Melatonin Alleviates Microglia-Mediated Neuroinflammation By Suppressing NLRP3 Inflammasome-Mediated Pyroptosis Via ROS/mtDNA/STING Pathway After Spinal Cord Injury

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Research

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Abstract

Background

Microglia pyroptosis-induced neuroinflammation has been one of the potential treatment targets for spinal cord injury (SCI). And melatonin is reported to have anti-neuroinflammation effect on SCI, but the underlying mechanism is largely unexplored. In addition, the potential regulatory role of stimulator of interferon genes (STING) mediated innate immune response in the SCI-induced neuroinflammation also remains unknown. The aim of this study is to identify the potential molecular mechanism of the anti-neuroinflammation effect of melatonin in SCI mice and to explore whether STING-mediated signal pathway is involved in this pharmacological process.

Methods

In vivo, the C57BL/6 female mice underwent SCI injury or Sham surgery (laminectomy alone). Melatonin and selective STING antagonist C-176 were administered intraperitoneally after injury in the SCI group once a day for 3 or 28 consecutive days for different experiments. The BMS score system was adopted to assess the motor function of mice. In vitro, the Lipopolysaccharide (LPS)/ATP was combinedly used to induce cell pyroptosis in BV2 microglia and the adenovirus was used to overexpress STING. A series of molecular experiments including Western blot (WB), quantitative real-time polymerase chain reaction (RT-qPCR), enzyme linked immunosorbent assay (ELISA) and immunofluorescence (IF) were performed in vivo and in vitro.

Results

Our results showed that melatonin effectively suppressed NLRP3 inflammasome-induced pyroptosis and STING-mediated pathway after SCI. In addition, C-176 also alleviated the NLRP3 inflammasome-mediated pyroptosis and promoted functional recovery in vivo. In vitro, we also found that melatonin abrogated NLRP3 inflammasome activation in LPS/ATP-induced BV2 cells, while overexpression of STING reversed the anti-pyroptotic role of melatonin. Subsequent results together indicated that the role of melatonin on STING-dependent NLRP3 inflammasome activation may be mediated by decreasing ROS production and cytosolic mtDNA release.

Conclusion

This study preliminarily demonstrated that melatonin exerts its anti-neuroinflammation role on SCI by alleviating the NLRP3 inflammasome-mediated pyroptosis, which was mediated by blocking the ROS/mtDNA/STING pathway. It provides us a better understanding of the pathological mechanism after SCI and offer experiment evidence to promote the use of melatonin for SCI.

1. Introduction

Spinal cord injury (SCI) is one of the most common and fatal injury of the spine, which leads to limb paralysis and other severe complications including sensory disorders, bedsores and lung infection[1]. There are approximately 2.5 million SCI patients around the world and an increase of 130,000 annually[2]. Unfortunately, there is no ideal or effective treatments for SCI in clinic for its complex pathological mechanism[3, 4]. Nowadays, it is widely accepted that the neuroinflammation-mediated extensive neuronal damage during the secondary phase of SCI is the main cause of persistent destruction after injury [5, 6]. Therefore, how to attenuate the neuroinflammation has become a hot issue for the research of SCI.

The NOD-like Receptor protein 3 (NLRP3) inflammasome, a cytosolic molecular platform comprised by NLRP3, apoptosis associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase-1, can be activated by both the pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)[7, 8]. Once NLRP3 inflammasome activated, the caspase-1-dependent Gasdermin D (GSDMD) cleavage leads to the pore formation on the cell membrane and subsequently promotes the secretion of mature proinflammatory cytokines (IL-1 β and IL-18), which will greatly exaggerate the inflammation response. This kind of programmed cell death is also known as cell pyroptosis[9]. Recent researches have shown that NLRP3-mediated pyroptosis plays crucial roles in various diseases including intestinal diseases[10], cardiovascular diseases[11, 12], neurological disease[13] as well as the age-related diseases[14]. Importantly, some researchers and we previously reported that NLRP3 inflammasome-mediated pyroptosis is also involved in SCI and the inhibition of pyroptosis may be one of the potential treatment targets for SCI[15–17].

Cyclic GMP-AMP synthase (cGAS)-stimulator of interferon genes (STING) axis, a newly identified signaling pathway, functions as an innate immune defensive system by sensing cytosolic DNA[18, 19]. In brief, DNA in the cytosol activates cGAS and produces cGAMP (cyclic GMP-AMP), which functions as a secondary messenger that binds and activates the STING. Then the downstream kinases such as tank-binding kinase 1 (TBK1) will be recruited to phosphorylate and facilitate nuclear translocation of the transcription factors (IRF3 and NF- κ B) that play corresponding roles[20, 21]. Recently, increasing evidences pointed that STING participates in multiple diseases by regulating NLRP3 inflammasome[22, 23]. Li N[24] unveiled that STING could activate the NLRP3 inflammasome and pyroptosis in cardiomyocytes. Notably, they also reported that STING was involved in the development of LPS-induced acute lung injury by promoting the activation NLRP3 inflammasome and pyroptosis[25]. However, whether STING can regulate NLRP3 inflammasome-mediated pyroptosis in SCI has not been well investigated till now.

