ORIGINAL RESEARCH

Melatonin Promotes Mitochondrial Biogenesis and Mitochondrial Degradation in Hepatocytes During Sepsis

Bin Hu, MS; Zhijiang Chen, PhD; Lili Liang, MS; Meiyu Zheng, MS; Xinxin Chen, PhD; Qiyi Zeng, PhD

ABSTRACT

Objective • This study aimed to investigate the protective mechanisms of melatonin in an in vitro model of sepsis-induced hepatocyte injury, specifically focusing on mitophagy and mitochondrial biogenesis.

Methods • In this study, we utilized lipopolysaccharide (LPS)-treated AML12 cells to establish an *in vitro* model of sepsis-induced hepatocyte injury. The effects of melatonin pretreatment were examined through various analyses, including assessments of oxidative stress, inflammation, mitophagy, mitochondrial biogenesis, and adenosine triphosphate (ATP) levels.

Results • The results revealed that LPS-treated AML12 cells exhibited elevated levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6 protein, intracellular reactive oxygen species (ROS), and lipid peroxidation, specifically malondialdehyde (MDA). Moreover, the levels of key markers associated with mitophagy, including PTEN-induced putative kinase 1 (PINK1), parkin, and LC3, were significantly increased ($P < .05$). Similarly, markers of mitochondrial biogenesis, such as peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α), nuclear respiratory factor 1 (NRF1), and mitochondrial transcription factor A (TFAM), were also significantly increased ($P < .05$). Conversely, superoxide dismutase (SOD) activity and ATP levels were significantly decreased in LPS-treated AML12 cells compared to the control group ($P < .05$). However, melatonin pretreatment led to a significant decrease in TNF-α and IL-6 protein levels, intracellular ROS, and MDA levels ($P < .05$), along with a significant increase in SOD activity, ATP levels, and markers of mitophagy and mitochondrial.

Conclusions • Our findings demonstrate that melatonin plays a role in regulating mitochondrial quality control in sepsis-induced hepatocytes. It achieves this result by promoting mitophagy and inducing mitochondrial biogenesis, thereby selectively eliminating dysfunctional mitochondria. (*Altern Ther Health Med.* 2023;29(7):284-289).

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INTRODUCTION

Sepsis is a significant global public health concern, contributing to high mortality rates in intensive care units.[1] According to the Sepsis-3 task force, sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection.[2] The liver plays a crucial role in defending against sepsis by clearing bacteria and mediating inflammatory responses. In critically ill patients, early hepatic dysfunction is a recognized risk factor for poor prognosis and increased mortality.[3] However, reducing liver injury and restoring liver function can effectively decrease the morbidity and mortality associated with sepsis.[4] Nevertheless, an ongoing clinical need remains to fully understand the molecular mechanisms underlying liver dysfunction during sepsis.

Mitochondria, as vital organelles, play a crucial role in the pathogenesis of sepsis.[5] Sepsis leads to ultrastructural damage and functional impairment in mitochondria, causing adenosine triphosphate (ATP) depletion, increased production of reactive oxygen species (ROS), oxidative stress-induced cell damage, and eventual organ failure.[6] A healthy mitochondrial network is essential for the recovery of cellular and organ function in patients with sepsis, relying on efficient mitochondrial quality control mechanisms. In mammals, mitochondrial quality control is regulated by various mechanisms, including mitophagy, mitochondrial biogenesis, and mitochondrial dynamics, which collectively contribute to mitochondrial turnover. Defective mitochondrial quality control mechanisms amplify organ failure during sepsis, while restoration of mitochondrial

quality control mechanisms can ameliorate organ dysfunction.[7] As mitochondrial damage and depletion are early manifestations of liver dysfunction during sepsis.[8] maintaining mitochondrial homeostasis in hepatocytes represents a potential therapeutic target in sepsis.

Melatonin (*N*-acetyl-5-methoxytryptamine), a hormone produced by the pineal gland, plays a vital role in various physiological functions, including stress response, regulation of sleep-wake rhythm, body temperature cycles, and circadian rhythms.[9] Melatonin exhibits a protective effect during sepsis by directly scavenging ROS or upregulating antioxidant enzymes to counteract oxidative stress and reduce inflammatory responses.[10] Additionally, melatonin has been shown to increase ATP levels.[11]

Recent findings suggest that melatonin may also regulate mitophagy, the selective degradation of damaged mitochondria, and promote mitochondrial biogenesis.[12] Sai et al.[13] demonstrated that melatonin induces mitophagy by upregulating Parkin expression. Furthermore, Pan et al.[14] found that melatonin enhances mitochondrial biogenesis and exerts protective effects in cadmium-induced hepatotoxicity. However, the relationship between melatonin and mitochondrial quality control in hepatocytes under septic conditions remains to be elucidated. This study aims to investigate the protective mechanisms of melatonin in an in vitro model of sepsis-induced hepatocyte injury, focusing on the roles of mitophagy and mitochondrial biogenesis.

