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Research article

The potential of melatonin in sepsis-associated acute kidney injury: Mitochondrial protection and cGAS-STING signaling pathway

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ABSTRACT

Melatonin (Mel) is known for various biological function, such as antioxidant and anti-inflammatory capabilities, as well as its ability to modulate immune responses, which can protect mitochondria and improve the prognosis of sepsis-associated acute kidney injury (SA-AKI). However, there is a multitude of theories regarding how Mel exerts its immune-modulating functions, with no consensus reached as of yet. We propose the protective effects of Mel on mitochondria are closely related to the cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) signaling pathway in the immune-inflammatory response. We intraperitoneally injected H151 and Mel into SA-AKI mouse models to interfere the cGAS-STING signaling pathway. By comparing behavioral, pathological, and molecular biology results, we discovered that Mel could reduce cGAS-STING signaling pathway while greatly relieving kidney damage and function. In addition, Mel-treated mice showed a significant increase in autophagosome formations, which might be linked to the cGAS-STING signaling pathway. Our findings suggest that Mel protection on kidney injury in SA-AKI mice is partially attributed to the inhibition of the cGAS-STING signaling pathway.

Abbreviations

Abbreviation	Full name
Mel	melatonin
SA-AKI	sepsis-associated acute kidney injury
cGAS	cyclic GMP-AMP synthase
STING	stimulator of interferon genes
cGAMP	cyclic GMP-AMP
TBK-1	TANK-binding kinase 1
IRF-3	interferon regulatory factor 3
mtDNA	Mitochondrial DNA
PINK1	PTEN-induced kinase 1
p62	SQSTM1/p62
LC3	light chain 3

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NF-κB	nuclear factor kappa B
TNF-α	tumor necrosis factor α
IL-1β	interleukin-1β
IL-6	interleukin-6
MSS	murine sepsis score
Scr	serum creatinine
BUN	blood urea nitrogen
LPS	lipopolysaccharide
TEM	Transmission electron microscope
ANOVA	One-way analysis of variance
ELISA	Enzyme-linked immunosorbent assay
qRT-PCR	Quantitative real-time PCR
WB	Western blot
H&E	Hematoxylin and Eosin staining

1. Introduction

SA-AKI is one of the most common complications of sepsis, severely affecting patient survival and prognosis [1,2]. Its pathogenesis is complex and multifactorial, involving metabolism, microcirculation, coagulation, and immune function [1]. In terms of SA-AKI treatment, interventions targeting immune dysregulation-induced inflammation have not yielded satisfactory results. Research indicated that the levels of Mel in peripheral blood of SA-AKI patients are positively correlated with disease prognosis [3,4]. However, most existing research has focused on the antioxidant, metabolic improvement, and anti-apoptotic effects of Mel to maintain renal function [5,6]. We hypothesize that Mel improves SA-AKI prognosis by protecting mitochondria and indirectly affecting the cGAS-STING signaling pathway.

Since the discovery of the cGAS-STING signaling pathway, it has received a lot of interest in academia. Over the past five years, research findings on this pathway have been increasing annually [7,8]. This pathway has been proven to be involved in inflammatory responses related to cancer, metabolism, immunity, and aging [9–11]. cGAS, a double-stranded DNA recognition protein, can promote the synthesis of cyclic GMP-AMP (cGAMP) from GTP and ATP upon activation [12]. cGAMP further activates STING protein on the endoplasmic reticulum, and by recruiting TANK-binding kinase 1 (TBK1), it can activate the downstream nuclear factor kappa B (NF-κB) pathway and interferon regulatory factor 3 (IRF3) pathway [13,14]. Given the particular identification of nucleic acids, we considered an organelle that contains a substantial amount of nucleic acids—mitochondria.

It is well known that mitochondria can synthesize mitochondria-specific nucleic acids and proteins [15]. Mitochondrial DNA (mtDNA) may activate the cGAS-STING signaling pathway in response to harmful stimuli, which is crucial in kidney injury [16,17]. Mitochondrial damage can also induce mitophagy. The stability of PTEN-induced kinase 1 (PINK1) on the outer mitochondrial membrane is enhanced, subsequently activating the E3 ubiquitin ligase Parkin. Parkin ubiquitinates membrane proteins as an mitophagy signal, leading to the aggregation of receptor protein SQSTM1/p62 (p62), which binds to the autophagosome structural protein microtubule-associated protein 1 light chain 3 (LC3) and is transported into the autophagosome [18–20]. Therefore, our study hypothesizes that the positive effects of Mel on SA-AKI prognosis may be linked to mitochondrial protection and autophagy, subsequently affecting the activity of the cGAS-STING signaling pathway.

This study combines molecular biology, pathology, and behavioral research to explore the effects of Mel on kidney function and nephritis in SA-AKI. We analyzed the impact of exogenously supplemented Mel on the cGAS-STING signaling pathway. Meanwhile, we found that compared to the STING-targeted inhibitor H151, Mel had a more significant effect on improving the prognosis of SA-AKI. Based on our results, we believe that Mel could serve as a potential therapeutic agent with broad application prospects in the treatment of SA-AKI, warranting further scientific research and clinical exploration.

2. Materials and methods

2.1. Animals

C57BL/6 mice (male, 16–20 g) were purchased at 6–8 weeks of age from Jiangsu Kerbio Medical Technology Group Co., Ltd (Jiangsu, China). All mice were housed in an environmentally controlled facility (≤5 mice per cage) with a temperature set at 22 °C–24 °C, humidity maintained at 40–60 %, and a 12-h light/12-h dark cycle to simulate an appropriate living environment. The mice had free access to food and water.

The ethics committee approval number is IAUCU23-0016. All procedures were approved by the Jiangsu Kerbio Medical Technology Group Co., Ltd.

2.2. Reagents

Antibody (cGAS, STING, NF-κB, p-NF-κB, IRF-3, p-IRF-3, SQSTM1/p62, LC3, PINK1) were provided by Cell Signaling Technology (Denver, USA). LPS was provided by Sigma Aldrich (St. Louis, USA). Mel and H-151 was provided by Medchemexpress (Monmouth

Junction, USA).