Melatonin ((N-acetyl-5-methoxytryptamine), a neuroendocrine hormone which was primarily synthesized in the pineal gland, has been frequently reported to have protective effects on various diseases for its diversity functions such as antioxidant, circadian

rhythm regulation, sleep promotion and immunomodulatory effects[26–31]. Of note, melatonin has been reported to own great anti-inflammatory function by suppressing NLRP3 inflammasome-mediated

pyroptosis[32–34]. Liu Z [35] reported that melatonin could prevent and treat systemic inflammatory response via alleviating NLRP3 inflammasome-induced pyroptosis by blocking NF- κ B/GSDMD signal in mice adipose tissue. Zhang Y [36] also pointed that melatonin replacement strategy might be considered as a new method to treat atherosclerosis for its essential role in against endothelial pyroptosis. However, fewer evidences were reported about the regulatory role of melatonin on NLRP3 inflammasome-mediated pyroptosis after SCI. In most recently, melatonin is reported to delay aging and neurodegeneration by inhibiting the cytosolic mtDNA-activated cGAS/STING/IRF3 pathway[37]. It greatly encouraged us to speculate that if mtDNA-activated STING pathway is also involved in the process of melatonin on regulating pyroptosis in SCI.

Therefore, the aims of this study were to determine the role of melatonin on NLRP3 inflammasome-mediated pyroptosis after SCI and to investigate whether the STING-mediated signaling pathway was involved in the underlying mechanism.

2. Materials And Methods

2.1 Animals

The C57BL/6 clean-grade female mice were purchased from the Animal center of Chinese Academy of Science (Shanghai, China), and housed in a humidity and temperature-controlled environment with free access to food and water (20-25°C, 65% humidity, 12h light/dark cycle). All animal experiments involved in this study followed the guidelines established by the Animal Ethical and Welfare Committee of Huashan Hospital, Fudan University.

2.2 Mouse model of SCI

The SCI mice model was prepared following the protocol as described in our previous research[5]. In brief, C57BL/6 female mice were weighed and anesthetized with intraperitoneal injection of 4% pentobarbital sodium (35mg/kg). The T8-T9 vertebrae were removed by laminectomy and the spinal cord was exposed. The mice in SCI group were inflicted by laterally compressing the cord using Dumont-type forceps with a spacer of 0.2mm for 20s. The mice in Sham group received laminectomy alone without injury to the spinal cord. All the wounds were washed with sterile saline and sutured layer by layer. Following surgery, Penicillin (20,000 U) was injected intramuscularly once a day for 3 consecutive days and bladder massage was conducted manually 3 times a day to assist urination.

After operation, the mice in SCI-Melatonin group received daily intraperitoneal injections of 10mg/kg melatonin (Sigma-Aldrich, St. Louis, MO, USA) for 3 days (for histological analysis) or 28 days (for BMS score analysis). In SCI-C176 group, the STING antagonist C-176 (MedChemExpress, 750nmol dissolved in 200 μ L corn oil) was firstly injected intraperitoneally at 1h post-injury [38], and then be used as the same way as melatonin.

2.3 Assessment for motor function of mice

The Basso Mouse Scale (BMS) score (0 to 9 points) was recorded at 1, 3, 5, 7, 14, 21 and 28 days after surgery to assess the locomotion function of the mice[39]. Briefly, the mice were allowed to move freely for 5 minutes in an open field, and the hindlimb movements, stepping and coordination points were assessed by two investigators who were blinded to treatment.

2.4 Cell culture and drug treatments

The murine BV2 microglia cell line was purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China) and cultured in DMEM medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and an antibiotic mixture (1% penicillin/streptomycin) (Invitrogen, Carlsbad, CA, USA) at 37 °C and 5% CO₂.

The cells were randomly assigned into the control and the LPS/ATP treatment groups. In LPS/ATP group, BV2 cells were exposed to the 1000 ng/ml LPS (Sigma-Aldrich, St. Louis, MO, USA) for 4h, followed by 5mM ATP (Sigma-Aldrich, St. Louis, MO, USA) for 2h to induce pyroptosis. In Melatonin group, the cells were pre-treated with melatonin at the concentration of 5μM or 10 μM for 24h before LPS/ATP stimulation.

The adenoviral infection was adopted to overexpress STING in BV2 cells according to the manufacturers' instructions. Adv-control or Adv-STING were designed and synthesized by Han Heng (Shanghai, China). Briefly, when cells reached 60% confluence, they were infected with Adv-control or Adv-STING at different multiplicity of infection (MOI), followed by incubation for 6 h. Then, the culture medium was replaced with fresh completed DMEM.

