Melatonin exerts neuroprotective effects in mice with spinal cord injury by activating the Nrf2/Keap1 signaling pathway via the MT2 receptor

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Abstract. Spinal cord injury (SCI) is a devastating event that often leads to severe disability, and effective treatments for SCI are currently limited. The present study investigated the potential effects and specific mechanisms of melatonin treatment in SCI. Mice were divided into Sham (Sham), Vehicle (Veh), Melatonin (Mel), and Melatonin + 4-phenyl-2-propionamidotetralin (4P-PDOT) (Mel + 4PP) groups based on randomized allocation. The expression of MT2 and the nuclear factor-erythroid 2-related factor 2 (Nrf2)/Keap1 signaling pathways were examined, along with oxidative stress indicators, inflammatory factors and GFAP-positive cells near the injury site. The polarization of microglial cells in different inflammatory microenvironments was also observed. Cell survival, motor function recovery and spinal cord tissue morphology were assessed using staining and Basso Mouse Scale scores. On day 7 after SCI, the results revealed that melatonin treatment increased MT2 protein expression and activated the Nrf2/Keap1 signaling pathway. It also reduced GFAP-positive cells, mitigated oxidative stress, and suppressed inflammatory responses around the injury site. Furthermore, melatonin treatment promoted the polarization of microglia toward the M2 type, increased the number of neutrophil-positive cells, and modulated the transcription of Bax and Bcl2 in the injured spinal cord. Melatonin treatment alleviated the severity of spinal injuries and facilitated functional recovery in mice with SCI. Notably, blocking MT2 with 4P-PDOT partially reversed the neuroprotective effects of melatonin in SCI, indicating that the activation of the MT2/Nrf2/Keap1 signaling pathway contributes to the neuroprotective properties of melatonin in SCI. The therapeutic and translational potentials of melatonin in SCI warrant further investigation.

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Introduction

Spinal cord injury (SCI) refers to the damage to the spinal cord caused by external factors, resulting in sensory, motor and functional impairments in the corresponding injured and lower segments (1). The incidence of traumatic SCI has been reported to be 569.7 per million of the population, with a significantly higher rate in men compared with women (753.6 vs. 387.7) (2). SCI can be categorized into primary and secondary injuries (3). Primary injury results in local necrosis and apoptosis of neurons and glial cells, while the subsequent inflammatory response of the nervous system leads to secondary damage. In the case of secondary injury, spinal cord ischemia and hypoxia trigger the generation and release of numerous free radicals and reactive oxygen species, further disrupting the microenvironment and impairing the functioning of the nervous system (4,5).

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a transcriptional regulatory factor that plays a crucial role in maintaining homeostasis by activating the expression of antioxidant and anti-inflammatory genes (6-9). Nrf2 binds to the antioxidant response element located in the promoter region of cytoprotective and antioxidant genes, thereby promoting their transcription (10-12). Under normal physiological conditions, Nrf2 is associated with Keap1, which inhibits Nrf2 activity (13,14). However, during oxidative stress, Nrf2 dissociates from the complex and translocates into the nucleus, where it stimulates the expression of anti-inflammatory and antioxidant genes (15-17). By reducing the levels of reactive oxygen species, Nrf2 can enhance nerve cell survival at the site of injury and improve the outcome of SCI.

Melatonin, also known as N-acetyl-5-methoxytryptamine, is a hormone that plays a crucial role in regulating biological rhythms due to its fluctuating concentrations throughout the day and night (18). In the past, the effects of melatonin were primarily attributed to its ability to promote sleep initiation and maintenance. However, emerging research indicates that melatonin possesses broader neuroprotective properties by inhibiting apoptosis, thereby improving neuronal survival. This has been observed in various neurological conditions, including Alzheimer's disease (19) and Parkinson's disease (20).