2.3. Drugs and treatments

Wild-type C57BL/6 mice were intraperitoneally injected with LPS (Sigma Aldrich Co., USA) at a dose of 15 mg/kg to establish a SA-AKI model. Control group mice were injected with an equal volume of 0.9 % saline solution. H-151-treated mice were intraperitoneally injected with H-151 (MedChemExpress, USA) at a dose of 10 mg/kg 3 h before LPS injection. The Mel-treated mice received intraperitoneal injections of Mel at a dose of 30 mg/kg 3 h before, and 6 and 12 h after LPS injection. Mice were euthanized 24 h after the LPS injection, and kidney tissues and blood samples were collected for further analysis.

2.4. Renal function tests

Renal function was evaluated by serum creatinine (Scr) and blood urea nitrogen (BUN). Mice were fasted for at least 12 h before the experiment to minimize the impact of food on kidney function. The collected blood was coagulated at room temperature for 40 min, then centrifuged at 4000 rpm for 15 min. The supernatant was aspirated and tested on a AU5800 automatic biochemical analyzer.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The collected blood was allowed to remain at room temperature for 40 min before being centrifuged at 4000 rpm for 10 min. The supernatant was carefully collected, and the serum levels of interleukin-1β (IL-1β) and interleukin-6 (IL-6) were detected using the Mouse IL-1β Quantikine ELISA Kit (R&D Systems, Minnesota, USA) and the IL-6 ELISA Kit (Invitrogen, New York, USA), respectively.

2.6. Murine sepsis score (MSS)

The MSS was used to assess the severity of septic disease in mice induced by LPS. The MSS scoring system covers seven key observational parameters: appearance, consciousness, activity, response, eye, respiratory rate, respiratory quality. Each variables range from 0 (normal) to 4 (severe), with a maximum total score of 28.The MSS is highly predictive and reliable in predicting sepsis progression and death, and is an important tool for assessing sepsis outcomes in different experimental models [21].

2.7. Quantitative real-time PCR (qRT-PCR)

Kidney tissues were placed in centrifuge tubes containing Trizol. Add chloroform, mix well and centrifuge. The supernatant was collected and ethanol was added to precipitate RNA. RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA). The cDNA samples were amplified using the CFX96 real-time PCR detection system (Bio-Rad) and TB Green Premix Ex Taq (Takara, Dalian, China). The CFX96 system was set with the following steps: Pre-denaturation at 95 °C for 30 s. Denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 20 s, repeated for 40 cycles. Short denaturation at 95 °C for 1 s and annealing at 65 °C for 15 s, followed by a final extension at 95 °C for 30 s. The relative mRNA expression levels were measured using GAPDH as the internal reference. The primers used are shown in Table 1.

2.8. Quantification of mitochondrial DNA

Total DNA were extracted and purified by using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). qRT-PCR was performed

Table 1
Primers used in qRT-PCR analysis.

Gene		Sequence
cGAS	Forward	CCAAGATGCTGTCAAAGTTTAGGA
	Reverse	CCTGGTTTTTCCTTCTCCACACT
STING	Forward	GGTTTATTCCAACAGCGTCTACG
	Reverse	TGTGACATGGCAAACAGGGTCT
IRF-3	Forward	TACGGCAGGACGCACAGAT
	Reverse	GCAGCTAACCGCAACACTTCT
NF-κB	Forward	GGACCTATGAGACCTTCAAGAGTATC
	Reverse	AGAAGTTGAGTTTCGGGTAGGC
IL-1β	Forward	GCAGGCAGTATCACTCATTGTGG
	Reverse	GAGTCACAGAGGATGGGCTCTTC
IL-6	Forward	GAGGAGACTTCACAGAGGATACCAC
	Reverse	TTGCCATTGCACAACTCTTTTC
TNF-α	Forward	TGACAAGCCTGTAGCCCACG
	Reverse	TTGTCTTTGAGATCCATGCCG
GAPDH	Forward	TCTTGTGCAGTGCCAGCCT
	Reverse	TGAGGTCAATGAAGGGGTCG

using the SyBR GreenER qPCR SuperMix (Thermo Fisher Scientific, Waltham, USA) and CFX96 Real-Time PCR Detection System. The primers used are shown in Table 2.

2.9. Western blot analysis (WB)

Kidney Tissue was broken up using the TGrinder H24 Tissue Milling homogenizer (TIANGEN, Beijing, China) and the protein was extracted using the total protein extraction kit (BestBio, Shanghai, China). After the protein is diluted to a suitable concentration, it is transferred to the polyvinylidene fluoride membrane by electrophoresis. The membrane is incubated in the antibody overnight. After incubation with the dilution of goat anti-rabbit antibody, the membrane was detected using Tanon 4600SF chemiluminescence imaging system.

2.10. Hematoxylin and eosin staining (H&E)

The kidney tissue fixed in formalin was embedded in paraffin blocks using an automatic dehydrator dakewehp300. Use a slicer to cut the paraffin blocks into 4–6 μm slices. The slices were sequentially stained with hematoxylin and eosin respectively and then sealed. Tissue damage such as renal tubular congestion and edema, degeneration, and vacuolated changes were assessed by microscopic observation and the relative percentage was calculated to assess the tubular damage score.

2.11. Apoptosis analysis

Tissue sections were deparaffinized in xylene and then rehydrated by soaking in anhydrous ethanol. TUNEL staining was performed using the One-step TUNEL In Situ Apoptosis Kit (Elabscience, Wuhan, China) to label fragmented DNA in apoptotic cells. Four fields of view were selected from each section, and apoptotic cells were counted using a fluorescence microscope. The average number of apoptotic cells was quantified.

2.12. Transmission electron microscope (TEM)

The samples were fixed with osmium acid fixative solution and 2.5 % glutaraldehyde fixative, treated with embedding agent and acetone, and sections of 70–90 nm were obtained with an ultrathin sectioning machine. The sections were first stained with lead citrate solution for 5–10 min, followed by staining with a 50 % ethanol saturated solution of hydrogen peroxide uranyl acetate for the same duration. The sections were air-dried and prepared for observation under a TEM.