MATERIALS AND METHODS

Study Design

The study design of this research involved conducting *in vitro* experiments using mouse hepatocyte AML12 cells to investigate the protective mechanisms of melatonin in sepsis-induced hepatocyte injury. Various analyses were performed to assess oxidative stress, inflammation, mitophagy, mitochondrial biogenesis, and ATP levels. The goal of the study was to elucidate the protective effects of melatonin on mitochondrial quality control mechanisms in sepsis-induced hepatocytes. The experimental design allowed for the investigation of melatonin's impact on mitophagy and mitochondrial biogenesis in the context of sepsis.

Cell Culture

Mouse hepatocyte AML12 cells were obtained from Shanghai Cell Bank (Chinese Academy of Sciences, Shanghai, China). The cells were cultured in DMEM/F12 medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma-Aldrich; St. Louis, MO, USA), insulin, transferrin, selenium (ITS) liquid media supplement (I3146, Sigma-Aldrich), and 40 ng/mL dexamethasone (Sigma-Aldrich). The cells were maintained at 37°C with 5% CO_2 in a humidified incubator.

Experimental Procedure

Melatonin (MEL) (Sigma-Aldrich) was dissolved in ethanol to achieve a final concentration of 0.01%. AML12 cells were cultured until they reached 80% confluence and were then divided into four groups: (1) Control group, where AML12 cells were treated with an equivalent volume of the vehicle; (2) MEL group, where AML12 cells were treated with 1 µM melatonin; (3) LPS group, where AML12 cells were treated with 25 µg/mL lipopolysaccharide (LPS) derived from Escherichia coli O55:B5 (Sigma-Aldrich) for 24 hours; and (4) MEL plus LPS group, where AML12 cells were pretreated with 1 µM melatonin for 1 hour, followed by exposure to 25 µg/mL LPS for 24 hours.

Assessment of Cell Viability

Cell viability was determined using the Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan). AML12 cells were seeded into 96-well plates at a density of 5×10^3 cells per well and treated with different concentrations of MEL (0, 0.01, 0.05, 0.1, 0.5, and 1 µM) and LPS (0, 1, 5, 10, and 25 µg/mL) for 24 hours. Afterwards, 10 µL of the CCK-8 reagent was added to each well, and the plates were incubated for 1 hour at 37°C. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Hercules, CA).

Measurement of Intracellular ROS Levels

Intracellular ROS levels were assessed using a commercially available kit (Beyotime Biotechnology, China), following the manufacturer's instructions. AML12 cells were trypsinized, centrifuged, and then incubated with 2',7'-dichlorofluorescein-diacetate (DCFH-DA) (10 µM in DMEM/F12). The quantification of ROS was performed by measuring the mean fluorescence intensity using a flow cytometer (BD Biosciences, San Jose, CA, USA) with excitation at 488 nm and emission at 525 nm.

Enzyme-Linked Immunosorbent Assay (ELISA)

The protein levels of TNF-α and IL-6 were determined in the cell-free culture supernatants of AML12 cells using a commercial ELISA kit (Neobioscience Technology Company, China), following the manufacturer's instructions.

Biochemical Analyses

The levels of malondialdehyde (MDA), superoxide dismutase (SOD) activity, and intracellular ATP in AML12 cells were measured using suitable kits: the lipid peroxidation MDA assay kit, the total SOD assay kit with WST-8, and the ATP assay kit (Beyotime Biotechnology, China), respectively. These analyses were conducted following the manufacturer's instructions.

Western Blot Analysis

AML12 cells were homogenized in radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease and phosphatase inhibitor cocktail (Beyotime Biotechnology, China). Total protein was extracted and quantified using a BCA protein assay kit (Beyotime Biotechnology). The proteins were then separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). After blocking with 5% BSA at room temperature for 1 hour, the membranes were incubated

overnight at 4°C with the following primary antibodies: PINK1 (1:1,000, Proteintech), Parkin (1:1,000, Proteintech), LC3 (1:1,000, Proteintech), PGC-1α (1:1,000, Proteintech), NRF1 (1:1,000, Proteintech), TFAM (1:1,000, Proteintech), and GAPDH (1:5,000, Proteintech). Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. Protein levels were quantified using densitometry and analyzed using Image J software.