2.5 Cell viability assay

The cell viability of BV2 cells after melatonin treatment was assessed by CCK-8 assay (Sigma-Aldrich, MO, USA) according to the manufacture's protocol. Briefly, BV2 cells were seeded in the 96-well plates and cultured for 24h, followed by a series concentration of melatonin (5, 10, 100, 500, 1000μM) treatment for 24h. Then the culture medium was removed and 110μL CCK8 work solution (containing 10μL CCK8 reagent) was added in each well. After incubation for 2h, the absorbance of each well was detected at 450nm.

2.6 Cell Morphological observation

Following drug treatment (LPS/ATP, melatonin), the medium was removed and the 4% formaldehyde was added for 30min. After that, cells washed with PBS for 3 times (10min per time), then the morphology of BV2 cells was photographed under light microscope.

2.7 Propidium iodide staining

After corresponding treatment, BV2 cells were stained with propidium iodide staining (PI) and Hoechst for 15min and the PI positive cells were observed under the fluorescence microscope (Leica, Germany).

2.8 Cell death assay

The secreted levels of lactate dehydrogenase (LDH) were detected using the LDH Cytotoxicity Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions.

2.9 Enzyme-linked immunosorbent assay (ELISA)

The levels of IL-1 β , IL-18 and TNF- α in cell culture supernatants and mouse serum were measured using commercial ELISA kits (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. The absorbance was detected by microplate reader (Thermo Scientific, Waltham, MA, USA) at 450 nm wavelength.

2.10 Measurement of cGAMP

cGAMP in spinal cord tissue and cell lysate was measured by chemiluminescent enzyme immunoassay (2'3'-cGAMP ELISA kit, Cayman Chemical, USA) according to manufacturers' protocols.

2.11 RNA extraction and quantitative real-time PCR

Total RNA was extracted from the spinal cord tissue or the harvested BV2 cells using Trizol reagent (Invitrogen, San Diego, CA, USA) and reverse-transcribed, then cDNAs were amplified according to the manufacturer's instruction. The SYBR Green PCR Master Mix Kit (Applied Biosystems, Shanghai, CHINA) was used to quantify the relative mRNA levels. β -actin served as the internal controls and the $2^{-\Delta\Delta CT}$ method was adopted to analyze the relative expression levels of mRNAs. Primer sequences are listed in Table.1.

2.12 Western blotting (WB) analysis

Total protein was extracted from the spinal cord tissues and BV2 cells using RIPA buffer containing phosphatase and protease inhibitor cocktail, and then quantified by the BCA Protein Assay kit. Protein samples were separated by 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocked with 5% non-fat milk at room temperature for 1 h, the membranes were incubated overnight at 4°C with primary antibodies: NLRP3 (1:1000, CST, 15101s), IL-1 β (1:1000, ABclonal, A16288), CASP-1 (1:1000, ABclonal, A18646), p65 (1:1000, CST, 8242), P-p65 (1:1000, CST, 3033), GSDMD (1:1000, Abcam, ab209845), ASC (1:1000, ABclonal, A11433), STING (1:1000, CST, 13647), TBK1 (1:2000, abcam, ab40676), P-TBK1 (1:1000, CST, 5483), IRF3 (1:1000, abcam, ab68481), P-IRF3 (1:1000, CST, 4947) and β -actin (1:2000, Abcam, ab8245). Following washed with TBST for 3 times, the HRP-conjugated secondary antibody (1:2000) was added and incubated at room temperature for 1 h. The protein bands were visualized using an enhanced chemiluminescence (ECL) kit, and blots were quantified by the gel imaging system (UVP LLC, Upland, CA, USA).

2.13 Histological analysis and immunofluorescence

On the third day after SCI, the mice (n=10) were sacrificed and the spinal cord segments near the lesion epicenter were harvested. The samples were then fixed with 4% paraformaldehyde overnight followed by embedded in paraffin. Hematoxylin and eosin (H&E) staining was performed according to the manufacturer's instructions. For Nissl staining, the sections were stained with toluidine blue following a standard protocol.

Immunofluorescence analysis was performed as previously described[5]. The sections of spinal cord tissue or BV2 cells in each group were fixed with 4% paraformaldehyde for 30 min and then blocked in 5% bovine serum albumin (BSA) containing 0.3% Triton X-100. Then the samples were incubated with primary antibodies overnight at 4°C: GSDMD (1:100, Abcam, ab209845), NLRP3 (1:100, ABclonal, A12694), STING (1:100, Abcam, ab210070), IRF-3(1:100, ABclonal, A0816), A. On the second day, the samples were incubated with appropriate fluorescently labeled secondary antibodies (1:250, Molecular Probes) and counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma-Aldrich). All images were observed with an optical or fluorescence microscope (Leica, Germany).