2.13. Statistical analysis

The data were presented as mean ± standard error of the mean (SEM) and analyzed with GraphPad Prism 9.0 software. Use Shapiro-Wilk to verify that the data is normally distributed. For the two sets of data conforming to the normal distribution, the differences between the data were verified using the Student's t-test. One-way analysis of variance (ANOVA) was used to verify that there were significant differences between groups, and Tukey's test was used to verify that there were differences between groups of data. To explore the correlation between various variables, Person correlation was calculated according to the distribution of variables. Statistical significance was considered at three levels ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

3. Result

3.1. Melatonin alleviates renal function decline in SA-AKI

As previously reported, sepsis can be induced by intraperitoneal injection of LPS [22]. To identify the period of most severe kidney injury, we administered LPS (15 mg/kg) intraperitoneally to mice and detected the levels of Scr and BUN in the peripheral blood at various time points (1, 6, 12, 24, and 48 h). Following LPS injection, both BUN and Scr levels gradually increased. Compared to Control group, the LPS-treated mice showed significantly elevated Scr and BUN at 24 h post-LPS injection. However, by 48 h, BUN and Scr had decreased (Fig. 1A). Additionally, the mice exhibited recovery in activity, responsiveness, and feeding behavior. Based on the kidney function curves, we detected the inflammatory cytokines IL-1β and IL-6 in peripheral blood using ELISA at the 24-h. The results showed that cytokine levels in LPS-treated mice were significantly higher than those in Control group (Fig. 1B). Therefore, we concluded that kidney injury is most severe at 24 h post-LPS injection, highlighting this time point as particularly valuable for studying the SA-AKI model.

Table 2
Primers used in qRT-PCR analysis.

Gene		Sequence
mtDNA	Forward	CCGCAAGGGAAAGATGAAAGAC
	Reverse	TCGTTTGGTTTCGGGGTTTC

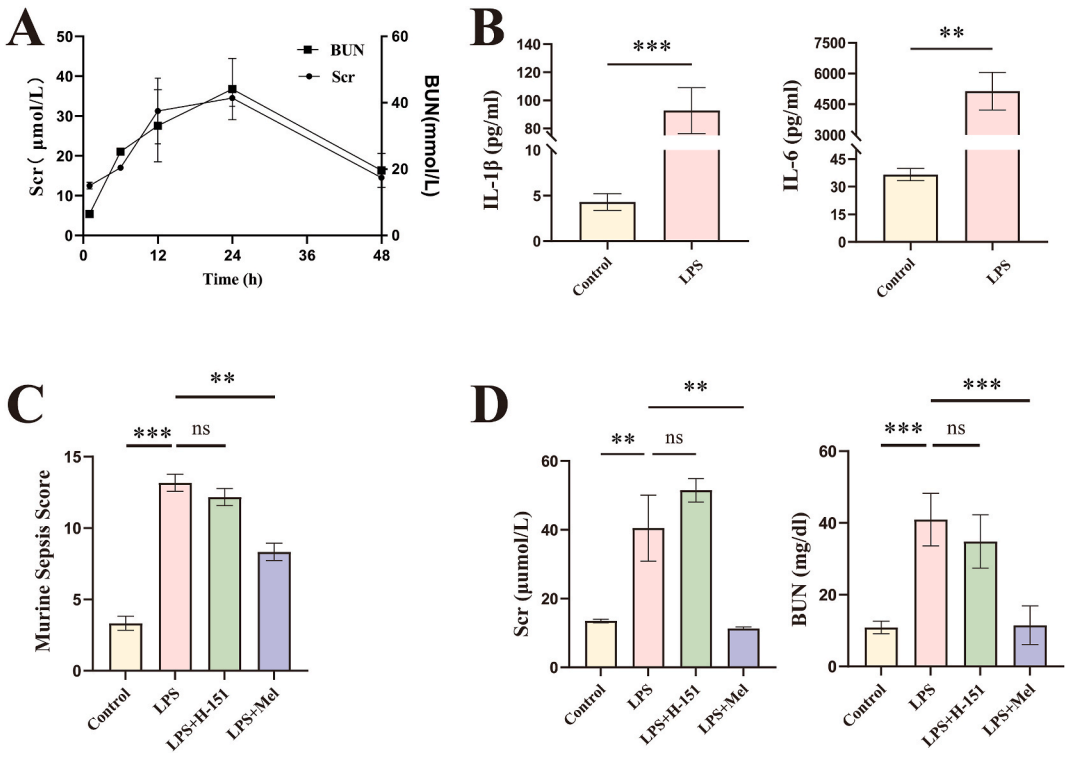

Fig. 1. Effects of Mel and H151 on serum levels of proinflammatory cytokines and renal function in LPS-treat mice.
(A) Changes in Scr and BUN of LPS-treat mice within 48 h; (B) IL-1β and IL-6 detected by Elisa (n = 4–6). (C) Murine Sepsis Score (n = 6); (D) Scr and BUN levels detected by Automatic Analyzer (n = 6). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. 'ns' indicates no significant difference.

We reproduced the method described above to establish the SA-AKI mouse model and chose 24 h after LPS injection as the time point for detecting relevant indicator changes. To evaluate the effects of Mel and H-151 on inflammation and kidney function in SA-AKI mice, we compared kidney function indicators and assessed changes in consciousness, activity, and responsiveness in four groups of mice at 24 h post-LPS injection. According to the MSS, Mel-treated mice (LPS + Mel group) showed significant improvements in response to external stimuli and consciousness compared to the SA-AKI group (LPS group), including drinking activity. In contrast, the H-151 group (LPS + H-151 group) exhibited heavy breathing and white secretions around the eyes, preventing them from opening their eyes, and their condition was similar to that of the SA-AKI group (Fig. 1C). In terms of kidney function, the mean levels of Scr and BUN in SA-AKI group were 40.50 μmol/L and 40.90 mmol/L, respectively. Unfortunately, there was no significant improvement in the H-151-treated group. Surprisingly, the kidney function of the Mel-treated group was virtually identical to that of Control group. (Fig. 1D). This indicates that Mel can significantly improve kidney function in mice with SA-AKI.

3.2. *Suppression of the STING ameliorates kidney inflammation in SA-AKI mice*

According to previous reports, the cGAS-STING signaling pathway can induce inflammatory responses. To validate our hypothesis, we first explored the role of the cGAS-STING signaling pathway in SA-AKI. We used WB to detect the expression of key proteins in this pathway in mouse kidney tissue (Fig. 2A). Compared to Control group, the SA-AKI group (LPS group) showed increased expression of STING and downstream inflammatory key molecules NF-κB and IRF3, along with their activated forms (p-IRF3) in kidney tissue. Notably, STING and IRF3 proteins were significantly elevated, whereas p-NF-κB did not show significant changes (Fig. 2B). Consistent with the WB results, qRT-PCR results recorded significantly higher relative mRNA expression levels of STING and IRF-3 in the SA-AKI group compared to Control group. Additionally, the inflammatory cytokines IL-1β, IL-6, and tumor necrosis factor α (TNF-α) were significantly elevated in kidney tissue. These findings indicate an exacerbation of kidney inflammation in the SA-AKI group (Fig. 2C–D).