Statistical Analysis

Statistical analysis was conducted using SPSS version 20.0 (IBM, Armonk, NY, USA). The data were presented as the mean ± standard deviation (SD) from three independent experiments, and GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA) was used for data summarization. Comparisons between groups were performed using a one-way analysis of variance (ANOVA), followed by a post hoc pairwise comparison using the least significant difference (LSD) test. A significance level of $P < .05$ was considered statistically significant.

RESULTS

Cell Viability

To assess the cytotoxic effects of LPS and melatonin on AML12 cells, a CCK-8 assay was performed. AML12 cells were incubated with various concentrations of melatonin (0.01 μM, 0.05 μM, 0.1 μM, 0.5 μM, and 1 μM) for 24 hours. The results indicated that the increased doses of melatonin did not significantly affect the viability of AML12 cells ($P > .05$), suggesting that melatonin was not cytotoxic to AML12 cells at the investigated concentrations in this study. In contrast, the viability of AML12 cells was significantly reduced after incubation with various concentrations of LPS (≤25 μg/mL) for 24 hours (Figure 1a and Figure 1b). Subsequent experiments were conducted using 25 μg/mL LPS treatment with or without 1 μM MEL for 24 hours.

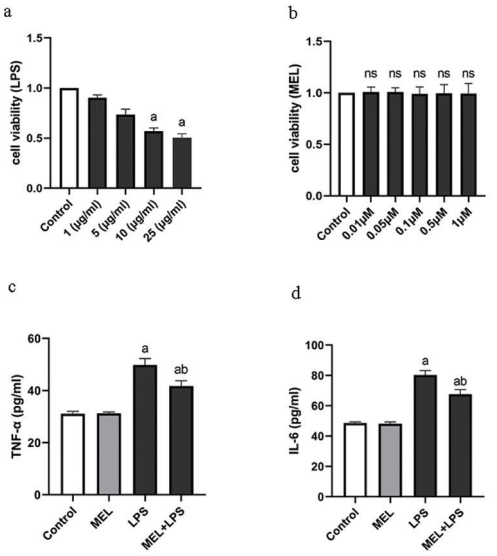
Melatonin Attenuates LPS-Induced Inflammatory Response in AML12 Cells

Given the increased levels of proinflammatory cytokines observed in sepsis, which can potentially harm hepatocytes, the protein levels of TNF-α and IL-6 were assessed in cell-free culture supernatants to investigate the impact of melatonin on the LPS-induced inflammatory response in hepatocytes. TNF-α and IL-6 protein levels were significantly elevated compared to the control group in cell-free culture supernatants from LPS-treated AML12 cells. However, melatonin pre-treatment significantly decreased the levels of TNF-α and IL-6 in cell-free culture supernatants from LPS-treated AML12 cells ($P < .05$); see Figure 1c and Figure 1d.

Melatonin Attenuates LPS-Induced Oxidative Stress in AML12 Cells

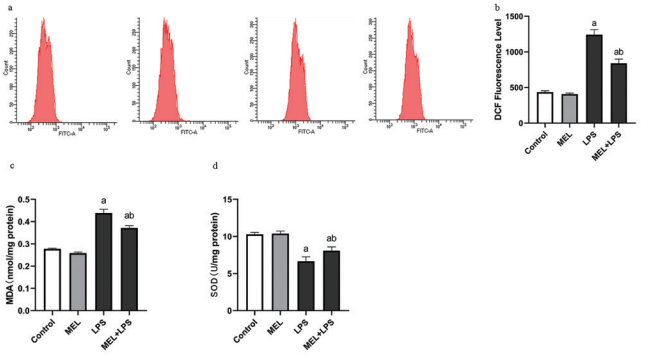
To assess the redox status of hepatocytes during sepsis, we measured the levels of intracellular ROS, See Figure 2a and Figure 2b. Additionally, we determined lipid peroxidation levels by examining MDA levels, as shown in Figure 2c. Furthermore,

Figure 1. Melatonin attenuated the LPS-induced inflammatory response in AML12 cells.