Currently, two melatonin receptors have been identified in mammals: MT1 (Mel1a) and MT2 (Mel1b), along with

nuclear binding sites ROR and RZR (21). Melatonin receptors are widely distributed in the brain, particularly in the hypothalamus, hippocampus and pineal gland, as well as in the retina and various peripheral tissues including the liver and adipose tissue (22). The MT2 receptor has been found to regulate stem cell activity and attenuate oxidative stress-induced damage, thereby reducing traumatic injury (14). Importantly, the functionality of the MT2 receptor partially overlaps with the Nrf2 signaling pathway.

Nonetheless, the precise mechanism underlying the effects of melatonin in SCI remains unclear, and the relationship between melatonin and the Nrf2 signaling pathway in SCI has yet to be elucidated. It was postulated that the melatonin MT2 receptor and the Nrf2 signaling pathway collectively contribute to the secondary injury mechanism in SCI, given that inflammatory responses and oxidative stress injury are recognized as highly detrimental factors in this context. In the present study, 4-phenyl-2-propionamidotetralin (4P-PDOT) was used, a specific antagonist targeting melatonin and MT2 receptors, to treat SCI mice and investigate the neuroprotective role of melatonin along with its associated signaling pathways.

Materials and methods

Animal studies. In the present study, male C57BL/6 mice aged 8-12 weeks and weighing between 22-28 g were obtained from the Animal Research Center of Zhengzhou University (Henan, China). The mice were housed in a standard animal facility with controlled environmental conditions of 26˚C temperature, 38.5% humidity, and a 12/12-h light-dark cycle. Mice had *ad libitum* access to food and water. A total of 112 mice were included in the present study, and 8 mice succumbed during the course of the experiment. All experimental procedures involving animals were conducted in accordance with the guidelines and regulations approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University (approval no. ZZUIRB 2022-145) (Zhengzhou, China). A conceivable effort was made to minimize animal suffering and the minimum number of animals necessary to achieve our research objectives, was employed.

Establishment of models. Operation was conducted following previously described protocols (23). Prior to the surgical procedure, mice were intraperitoneally injected with pentobarbital sodium (40 mg/kg) for anesthesia. Following anesthesia, the back skin was prepared and disinfected using iodophor solution. The mice were then positioned in the prone position on the experimental table, with the skin fixed. A 2-cm incision was made along the midline of the mouse's back after proper preoperative preparation. The fascia and paraspinal muscles were carefully separated to expose the T9-T11 vertebrae segment. SCI was induced using a modified mouse Allen method, wherein a 5-g hammer was dropped from a height of 3 cm onto the T10 spinal cord segment. Successful modeling was confirmed by hindlimb twitching. Following this, the muscle and skin layers were sutured meticulously and the area was disinfected again with iodophor solution. For the next three days after the surgery, gentle abdominal massages were administered to the mice twice daily, and gentamicin (40 mg/kg) was administrated to prevent urinary tract infection.

Experimental groups. Mice were randomly assigned to one of the four groups using a random number table method, as previously described (24,25) (http://www.randomization.com): i) Sham group, in which the vertebral plate of the corresponding segments was surgically removed without any further treatment; ii) Vehicle group (Veh), receiving intraperitoneal injections of normal saline (10 mg/kg) immediately after SCI, once daily for 7 days; iii) Melatonin group (Mel), receiving intraperitoneal injections of melatonin (cat. no. M813985; Shanghai McLean Biochemical Technology Co., Ltd.) (10 mg/kg) immediately after SCI, once daily for 7 days; iv) Mel + 4PP group, receiving immediate intraperitoneal injections of melatonin (10 mg/kg) after SCI, followed by intragastric administration of 4P-PDOT (cat. no. P910644; Shanghai McLean Biochemical Technology Co., Ltd.) (10 mg/kg), once daily for 7 days. Melatonin was dissolved in absolute ethanol and then diluted to a concentration of 1 mg/ml using PBS. 4P-PDOT was dissolved in DMSO and also diluted to a concentration of 1 mg/ml using PBS. The concentrations of melatonin and 4P-PDOT were determined based on preliminary experiments and previous studies (26-30).