2.14 Measurement of ROS production

Total ROS production was detected by commercial fluorescence probe. Briefly, BV2 cells pretreated with or without melatonin were stimulated by LPS/ATP and then incubated in the dark with 10 μ M DCFH-DA (MedChemExpress, USA) at 37°C for 30 min. Then cells were washed with PBS to remove the extracellular DCFH-DA followed by observing under the fluorescence microscope (Leica, Germany). Relative ROS production was quantified by the change of fluorescence intensity comparing to the control cells at isosmolar condition.

2.15 mtDNA DNA isolation and mtDNA copy number analysis

mtDNA was evaluated by RT-qPCR as previously described[40]. In brief, BV2 cells were harvested after corresponding treatment in different groups. Thereafter, cytosolic fractions were isolated by centrifugation, and the DNA was isolated from the whole cell extracts and cytosolic fractions respectively (Qiaquick nucleotide removal columns, Qiagen). The whole cell extracts served as control for total mtDNA.

2.16 Statistical analysis

All results are presented as means $\pm$ standard deviation. Student's unpaired t tests and one-way ANOVA analysis of variance (ANOVA) followed by Dunnett's test were used to analyze all data. P $\leq$ 0.05 was considered to be statistically significant. All statistical analysis was performed with the SPSS 22.0 software (IBM, NY).

3. Results

3.1 Melatonin reduced NLRP3 inflammasome-mediated pyroptosis in mice after SCI

As shown in Fig. 1A, all mice were randomly divided into three groups (Sham, SCI and SCI + MT). 10 mice in each group were sacrificed for histological analysis at 3 days post-injury and the other 10 mice were used to assess the functional recovery using BMS score system at 1, 3, 7, 14, 21, 28 days post-SCI. We can easily see in Fig. 1B that higher BMS scores were exhibited in SCI-MT group compared to SCI group at each time points after SCI. As expected, the protein level of NLRP3 inflammasome and pyroptosis-related proteins (including NLRP3, GSDMD, GSDMD-NT, Cleaved Caspase-1) were upregulated obviously in the SCI group as demonstrated by the western blot (WB) assay in Fig. 1C-D. Similarly, the level of serum LDH in SCI mice was also obviously higher than that in the mice of Sham group, which was assessed by the commercial LDH kits (Fig. 1E). In addition, the results of ELISA indicated that the level of released pro-inflammatory cytokines (TNF- α , IL-1 β and IL-18) in serum were all significantly elevated after SCI (Fig. 1F). The increased expression of NLRP3 was confirmed by immunofluorescence (Fig. 1G). Notably, all of these above changes were obviously reversed by melatonin administration (10mg/kg, i.p) (Fig. 1C-G). It preliminarily demonstrated that melatonin has the ability to mitigate the pyroptosis-mediated neuroinflammation and could promote the motor function recovery after SCI in mice.

3.2 Melatonin suppressed the activation of STING-mediated signaling pathway after SCI in mice

To investigate whether the STING-mediated signaling pathway participated in the pathological process after SCI, we detected the expression level of STING pathway in the spinal cord samples by WB and RT-qPCR assays. As indicated in Fig. 2A-C, the mRNA and protein level of STING were both increased in SCI group comparing to Sham group. And although there is no significant difference on the mRNA level of TBK1 and IRF3 among three groups, the protein phosphorylation levels of TBK1 and IRF3 (two main downstream effectors of the STING-mediated pathway) were obviously elevated in the SCI group than that in the Sham group. Moreover, the higher level of cGAMP production was detected in the SCI group (Fig. 2D). Importantly, the results of immunofluorescence (IF) staining in Fig. 2E showed that the STING was co-localized with Iba-1 (a microglia marker), which implied that STING pathway activation was mainly located in the microglia cell after SCI. Unsurprisingly, the IF intensity expression pattern of STING was identical to the results of WB and RT-qPCR. Of note, all the increased markers that representing the STING pathway activation in SCI group were all obviously decreased in SCI-MT group, which fully demonstrated that the melatonin could effectively blocked the activation of the STING signaling pathway after the spinal cord injury.