To further verify the role of the cGAS-STING signaling pathway in inducing nephritis, we attempted to inhibit STING and its downstream pathways using the STING-targeted inhibitor H151 (10 mg/kg). Prior to LPS injection, one group of mice was pre-injected with H151, establishing the H151 group (LPS + H151 group). We then repeated the aforementioned molecular biology assays. The results showed that, compared to the SA-AKI group, the expression levels of pathway proteins, including STING, were significantly reduced in the H151 group. Notably, the levels of IRF3 and p-IRF3 decreased more markedly than those of NF-κB and p-NF-κB (Fig. 2A–C). Additionally, the levels of inflammatory cytokines in the kidneys were significantly controlled in the H151-treated group compared to the SA-AKI group (Fig. 2D). These results indicate that the cGAS-STING signaling pathway contributes to the exacerbation

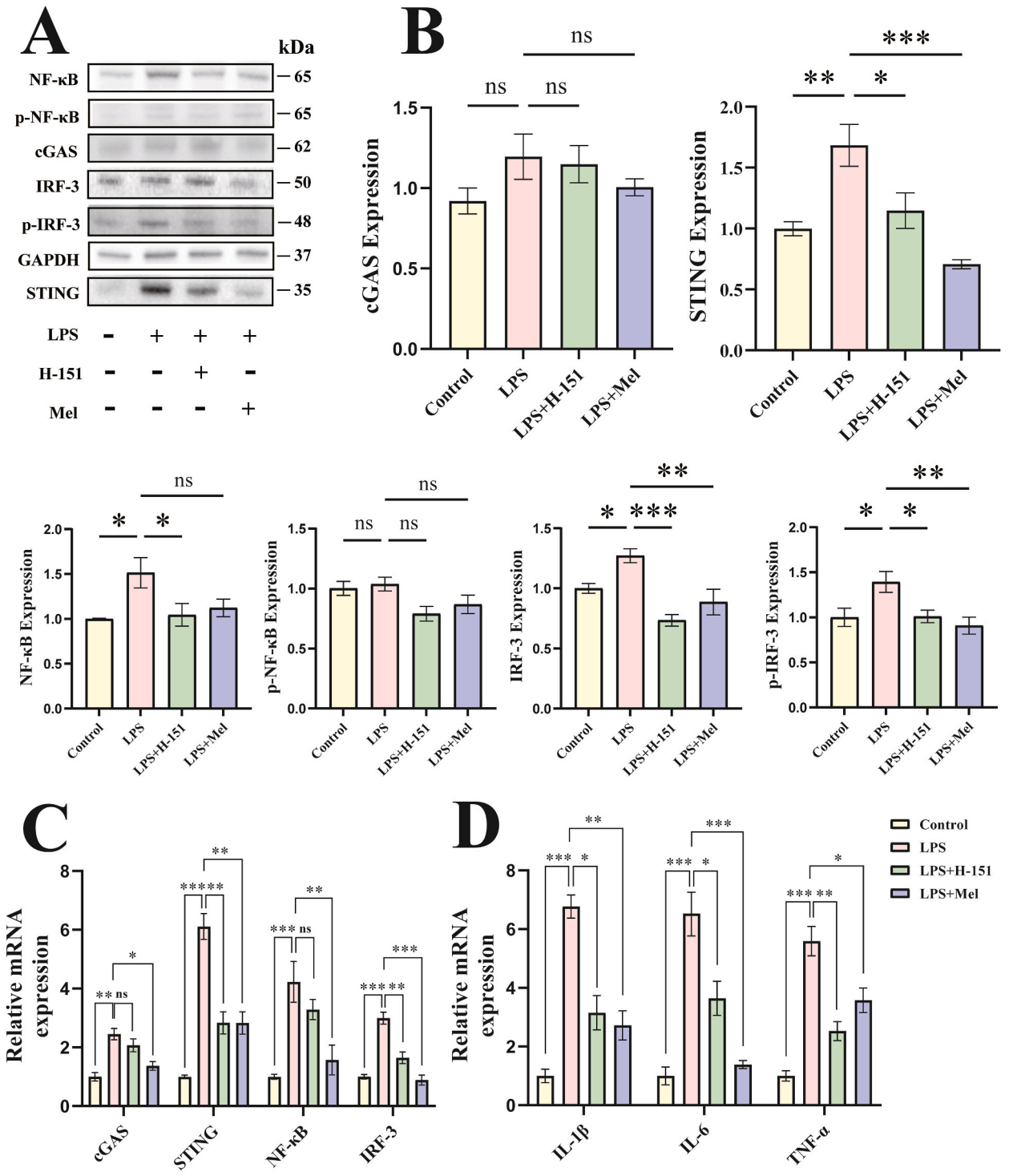

Fig. 2. Effects of Mel and H-151 on the cGAS-STING signaling pathway in a mouse model of SA-AKI.
(A) Representative western blot; (B) Expression of cGAS, STING, NF-κB, p-NF-κB, IRF-3, and p-IRF-3 in the kidney tissues of LPS-treated mice with Mel or H-151 detected by western blot (n = 6); (C) Expression of cGAS, STING, NF-κB, IRF-3detected by qRT-PCR (n = 6). (D) Expression of proinflammatory cytokines (IL-1β,IL-6,TNF-α) detected by qRT-PCR (n = 6).*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. 'ns' indicates no significant difference.

of kidney injury and nephritis in SA-AKI mice.