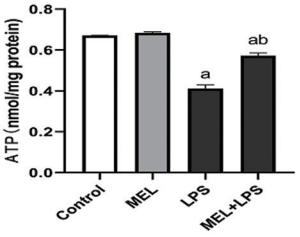
Note: Figure 1a and 1b: AML12 cell viability following treatment with different concentrations of LPS or MEL for 24 hr. Data are presented as the *mean* ± SD of three independent experiments. Statistical significance was indicated as $^{a}P < .05$ versus Control and $^{ns}P > .05$ versus Control. Figure 1c and 1d: TNF-α and IL-6 protein levels in cell-free culture supernatants. Data are presented as the *mean* ± SD of three independent experiments. Statistical significance was indicated as $^{a}P < .05$ versus Control and $^{b}P < .05$ versus LPS.

Figure 2. Melatonin attenuated LPS-induced oxidative stress in AML12 cells.



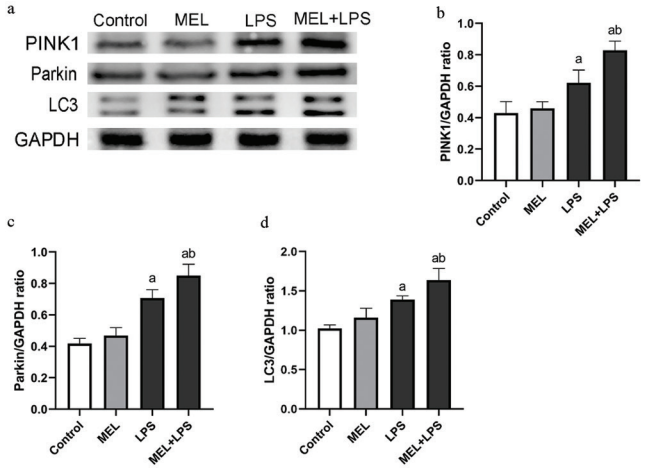
Note: Figure 2a: AML12 cells were stained with DCFH-DA followed by flow cytometric analysis to measure intracellular ROS levels; Figure 2b: The quantification of intracellular ROS levels were presented as mean fluorescence intensity; Figure 2c MDA levels; and Figure 2d: SOD activity were assessed using a lipid peroxidation MDA assay kit and a total SOD assay kit with WST-8, respectively. Data are presented as the *mean* ± SD of three independent experiments. Statistical significance was indicated as $^{a}P < .05$ versus Control and $^{b}P < .05$ versus LPS.

Figure 3. Effect of melatonin on mitochondrial function in AML12 cells.



Note: Intracellular ATP levels were measured in AML12 cells to assess mitochondrial function. Data are presented as the *mean* ± SD of three independent experiments. Statistical significance was indicated as $^{a}P < .05$ versus Control and $^{b}P < .05$ versus LPS.

Figure 4. Melatonin upregulated mitophagy-related protein levels in AML12 cells.



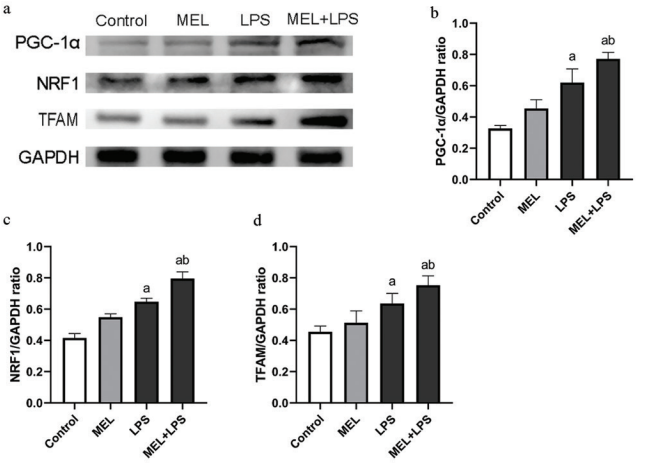
Note: Figure 4a: Western blot analysis assessed the levels of mitophagy-related proteins (PINK1, Parkin, and LC3); Figure 4b and 4c: The quantification of protein levels was normalized to the GAPDH level. Data are presented as the *mean* ± SD of three independent experiments (n = 3 in each group). Statistical significance was indicated as $^{a}P < .05$ versus the Control group and $^{b}P < .05$ versus the LPS group.

we evaluated SOD activity, See Figure 2d. Compared to the control group, LPS-treated AML12 cells exhibited significantly elevated intracellular ROS and MDA levels, indicating increased oxidative stress. However, melatonin pre-treatment significantly reduced intracellular ROS and MDA levels in LPS-treated AML12 cells ($P < .05$). Conversely, SOD activity, an antioxidant enzyme, was significantly decreased in LPS-treated AML12 cells compared to the control group. However, melatonin pre-treatment significantly increased SOD activity in LPS-treated AML12 cells ($P < .05$).