3.3 C-176 attenuated neuroinflammation and promoted functional recovery of locomotion by alleviating NLRP3 inflammasome and pyroptosis after SCI in mice

As mentioned above that the STING pathway activation was located in microglia, and we have reported NLRP3 inflammasome-mediated pyroptosis is also mainly occurs in microglia after SCI in our previous study[16], so we adopted the specific small-molecule inhibitor of the STING, C-176, to further clarify the

relationship between the pyroptosis and the STING pathway in the microglia during SCI. As shown by WB (Fig. 3A), the protein level of pyroptosis-related proteins (NLRP3, GSDMD, GSDMD-NT and Cleaved Caspase-1) were all significantly inhibited by C-176 after SCI. Furthermore, the increased LDH level and the pro-inflammatory cytokines (TNF- α , IL-1 β and IL-18) after SCI were all attenuated by C-176, which were assessed by commercial kits, respectively (Fig. 3B-C). The higher BMS score (Fig. 3D) in the SCI + C-176 group indicated that mice recovered faster with the C-176 treatment. What's more, the HE staining presented the reduced inflammatory response after SCI in the presence of C-176 (Fig. 3E). The more Nissl body in Nissl staining in C-176 group also proved that C-176 could attenuated neuronal loss (Fig. 3E). Additionally, the negative effect of C-176 on the pyroptosis after SCI was further illustrated by the results of TUNEL staining as well (Fig. 3F). In short, these above results fully revealed that C-176 exerted its positive role on SCI depending on inhibiting the NLRP3 inflammasome-induced pyroptosis, which greatly indicated that the STING-mediated signaling pathway likely lies in the upstream of the NLRP3 inflammasome-mediated pyroptosis after SCI.

3.4 Overexpression of STING abolished the anti-pyroptotic effects of melatonin in LPS/ATP-induced BV2 cells

The BV2 cell (a type of microglia cell line) were used to further elucidate the relationship between NLRP3 inflammasome-mediated pyroptosis and the STING signaling pathway in vitro. To simulate the microenvironment during spinal cord injury, the LPS (1000ng/ml, 4h) and ATP (5 mM, 2h) were used in combination to establish a cell pyroptosis model in vitro. Firstly, to determine the appropriate intervention concentration, the BV2 cells were treated with a series concentration of MT (5, 10 100, 500 and 1000 μ M). The results of cell viability (Fig. 4A) showed that 5 and 10 μ M could be more appropriate. In Fig. 4B, the typical morphological features of pyroptosis (cell swelling, prominent bubble-like vesicles as indicated by red arrows) was obvious under the light microscope in LPS/ATP group. To further confirm pyroptosis, we detected the key pyroptotic proteins (NLRP3, GSDMD, GSDMD-NT, and Cleaved caspase-1) by WB assay. Immunoblot results revealed that all of these proteins increased significantly in LPS/ATP-treated group (Fig. 4C). Also, the markedly increased level of LDH and the pro-inflammatory cytokines (TNF- α , IL-1 β , IL-18) in the cell culture supernatant, as well as the higher PI-positive cell ratio also confirmed the loss of cell membrane integrity (Fig. 4D-E).

To verify the anti-pyroptotic role of melatonin in vitro, the BV2 cells were pre-treated with melatonin (5 μ M and 10 μ M) for 24h prior to the LPS/ATP stimulation. As evidenced by the results in Fig. 4B-E, the BV2 cell pyroptosis was successfully suppressed by melatonin (5 μ M and 10 μ M).

Besides, the activated STING signaling pathway (increased STING-associated protein and higher cGMP level) was also blocked by melatonin as presented in Fig. 5A-B. Other than that, the lower fluorescence intensity of STING and IRF-3 in MT pre-treated group (5 μ M and 10 μ M) in the IF staining results also corroborating the above results (Fig. 5C-D).

Then to test if the melatonin played its role depending on STING, the level of STING in BV2 cells was upregulated by adenovirus. Intriguingly, the Ad-STING greatly reversed the anti-pyroptotic role of

melatonin in LPS/ATP-induced BV2 cells (Fig. 6A). Consequently, the level of LDH and the pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) all showed the similar change trend to the results of WB (Fig. 6B-C). In addition, the results of PI staining (Fig. 6D) further demonstrated that STING overexpression reversed the anti-pyrototic effects of melatonin. Also, the IF staining of NLRP3 showed the weaker fluorescence intensity with the pre-treatment of melatonin, while the intensity increased again as the STING were upregulated (Fig. 6E). Overall, the results above jointly demonstrated that melatonin alleviated the LPS/ATP-induced pyroptosis in a STING-dependent way in BV2 cells.

3.5 Melatonin suppressed STING pathway by reducing ROS production and mtDNA release in LPS/ATP exposed BV2 cells

ROS overproduction triggers the release of cytosolic mitochondrial DNA, which has been reported to be associated with STING signaling pathway activation. Therefore, we sought to identify the possible mechanism on how the melatonin regulating the STING/NLRP3 pathway. A DCFH-DA fluorescence probe was used to measure the production of ROS in LPS/ATP-induced BV2 cells. The representative fluorescence image in Fig. 7A showed that LPS/ATP provoked a robust increase of ROS production in BV2 cells. Importantly, the relative fluorescence intensity of ROS was significantly suppressed by melatonin (5 μ M and 10 μ M). Correspondently, the higher level of cytosolic mtDNA in LPS/ATP group were all decreased greatly in melatonin pre-treatment groups (5 μ M and 10 μ M). Taken together, these results implied that melatonin suppressed the activation of STING/NLRP3 pathway via decreasing the production of ROS and the release of cytosolic mtDNA.