3.3. Melatonin can ameliorate cGAS-STING signaling pathway-mediated SA-AKI nephritis

Considering the correlation between the cGAS-STING signaling pathway and SA-AKI, as well as reports that Mel can significantly improve the prognosis of SA-AKI patients [4], we sought to investigate the interaction between Mel and the cGAS-STING signaling pathway. We injected Mel (30 mg/kg) intraperitoneally into SA-AKI mice to establish a Mel-treated group (LPS + Mel group). Within 24 h post-LPS stimulation, the renal function of this group improved significantly (Fig. 1D). Additionally, we examined the gene and protein expression of molecules in the inflammatory pathway. Compared to SA-AKI group, the Mel-treated group exhibited significantly reduced expression of STING, NF-κB, IRF3, and their phosphorylated proteins (Fig. 2A–B). Levels of inflammatory factors were also significantly controlled (Fig. 2D). Furthermore, kidney histopathology revealed that the Mel-treated group had reduced congestion and edema in the renal interstitium, decreased inflammatory cell infiltration, and fewer apoptotic necrotic cells (Fig. 3A–B). Based on these results, we conclude that Mel can improve the prognosis of SA-AKI, and this improvement is attributed to the inhibition of the cGAS-STING signaling pathway.

3.4. Melatonin supplement can promote autophagosome formations in renal tubular epithelial cells

As previously mentioned, the cGAS-STING signaling pathway can only recognize nucleic acid substances [12]. The leakage of mtDNA from damaged mitochondria may be a key factor in the activation of this pathway [23]. Previous studies have found that mitophagy can recognize and clear damaged or dysfunctional mitochondria [18]. Therefore, we hypothesize that Mel can down-regulate the cGAS-STING signaling pathway possibly because it can promote mitophagy.

We examined the key proteins involved in the mitophagy pathway—PINK1, p62 and LC3 (LC3I and LC3II)—in the kidney tissues of Control group, SA-AKI group, and Mel-treated group. These proteins correspond to different stages of autophagy: autophagosome formation, recognition, and maturation, respectively [24]. We found that, compared to Control group, the SA-AKI group showed increased expression levels of PINK1, LC3II, and p62, although these increases were not significant. However, with Mel intervention, the increases in PINK1 and LC3II/I were more pronounced, while p62 expression decreased compared to the SA-AKI group and was similar to that of Control group (Fig. 4A–B).

We utilized TEM to observe mitochondrial alterations in renal tubular epithelial cells. In Control group, most renal tubular mitochondria exhibited a tubular morphology, with well-defined mitochondrial cristae. Conversely, renal tubules stimulated by LPS displayed numerous swollen and distorted mitochondria, along with vacuolar alterations. Additionally, some mitochondrial cristae exhibited signs of fragmentation damage. In the Mel-treated group, renal tubular cell mitochondria exhibited less deformation compared to SA-AKI group, alongside the emergence of several free membrane structures on the damaged mitochondrial surface. We quantified the numbers of autophagosomes and autolysosomes in the field of view. The results revealed a significant increase in both autophagosomes and autolysosomes following Mel intervention (Fig. 4C–D). However, WB analysis revealed that p62 and LC3 expression were not significantly changed. We believe that, while melatonin promotes autophagosome formation, autophagy flux activation is unlikely to be dramatic.We believe that the cGAS-STING signaling pathway may have a close connection with autophagosome. We performed Person correlation to analyze the correlation between autophagosome-related indicators and cGAS-STING signaling pathway indicators (Fig. 4E). The results showed a significant negative correlation between the average number of autophagosomes and the mRNA and protein expression of cGAS, STING, IRF-3, and p-IRF-3 ($p < 0.05$). To sum up, Mel might stimulate the process of mitophagy, as reflected by the increased number of autophagosomes. Moreover, there could be a negative correlation between the cGAS-STING signaling pathway's activation and the augmentation of mitophagy.

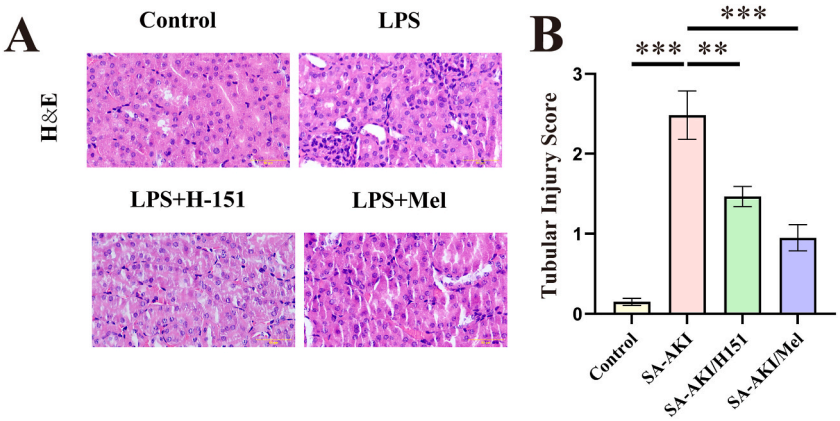

Fig. 3. H&E staining of renal tubular epithelial tissue.
(A) Image of kidney tissue sections stained with H&E (scale bar: 50 μm); (B) Tubular Injury score based on H&E staining (n = 6). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