Melatonin Attenuates LPS-Induced Mitochondrial Dysfunction in AML12 Cells

Intracellular ATP levels were measured to examine mitochondrial function in hepatocytes during sepsis. LPS-treated AML12 cells exhibited a significant decrease in intracellular ATP levels compared to the control group. However, pre-treatment with melatonin significantly increased intracellular ATP levels in LPS-treated AML12 cells ($P < .05$), refer to Figure 3.

Figure 5. Melatonin upregulated mitochondrial biogenesis-related protein levels in AML12 cells.



Note: Figure 5a: Western blot analysis was performed to assess the levels of mitochondrial biogenesis-related proteins (PGC-1α, NRF1, and TFAM); Figures 5b, 5c, and 5d: The quantification of protein levels were normalized to the GAPDH level. Data are presented as the *mean* ± SD of three independent experiments (n = 3 in each group). Statistical significance was indicated as $^{a}P < .05$ versus the Control group and $^{b}P < .05$ versus the LPS group.

Melatonin Upregulates Mitophagy-Related Protein Levels

The PINK1-Parkin pathway is a key regulator of mitophagy. Western blot analysis revealed that PINK1, Parkin, and LC3 protein levels were significantly elevated in LPS-treated AML12 cells compared to the control group. Furthermore, melatonin pre-treatment significantly increased the protein levels of PINK1, Parkin, and LC3 in LPS-treated AML12 cells ($P < .05$), refer to Figure 4.

Melatonin Upregulates Protein Levels Related to Mitochondrial Biogenesis

PGC-1α, NRF1, and TFAM are crucial proteins involved in mitochondrial biogenesis. Western blot analysis revealed a significant increase in the protein levels of PGC-1α, NRF1, and TFAM in LPS-treated AML12 cells compared to the control group. Furthermore, melatonin pre-treatment significantly enhanced the protein levels of PGC-1α, NRF1, and TFAM in LPS-treated AML12 cells, as demonstrated by Western blot analysis ($P < .05$), refer to Figure 5.

DISCUSSION

Liver dysfunction is commonly observed in patients with sepsis, particularly in the early stages, and reducing liver injury and restoring liver function have been shown to

decrease sepsis-associated mortality.[4] While the complete molecular mechanisms underlying sepsis-induced liver injury remain elusive, evidence suggests the involvement of mitochondrial dysfunction.[15] Mitochondrial dysfunction is closely associated with hepatocyte injury during sepsis, contributing to inflammation and oxidative stress.[16] Therefore, restoring mitochondrial function may be a promising therapeutic approach for sepsis-induced injury.[17]

Activation of mitophagy and mitochondrial biogenesis has been shown to improve mitochondrial function and alleviate injury during sepsis.[17-18] Melatonin, known for its high concentrations in mitochondria, appears to target mitochondria as its primary site of action.[18] Furthermore, melatonin has demonstrated anti-inflammatory and antioxidative stress functions by directly scavenging ROS or enhancing antioxidant enzyme activity, thereby exerting a protective role in sepsis pathogenesis.[19] In our study, we utilized LPS to establish an *in vitro* model of sepsis-induced hepatocyte injury. Melatonin pretreatment was observed to enhance mitophagy and mitochondrial biogenesis in LPS-treated cells.

Sepsis is characterized by metabolic imbalance, mitochondrial dysfunction, and excessive production of ROS, which can accumulate and cause damage to hepatocytes.[20] Mitochondria are particularly susceptible to ROS-induced damage within cells. The production of mitochondrial ROS primarily occurs in the electron transport chain during oxidative phosphorylation. Disrupted mitochondrial function can lead to excessive ROS production, resulting in damage to mitochondrial proteins and DNA, thus establishing a self-perpetuating cycle of ROS generation.[21]

Mitophagy, a process of selective autophagy targeting damaged mitochondria, plays a crucial role in eliminating dysfunctional mitochondria and preventing excessive production of reactive oxygen species.[6] The PINK1-Parkin pathway is a key regulator of mitophagy. During sepsis, PINK1 accumulates on the outer membrane of dysfunctional mitochondria, facilitating the translocation of Parkin from the cytoplasm to the outer mitochondrial membrane. This recruitment of autophagic receptors and the binding of Parkin to LC3B II trigger the initiation of mitophagy.[7] Accumulating evidence suggests that mitophagy may play a protective role in sepsis-induced injury. Studies have demonstrated that enhancing mitophagy reduces LPS-induced cardiomyocyte injury, oxidative stress, and inflammation.[22]