4. Discussion

In this study, we proved the anti-pyrototic effect of melatonin on SCI, and initially identify the role of STING/NLRP3 signal axis in the pharmacological process of melatonin after SCI.

At present, spinal cord injury (SCI) is still one of the most catastrophic neurological injury diseases and no effective therapies yet are available clinically. Nowadays, a large amount evidence has shown that microglia-mediated neuroinflammation is responsible for the development of SCI[6, 41, 42]. Therefore, attenuating neuroinflammation would be a prospective strategy for treating SCI. Based on many previous researches, melatonin seems to be a promising candidate to treat SCI for its various therapeutic effects including lowering edema[43], inhibiting scar formation[44], attenuating microgliosis[45]. In addition, Zhang Y [46] reported that melatonin could reduce neuroinflammation by promoting M2 microglia polarization, subsequently improved functional recovery after SCI. Besides, the microglia pyroptosis has also emerged as another important resource of neuroinflammation in SCI. In our previous studies, we have identified the crucial role of microglia pyroptosis in SCI[15]. It is a novel kind of inflammatory programmed cell death characterized by cell swelling and lysis, leading to the secretion of proinflammatory cytokines (including IL-1 β , IL-18 and TNF- α). As we know, inflammasomes activation is the crucial initial event to mediate the pyroptosis. And in general, five kinds of inflammasomes (NLRP1, NLRC4, AIM2, RIG-1 and NLRP3) has been identified while the NLRP3 inflammasome is the most widely

investigated in SCI. However, few studies focused on exploring whether melatonin exerts its regulatory role through regulating the NLRP3 inflammasome after SCI. And no reports are published about the role of STING pathway on regulating NLRP3 inflammasome during the pathological process after SCI.

In the present study, we found that melatonin effectively inhibited the expression of NLRP3 inflammasome associated proteins: NLRP3, ASC, Cleaved Caspase-1 and GSDMD (the executor of pyroptosis), which were all greatly increased after SCI. Moreover, it is notable that the protein level of STING and its downstream molecule (P-TBK1 and P-IRF3) were also upregulated after SCI. The STING-mediated pathway, as a part of innate immune system, has been found to play a significant role in modulating infection and inflammation[47, 48]. Increasing evidences have indicated the regulating role of STING signaling pathway in neuroinflammation and neurological diseases[49–51]. Sliter [52] showed that STING deficiency eliminated inflammatory phenotypes in Parkin- and PINK1-deficient mice. Jauhari A [37] also reported the increased level of cytosolic DNA and inflammation with the activated cGAS/STING/IRF3 in HD patients. And to our knowledge, it is the first time that STING-mediated pathway participated in the SCI has been reported. Importantly, our results in vivo and in vitro together indicated that melatonin effectively blocked the activation of STING signaling pathway after SCI.

In recent years, the interplay between STING and NLRP3 inflammasome has been gradually revealed. In an LPS-induced cardiac injury model, the NLRP3 inflammasome was activated by the stimulation of STING, and the protection role of STING knockdown was reversed by NLRP3 overexpression[24]. What's more, Li N[25] also proved that STING deficiency inhibited NLRP3 inflammasome and pyroptosis in LPS-treated Lung. Here, we also discovered that downregulation of STING by C-176 obviously reduced the NLRP3 inflammasome activation in vivo. On the other hand, overexpression of STING abolished the anti-pyroptotic effect of melatonin in LPS/ATP treated BV2 cells. These results are consistent with the previous published studies and together indicated that the anti-pyroptotic role of melatonin is mediated by the blockage of STING-mediated pathway.

It is well-known that NLRP3 inflammasome activation is related to multiple signal molecules including DAMPs, oxidant stress, mitochondrial dysfunction as well as reactive oxygen species (ROS)[53–55]. Wang BR[56] demonstrated that ROS was essential in PM2.5-induced IL-1 β production and NLRP3 inflammasome activation in oA β -stimulated microglia. What's more, ROS-mediated the release of mtDNA into cytoplasm has been reported to be responsible for the activation of cGAS/STING pathway[57]. On the other hand, it is also acknowledged that melatonin plays a crucial role in multiple diseases as a mitochondria-targeted antioxidant[58, 59]. Hence, we speculated melatonin suppressed STING/NLRP3 axis by alleviating oxidative stress-related factors. In agreement with the previous published studies, we found that the increased ROS and cytosolic mtDNA was detected in LPS/ATP induced microglia pyroptosis. And the reduced ROS production and mtDNA release in the melatonin pre-treatment groups demonstrated that melatonin exerts its protection role by reducing pyroptotic neuroinflammation via blocking the ROS/mtDNA-activated STING/NLRP3 signaling axis. Of course, a shortcoming in our study is the lack of STING knock-out mice, it would provide more powerful evidence to our conclusion. In

addition, the detail molecular mechanism of the activation of STING-mediated pathway in SCI is worth exploring in our further studies.