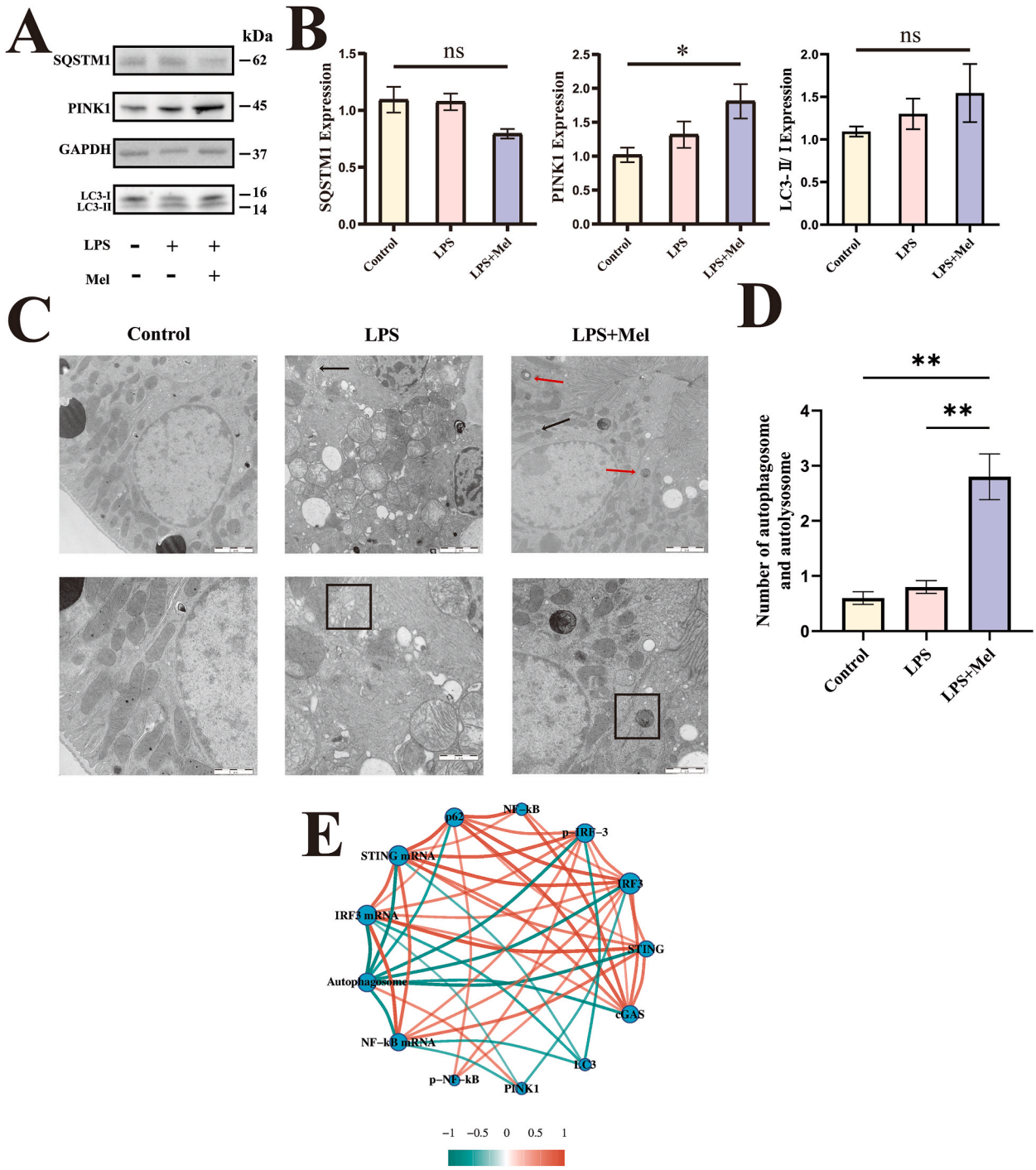

Fig. 4. Effects of Mel on mitophagy in a mouse model of sepsis-associated acute kidney injury.
(A) Representative western blot (n = 6); (B) Expression of SQSTM1, PINK1 and LC3-II/I in the kidney tissues of LPS-treat mice with Mel detected by western blot (n = 6); (C) Representative ultrastructure images of mouse renal epithelial cells observed by transmission electron microscopy (black arrow: autophagosome; red arrow: autolysosome; upper panel: scale bar: 2 μm; lower panel: scale bar: 1 μm, n = 3). (D) Number of autophagosomes and autolysosomes was calculated. (E) Correlation analysis between the expression of the cGAS-STING signaling pathway and mitophagy markers (SA-AKI group vs. LPS + Mel group). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. 'ns' indicates no significant difference.

3.5. Melatonin supplement can alleviate mitochondrial damage and apoptosis in renal tissues

When mitochondria are damaged, mtDNA may leak into the cytoplasm. The content of mtDNA in kidney tissue was detected by PCR. In this experiment, we found that the mtDNA in kidney tissue of SA-AKI group was significantly higher than that of Control group. However, with the intervention of Mel, the mtDNA content was alleviated (Fig. 5A). Meanwhile, DNA fragmentation labeled by TUNEL staining could be observed surrounding the cytoplasm, overlapping with the cells stained with DAPI (Fig. 5C). This indicates that the cytoplasm of necrotic cells is filled with scattered nucleic acid material. In the meanwhile, the renal tubular epithelial tissue of SA-AKI mice given Mel-treated demonstrated a significant decrease in apoptotic cells (Fig. 5B).

According to previous research results, the leakage of mtDNA may be one of the sources of activation of the cGAS-STING signaling pathway [23]. Combining PCR, WB, and electron microscopy results, Mel can promote autophagosome formations in the renal tubules of the SA-AKI mouse model while simultaneously reducing the mtDNA content in the tissue. Additionally, histopathological examination of kidney sections revealed that the Mel intervention group exhibited significantly less interstitial congestion, edema, inflammatory cell infiltration, and fewer apoptotic and necrotic cells compared to the LPS group (Fig. 3A–B). This demonstrates that Mel-mediated mitophagy indeed ameliorates kidney tissue damage to a certain extent.

4. Discussion

Mel has a variety of biological roles, including antioxidant, anti-inflammatory, and immune-regulating effects [25]. Research has demonstrated that Mel can successfully lower inflammatory responses and enhance prognosis in SA-AKI patients [4]. Simultaneously,