Conversely, inhibition of mitophagy in Parkin-knockout mice leads to worsened cardiac function and sustained degradation of mitochondrial metabolic functions compared to wild-type mice during sepsis.[23] However, the regulatory role of melatonin in mitophagy and mitochondrial biogenesis in hepatocytes during sepsis remains to be elucidated. In our present study, we observed induction of mitophagy in hepatocytes exposed to LPS compared to the control group, as evidenced by increased expression of mitophagy-related proteins. Furthermore, melatonin pretreatment enhanced the effects of mitophagy, suggesting that melatonin may potentiate mitophagy in hepatocytes during sepsis.

Sepsis has detrimental effects on the mitochondrial network, leading to the depletion of healthy mitochondria and energy deficiency. To maintain a healthy population of mitochondria, mitochondrial biogenesis is crucial. The PGC-1α-NRF1-TFAM pathway primarily regulates mitochondrial biogenesis. PGC-1α, a transcriptional co-activator, promotes mitochondrial DNA replication and transcription by activating the transcription factor NRF1, subsequently upregulating the expression of TFAM, which is responsible for regulating mitochondrial DNA transcription and replication.[7]

The process of mitochondrial biogenesis also plays a significant role in sepsis. In astrocytes, sepsis can stimulate mitochondrial biogenesis, increase the mitochondrial population within cells, and restore mitochondrial ultrastructure to meet the heightened energy demands imposed by septic conditions.[24,25] Pharmacological interventions that enhance mitochondrial biogenesis have shown promise in alleviating sepsis-induced injury.[17] Our study revealed elevated protein levels of PGC-1α, NRF1, and TFAM in LPS-treated hepatocytes compared to the control group. Notably, melatonin pretreatment further enhanced these effects, suggesting that melatonin may promote mitochondrial biogenesis in hepatocytes during sepsis.

Mitochondrial dysfunction is considered an early event and is closely associated with oxidative stress and inflammation during sepsis. Restoring mitochondrial function has been shown to attenuate oxidative stress and the inflammatory response,[6] highlighting the significance of improving mitochondrial function in preventing and treating sepsis. Mitochondria play a crucial role in generating cellular energy through ATP. Cellular ATP levels serve as markers of mitochondrial function. Our study showed a reduction in intracellular ATP levels in LPS-treated hepatocytes compared to the control group. However, this effect was reversed by melatonin pretreatment.

Furthermore, melatonin demonstrated a protective effect in our in vitro model of sepsis-induced hepatocyte injury. It was evident from the decreased release of inflammatory cytokines induced by LPS in hepatocytes and the alleviation of LPS-induced oxidative stress. These findings suggest that melatonin has the potential to improve mitochondrial function, attenuate oxidative stress, and mitigate the inflammatory response in hepatocytes during sepsis.

Study Limitations

Several limitations should be acknowledged in this study. Firstly, our study utilized an *in vitro* model of sepsis-induced hepatocyte injury, which may not fully recapitulate the complex pathophysiology of sepsis in vivo. Therefore, caution should be exercised when extrapolating the findings to clinical settings. Additionally, the concentrations of melatonin used in our experiments were based on preliminary studies and may not reflect physiological levels in patients. Further studies are needed to determine the optimal dosage and timing of melatonin administration in sepsis.

Moreover, the mechanisms underlying the effects of melatonin on mitophagy and mitochondrial biogenesis in hepatocytes during sepsis remain incompletely understood. Future investigations should focus on unraveling the specific molecular pathways involved. Lastly, while our study demonstrated the protective effects of melatonin on hepatocytes during sepsis, it is important to note that sepsis is a complex systemic condition that involves multiple organ systems. The impact of melatonin on other organs and overall sepsis outcomes requires further exploration.

CONCLUSION

Our study has provided valuable insights into the role of melatonin in regulating mitochondrial quality control in hepatocytes during sepsis. Melatonin demonstrated its potential as a therapeutic intervention for sepsis-induced liver injury by promoting mitophagy and stimulating mitochondrial biogenesis. The findings highlight the importance of targeting mitochondrial function in the management of sepsis. Further research is warranted to explore the precise mechanisms underlying melatonin's effects and to evaluate its therapeutic efficacy in clinical settings.

DATA AVAILABILITY

The data used to support this study is available from the corresponding author upon request.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally, read and approved the final manuscript.

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