5. Conclusion

In conclusion, as depicted in Fig. 8, we reported for the first time that the ROS/mtDNA/STING pathway undertook an important role in NLRP3 inflammasome-mediated pyroptosis after SCI in mice. It was also a novel mechanism that interpreting the anti-pyroptotic effect of melatonin on SCI and thus provided potential therapeutic target for treating SCI.

Abbreviations

SCI: Spinal cord injury;

MT: Melatonin;

PAMPs: Pathogen-associated molecular patterns;

DAMPs: Damage-associated molecular patterns;

cGAS: Cyclic GMP-AMP synthase;

STING: Stimulator of interferon genes;

GSDMD: Gasdermin D;

NLRP3: NLR family pyrin domain-containing-3;

ASC: Apoptosis-associated speck-like protein;

i.p.: Intraperitoneal injection;

CCK-8: Cell Counting Kit-8;

PI: Propidium Iodide;

WB: Western Blot;

RT-qPCR: real-time quantitative polymerase chain reaction;

ELISA: Enzyme-linked immune-sorbent assay;

H&E: Hematoxylin and eosin;

ROS: Reactive oxygen species.

Declarations

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Authors' contributions

All authors read and approved the final manuscript. Jin Wang, Haiyuan Yang and Fan Zhang performed the experiments and drafted the manuscript; Jin Wang, Fan Zhang and Minghao Shao conducted the data analysis; Haiyuan Yang and Haocheng Xu reviewed and edited the manuscript. Shun Xu and Feizhou Lyu conceived and designed the study. The authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Ethics Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Ethics Committee of Experimental Research, Huashan Hospital, Fudan University (Shanghai, China).

Consent for publication

Not applicable.

Competing interests

The authors have declared that no competing interests exist.

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Tables

Table. 1 Primers used for qRT-PCR in this study.

Target genes	Forward primer	Reverse primer
STING	CCGAAGACTGTACATCCTCTTT	AGCATATCTCGGAATCGAATGT
TBK1	ACTGGTGATCTCTATGCTGTCA	TTCTGGAAGTCCATACGCATTG
IRF3	AGGCTTCTGGGCCTTATGTG	TGCTTCTCTCGCCAGGAATAC
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT

Figures

Figure 1

Melatonin inhibited the NLRP3 inflammasome-mediated pyroptosis and promoted motor function recovery after SCI in mice. A. The schematic diagram of the treatment of mice in three groups (Sham, SCI and SCI+MT) and the corresponding analysis after SCI. B. The locomotion function of mice in three

groups was evaluated by BMS score at 1, 3, 7, 14, 21, 28 days after surgery. C-D. Representative immunoblotting and quantitative analysis of pyroptosis-associated proteins in three groups. E. The released level of serum LDH in mice was detected by LDH Assay Kit. F. The level of serum pro-inflammatory cytokines (TNF- α , IL-1 β and IL-18) in three groups was measured by ELISA assay. G. The representative immunofluorescence image of IBA-1 and NLRP3 of spinal cord in three groups * $p < 0.05$, ** $p < 0.01$ versus Sham group, # $p < 0.05$ versus SCI group. All data were shown as the mean $\pm$ SD from 3 independent experiments.

Figure 2

Melatonin impeded the activation of STING-mediated pathway after SCI in mice. A-B. Representative immunoblotting and quantitative analysis of STING pathway-associated proteins (STING, TBK1, p-TBK1, IRF3 and P-IRF3) in three groups. C. The quantitative real-time PCR results of STING, TBK1 and IRF3 in three groups. D. The level of cGAMP in three groups was measured by 2'3'-cGAMP ELISA kit. E. The representative two-photon excitation images of immunofluorescence of Iba-1 (red) and STING (green) acquired from spinal cord tissue in mice at 3 days after SCI in three groups. Nuclei are stained with DAPI (blue). Scale bar: 50 μ m. * $p < 0.05$, ** $p < 0.01$ versus Sham group, # $p < 0.05$ versus SCI group. All data were shown as the mean $\pm$ SD from 3 independent experiments.