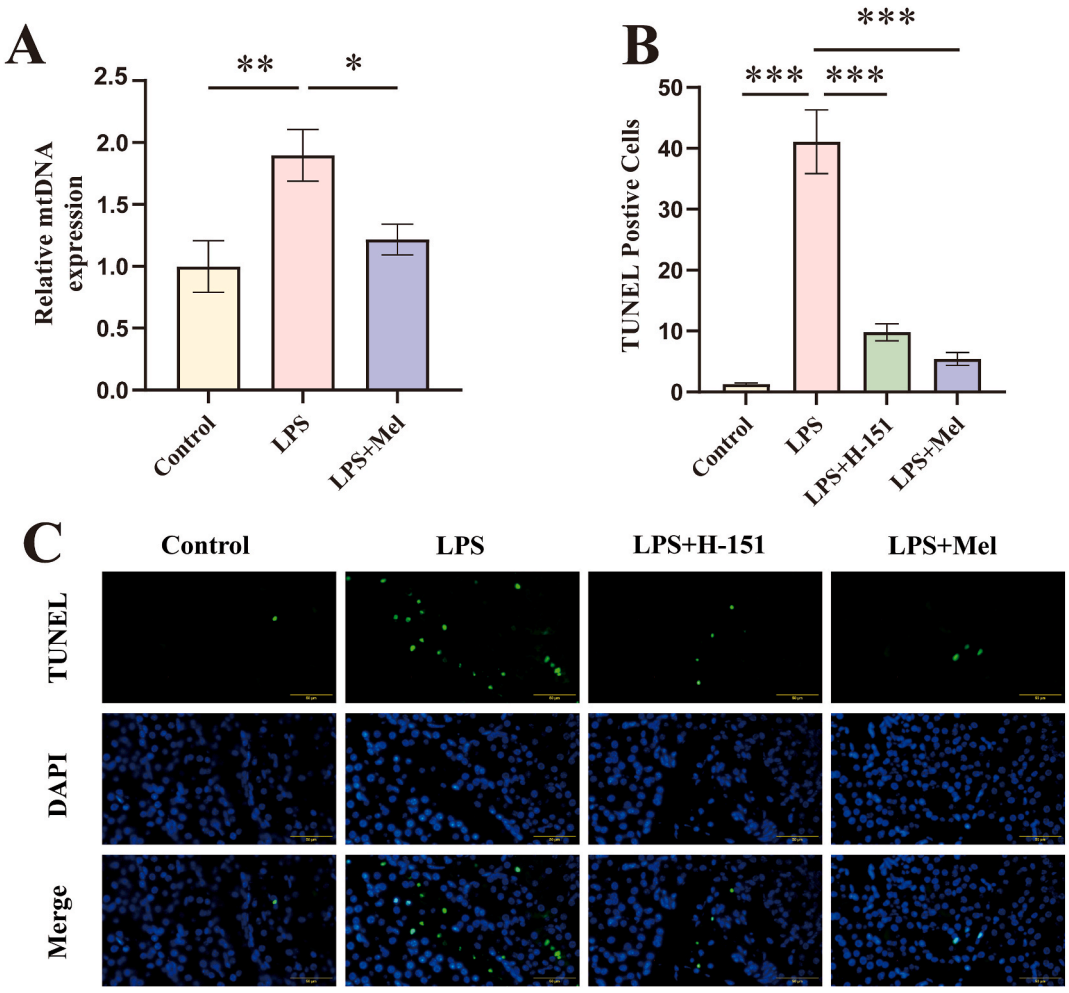

Fig. 5. Effects of H-151 and Mel on the renal pathology in mice model of SA-AKI.
(A) Expression of mtDNA detected by qRT-PCR (n = 6); (B)Tubular Injury score based on H&E staining (n = 6); (C) Apoptotic cells in kidney tissue sections were detected by TUNEL staining and counted(n = 6); (D) Image of kidney tissue sections stained with H&E (scale bar: 50 μm) and TUNEL staining (scale bar: 50 μm). Statistical significance was assessed by one-way ANOVA followed by Tukey Honestly Significant Difference Test.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

current studies have demonstrated the important role the cGAS-STING signaling pathway plays in immune surveillance and inflammatory damage [7]. Based on this research background, the main findings of this study are as follows: (I) LPS-induced activation of the cGAS-STING signaling pathway exacerbates renal inflammatory injury in SA-AKI. (II) H-151 can inhibit STING pathway and ameliorate nephritis; (III) Mel can stimulate autophagosome formations and reduce the content of mtDNA in renal tissue; (IV) Mitophagy has a negative correlation with the cGAS-STING signaling pathway's activity and lessen SA-AKI renal tubular cell injury. In conclusion, this study explored that Mel can inhibit the cGAS-STING signaling pathway to lessen kidney injury in the SA-AKI model. We hope that this discovery provides a novel therapeutic avenue for attenuating the inflammatory associated with SA-AKI.

Inflammation and mitochondrial damage have been pivotal factors in the pathogenesis of SA-AKI [1]. According to previous studies, Mel in peripheral blood of patients with sepsis were positively correlated with patient prognosis [4]. Taking into consideration the antioxidative function of Mel and thereby ameliorating inflammation, this result is not surprising [5,6,26]. However, with further investigation into the cGAS-STING signaling pathway, we believe it might offer a more profound explanation for the Mel-mediated improvement in SA-AKI prognosis.

cGAS-STING signaling pathway functioning as an immune pathway is involved in autoimmune, neurological, metabolic, cardiovascular and inflammatory diseases [27–30]. Although STING pathway is associated with the development of various kidney disorders, including diabetic nephropathy, hypertensive nephropathy, and drug-induced kidney damage [31–33]. However, there is still insufficient evidence regarding its role in SA-AKI. In light of this situation, our experiments first confirmed the role of the cGAS-STING signaling pathway in the development of SA-AKI. In our investigations, cGAS, STING, and the downstream inflammatory pathway were all considerably overexpressed in renal tissues of SA-AKI mice, together with increased inflammatory markers. To further confirm their relationship, we employed H-151 to selectively inhibit STING, resulting in improved levels of renal damage and reduced inflammation.This research demonstrated that the activation of STING signaling pathway was positively associated with renal inflammation.

There are multiple studies indicating a correlation between cGAS-STING signaling pathway activation and mitochondrial damage. In SA-AKI, mitochondrial damage is considered a key factor in renal tubular cell necrosis [34]. Mitochondrial damage may lead to leakage of mtDNA, disrupting mitochondrial bioenergetic metabolism and exacerbating cell death pathways [35]. The leakage of nucleic acid substances may activate various immune-inflammatory pathways, including the cGAS-STING signaling pathway, further exacerbating renal tissue damage [23]. We indeed observed a significant presence of fragmented DNA in the cytoplasm of cells in the LPS group through TUNEL staining. Therefore, to rectify excessive mitochondrial damage, cells will initiate regulatory mechanisms such as mitophagy to make sure the stabilization of mitochondrial quantity and quality [36].

According to previous studies, Mel, as a mitochondrial protective agent, can affect the process of mitophagy to some extent [4,37]. In study of autophagy and cancer, Wang's team found Mel promoted the initiation of autophagy in normal cells, but exhibited inhibition of autophagosome degradation in oral squamous cell carcinoma cells [38]. In terms of neuroinflammation and depression, it has been found that melatonin does not show a significant effect on mitophagy in healthy mice. However, it improved LPS-induced autophagy and reduced neuroinflammation by activating the FOXO3α signaling pathway [39]. In addition, kidney disease research has shown that Mel can promote mitophagy by activating SIRT3 and enhancing the deacetylation of TFAM's K154 site, which in turn regulates mtDNA transcription and mitochondrial function [4]. Therefore, the effect of melatonin on mitophagy varies with cell type and environment and cannot be generalized.

