrf2 pathway, and modulates endoplasmic reticular stress and HSP in rats with chronic hepatotoxicity. World J Gastrointest Pharmacol Ther 2022; 13: 11-22.
[12] Hosokawa Y, Hosokawa I, Shindo S, Ozaki K and Matsuo T. IL-29 enhances CXCL10 production in TNF-alpha-stimulated human oral epithelial cells. Immunol Invest 2017; 46: 615-624.
[13] Tang L, Zhang C, Lu L, Tian H, Liu K, Luo D, Qiu Q, Xu GT and Zhang J. Melatonin maintains inner blood-retinal barrier by regulating microglia via inhibition of PI3K/Akt/Stat3/NF-kappaB signaling pathways in experimental diabetic retinopathy. Front Immunol 2022; 13: 831660.
[14] Xi Z, Yuan XQ and Zhang XM. Melatonin reduces inflammation in intestinal cells, organoids and intestinal explants. Inflammopharmacology 2021; 29: 1555-1564.
[15] Qiu X, Liang T, Wu Z, Zhu Y, Gao W, Gao B, Qiu J, Wang X, Chen T, Deng Z, Li P, Chen Y, Zhou H, Peng Y, Xu C, Su P, Liang A and Huang D. Melatonin reverses tumor necrosis factor-alpha-induced metabolic disturbance of human nucleus pulposus cells via MTNR1B/Galphai2/YAP signaling. Int J Biol Sci 2022; 18: 2202-2219.
[16] Cai J, Yang J, Chen X, Zhang H, Zhu Y, Liu Q and Zhang Z. Melatonin ameliorates trimethyltin chloride-induced cardiotoxicity: the role of nuclear xenobiotic metabolism and Keap1-Nrf2/ARE axis-mediated pyroptosis. Biofactors 2022; 48: 481-497.
[17] Yin Y, Xu L and Steinway SN. Editorial: 3D organoid and organ-on-a-chip and their applications for virology and antiviral research. Front Microbiol 2023; 14: 1266136.
[18] Yu GM and Tan W. Melatonin inhibits lipopolysaccharide-induced inflammation and oxidative stress in cultured mouse mammary tissue. Mediators Inflamm 2019; 2019: 8597159.
[19] Mohammadi N, Alizadeh M, Akbarzadeh S, Rezaei M, Mahmoodi M, Netticadan T and Movahed A. Melatonin administered postoperatively lowers oxidative stress and inflammation and significantly recovers heart function in patients undergoing CABG surgery. Eur J Med Res 2025; 30: 585.




**Supplementary Figure 1.** Effects of Nrf2 on expression of HO-1 in HOEC and HOK cell lines.