Figure 3

The STING small-molecule inhibitor C-176 alleviated NLRP3 inflammasome-induced pyroptosis and enhance locomotion recovery after SCI in mice. A. Representative Western blotting of STING and pyroptosis-related proteins and quantification analysis 3 days after SCI or Sham surgery; B. The release level of serum LDH in different groups detected by LDH assay kits. C. The level of serum pro-inflammatory cytokine (TNF- α , IL-1 β and IL-18) of mice in different groups assessed by commercial

ELISA kits, respectively. D. The locomotion function of mice in four groups (Sham+Saline, Sham+C-176, SCI+Saline and SCI+C-176) assessed by BMS score at 1, 3, 5, 7, 14, 21,28 days post-injury. E. The representative hematoxylin-eosin (HE) and Nissl staining of different groups 3 days after SCI or sham surgery. Scale bar: 50μm; F. The Representative images of TUNEL staining (green) in spinal cord tissue of each group. Nuclei are stained with DAPI (blue). Scale bar: 50μm. *p < 0.05 versus Sham+Saline group, #p < 0.05 versus SCI+Saline group. All data were shown as the mean ± SD from 3 independent experiments.

Figure 4

Melatonin inhibited the LPS/ATP induced NLRP3 inflammasome-dependent pyroptosis in BV2 cells. A. The cell viability of BV2 cells exposed to a series concentration of melatonin (5, 10, 100, 500 and 1000 μ M); B. The representative images of cell morphology in different groups. (Cell swelling and bubble-like vesicle were indicated by red arrows), scale bar: 100 μ m; C. The representative Western blotting of pyroptosis-associated proteins (NLRP3, GSDMD, GSDMD-NT, Cleaved Caspase-1) and quantitative analysis in different treatment groups, respectively; D. The relative LDH level and the level of serum pro-inflammatory cytokine (TNF- α , IL-1 β and IL-18) in the cell culture supernatant of different groups assessed by commercial ELISA kits, respectively; E. The representative image of PI staining (red) in different groups, Nuclei were stained with Hoechst (blue), scale bar: 200 μ m. * $p < 0.05$ versus Control group, # $p < 0.05$ versus LPS/ATP group. All data were shown as the mean $\pm$ SD from 3 independent experiments.

Figure 5

Melatonin effectively inhibited STING-mediated signaling pathway in LPS/ATP induced BV2 cells. A. Representative Western blotting of STING pathway-associated proteins (STING, TBK1, P-TBK1, IRF3, P-IRF3) and quantitative analysis in different groups (Control, LPS/ATP, LPS/ATP+5 μ M MT and

LPS/ATP+10μM MT); B. The relative cGMP level in cell culture supernatant of four groups measured by 2'3'-cGAMP ELISA kit; C-D. The representative Immunofluorescence images of STING (E) and IRF-3 (F) in BV2 cells of different groups. Nuclei are stained with DAPI (blue). Scale bar: 10μm and 20μm, respectively. *p < 0.05, **p < 0.01 versus Control group, #p < 0.05 versus LPS/ATP group. All data were shown as the mean ± SD from 3 independent experiments.

Figure 6

Over-expression of STING reversed the anti-pyroptotic effect of melatonin (10 μ M) on LPS/ATP induced BV2 cells. A. The Representative Western blotting of pyroptosis-associated proteins (NLRP3, GSDMD, GSDMD-NT, Cleaved Caspase-1) and quantitative analysis in different groups (Control, LPS/ATP, LPS/ATP+10 μ M MT and LPS/ATP+10 μ M MT+ Ad-STING); B. The relative released LDH level in the cell culture supernatant of different groups assessed by LDH kits; C. The level of pro-inflammatory cytokine (TNF- α , IL-1 β and IL-18) in cell culture supernatant of different groups detected by commercial ELISA kits; D. The representative images of PI staining of BV2 cells in different groups, Nuclei are stained with Hoechst (blue), scale bar: 200 μ m; E. The representative immunofluorescence images of NLRP3 (green) in BV2 cells of different groups. Nuclei are stained with DAPI (blue), Scale bar: 20 μ m. *p < 0.05, *p < 0.01 versus Control group, #p < 0.05 versus LPS/ATP group, &p < 0.05 versus LPS/ATP+10 μ M MT group. All data were shown as the mean $\pm$ SD from 3 independent experiments.

Figure 7

Melatonin reduced the ROS production and the release of mtDNA in LPS/ATP-induced BV2 cells. A. The representative immunofluorescence images of ROS detected by DCFH-DA fluorescence probe in BV2 cells of four groups (Control, LPS/ATP, LPS/ATP+5μM MT and LPS/ATP+10μM MT). Scale bar: 200μm. B. The quantitative results of ROS production in different four groups. C. The relative level of cytosolic mtDNA in BV2 cells of different four groups. * $p < 0.05$ versus Control group, # $p < 0.05$ versus LPS/ATP group. All data were shown as the mean $\pm$ SD from 3 independent experiments.

Figure 8

The mechanism of anti-pyrototic effect of melatonin on spinal cord injury. Melatonin reduced the DAMPs/PAMPs induced ROS production and the mtDNA release in microglia cells, subsequently blocked the activation of STING pathway. Thereafter, the NLRP3 inflammasome-mediated pyroptosis were inhibited and the corresponding neuroinflammation were alleviated during the secondary phase of SCI.