A drop in p62 protein levels was detected, indicating that Mel promotes mitophagy breakdown, which is consistent with the findings of the current investigation.In our study, we found an upregulation in the expression levels of PINK1 and LC3II/I in the SA-AKI group. This suggests that the oxidative stress induced by LPS can stimulate mitophagy. And in the Mel-treated group, however, the expression levels of PINK1 and LC3II were even higher, indicating that Mel can further enhance the mitophagy response.

Some studies have demonstrated that degradation of p62 often occurs in mice with enhanced mitophagy. Lower expression of p62 indicates that damaged mitochondria are sent to lysosomes for degradation. It typically happens in the mid to late stages of mitophagy responses [40]. But p62 showed a slight increase in the SA-AKI group in our experiments. We considered it might be because p62, as a stress protein, has its expression level upregulated substantially when renal tubular epithelial cells receive oxidative stress and inflammatory factor stimulation [41]. This phenomenon is not difficult to be observed when exposed to poison such as LPS. However, under the influence of Mel, mitophagy activity is significantly enhanced. Excess p62, along with damaged mitochondria, is then delivered to lysosomes and degraded together with LC3. Therefore, the intervention of Mel results in a decrease in p62 compared to the SA-AKI group.

We validated our hypothesis of enhanced mitophagy by using TEM. We observed varying degrees of mitochondrial swelling, deformation, and inner membrane damage in the renal tubular epithelial cells of both the SA-AKI group and the Mel intervention group of mice. Additionally, under the microscope, both groups exhibited varying degrees of mitochondrial damage. However, in the Mel-treated group, several autophagosomes were notably observed surrounding damaged mitochondria. Conversely, in the SA-AKI group, besides varying degrees of mitochondrial damage, there was also a significant increase in mitochondrial numbers in the field of view, indicative of mitochondrial proliferation as a compensatory response. This observation helps explain the differences in mtDNA levels between the different groups. In summary, we can conclude that Mel effectively upregulates autophagosome formations in renal tubular epithelial cells under LPS treatment and reduces mtDNA leakage.

As previously discussed, mitochondrial damage-induced mtDNA is a key factor in activating cGAS-STING signaling pathway which induces escalating inflammation. mtDNA-cGAS-STING signaling pathway has been proved to participate in cell survival, proliferation, apoptosis, necrosis, cellular pyroptosis, and cellular senescence [23]. In addition, it has been found that the p62/SQSTM1-dependent autophagy pathway negatively regulates the cGAS-STING signaling pathway in response to TBK1 activation, reducing inflammatory responses and cellular damage [42]. In our experiment, the expression of cGAS, STING, and downstream key factors NF-κB and IRF-3 in

the Mel-treated group was significantly lower than that in the SA-AKI group. We can conclude that Mel significantly inhibits the activity of this signaling pathway. Pathological results demonstrate that under Mel intervention, interstitial congestion, edema, vacuolar changes, and the degree of inflammatory cell aggregation in mouse renal tubules are significantly lower than those in the LPS group. Additionally, TUNEL staining provides a more intuitive expression of significantly lower levels of cell apoptosis in renal tissues compared to the LPS group. These findings indicate that Mel could potentially alleviate inflammation by regulating mitophagy and diminishing mtDNA leakage, consequently ameliorating renal injury.

It is noteworthy that in diabetic nephropathy, some scholars have found that activation of cGAS often accompanies strong expression of STING-IRF-3-IFN, leading to tubular damage and proteinuria [31]. However, in drug-induced nephropathy, the results show that the STING-NF-κB inflammatory is a primary cause in exacerbating nephritis [23]. In our experiments, we found that in sepsis-induced kidney injury, both at the genetic and protein levels, the activation of IRF-3 was more significant than NF-κB. This suggests that STING-IRF-3-IFN may play a predominant role in exacerbating kidney injury in SA-AKI. Without considering errors resulting from human intervention, we believe that factors contributing to this discrepancy extend beyond differing pathogenic elements. Specific kidney locations, such as damage to the glomerulus or renal tubules, may also lead to this outcome. However, further experimentation is needed to substantiate this assertion. After dissecting the specific causes, we believe that targeting downstream cGAS-STING signaling pathway more precisely can improve kidney disease.

Regarding intervention for SA-AKI, this experimental outcome demonstrates that direct inhibition via H-151 and mitochondrial protection mediated by Mel can both effectively alleviate inflammation and renal tissue damage. However, despite comparable levels of STING and downstream key factor activity between the two groups, the Mel-treated group consistently exhibits significantly superior renal outcomes in terms of inflammation severity, pathological findings, and kidney function compared to the H-151-treated group. Mel is a complex and unique anti-inflammatory hormone. Both Mel itself and its metabolites possess antioxidant functions that limit peroxyl radical [43]. Taken together, we propose that Mel, by safeguarding mitochondria, possesses the capacity to ameliorate renal injury and attenuate inflammation. This effect is not only intricately linked with the cGAS-STING signaling pathway but also connected with anti-inflammatory and antioxidant properties inherent to Mel-induced mitophagy [44,45].

In conclusion, our results suggest that Mel can effectively up-regulate autophagosome formations in LPS-induced SA-AKI, which is associated with the downregulation of the cGAS-STING signaling pathway. This process can ameliorate kidney injury and improve kidney function. We conclude that Mel, as one of the commonly used clinical hormones, has huge therapeutic potential in SA-AKI-related inflammatory injury.

CRediT authorship contribution statement

Yuchun Cao: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Xiaofang He:** Resources, Investigation, Formal analysis. **Zeyuan Liu:** Software, Resources, Data curation, Conceptualization. **Liying Miao:** Writing – review & editing, Validation, Supervision. **Bin Zhu:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition.

Ethics approval statement

Animal ethics has been approved by the Jiangsu Kerbio Medical Technology Group Co., Ltd (IAUCU23-0016).

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper or the supplementary materials.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Bin Zhu reports financial support was provided by Changzhou Municipal Health Commission. Bin Zhu reports financial support was provided by Changzhou Science and Technology Bureau. Bin Zhu reports financial support was provided by Medical young talent of Jiangsu. Bin Zhu reports financial support was provided by Top Talent of Changzhou medical innovation talent training. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e41501.

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