Assessment of motor function. The motor function of the hindlimbs in mice was assessed using the Basso Mouse Scale (BMS) score at 1, 3, 7, 14, 21 and 28 days after SCI, with 6 mice in each group. The BMS score ranges from 0 to 9, where 0 indicates complete paralysis and 9 indicates normal functioning of the hindlimbs. Prior to the assessment, the mice were given an opportunity to familiarize themselves with the open field environment. Two experimenters independently observed and analyzed the mice's behavior on a computer, assigning scores that were subsequently averaged (23). The detailed rules of BMS scoring are shown in Table I (31).

Tissue processing. Gene expression differences were found to be most significant 7 days after SCI (32). Therefore, on day 7 after the operation, mice were euthanized, and complete spinal cord tissue (T9-T11) was collected, with the injury site designated as the epicenter. Tissues intended for oxidative stress detection, reverse transcription-quantitative (RT-q) PCR, and western blot analysis were stored at -80˚C in a refrigerator for subsequent processing. Additionally, tissues designated for immunohistochemical and H&E staining were fixed in 4% paraformaldehyde and then dehydrated using a series of 10, 20 and 30% sugar water solutions in a refrigerator at 4˚C; each concentration was placed there for 24 h. The spinal cord tissues were sectioned into 15-μm slices using a cryostat. Once completely dried, the sections were stored at -80˚C in a refrigerator for follow-up experiments.

Immunohistochemical staining. Immunofluorescence staining was conducted following previously described protocols (33,34). The tissue sections were gradually rehydrated and washed with PBS for 5 min each time, followed by fixation with 4% paraformaldehyde. Subsequently, the sections were fixed in acetone for 10 min, and antigen retrieval was performed using a citric acid solution containing 0.05% Tween. The primary antibodies, including iNOS, inducible nitric oxide synthase (iNOS; cat. no. AF0199; Affinity Biosciences, Ltd.), Arginase 1 (Arg1; cat. no. DF6657;

Table I. Basso mouse scale.

Grading	Scoring standard
0	No ankle joint movement
1	Slight ankle joint movement
2	Extensive ankle joint mobility
3	No weight-bearing, or occasional, frequent, sustained forefoot standing without hindfoot standing
4	Occasional forefoot standing
5	Frequent, sustained forefoot standing with some coordination, but not coordinated; or frequent, sustained forefoot standing with some coordination, but toes rotate upon contact and lift-off
6	Frequent, sustained forefoot standing with some coordination, toes are steady upon contact; or frequent, sustained forefoot standing, highly coordinated, but toes rotate upon contact and lift-off
7	Frequent, sustained forefoot standing, highly coordinated, toes are steady upon contact but rotate during lift-off; or frequent, sustained forefoot standing, highly coordinated, toes are steady upon contact and lift-off, but significant trunk
8	Frequent, sustained forefoot standing, highly coordinated, toes are steady upon contact and lift-off, slight trunk instability; or frequent, sustained forefoot standing, highly coordinated, toes are steady upon contact and lift-off, trunk stable but tail droops or curls upwards and then droops
9	Frequent, sustained forefoot standing, highly coordinated, toes are steady upon contact and lift-off, trunk stable, tail raised

Affinity Biosciences, Ltd.), GFAP (cat. no. 16825-1-AP; Proteintech Group, Inc.) and NeuN (cat. no. DF6145; Affinity Biosciences, Ltd.), were diluted in PBST solution containing 0.05% Tween (1:200) and applied to the sections. The sections were then covered and placed in a humidified chamber, incubating overnight at 4˚C.

The sections were washed three times with PBS for 15 min each time, ensuring that the entire process was carried out in the absence of light. The secondary Goat Anti-Rabbit IgG (H+L) Fluor594-conjugated antibody (1:200; cat. no. S0006; Affinity Biosciences, Ltd.), was applied to the sections and incubated for 2 h.

After staining, the sections were sealed with 10 μg/ml DAPI (cat. no. C0065; Beijing Solarbio Science & Technology, Ltd.) staining solution and incubated for 15 min. The fluorescence microscope was used to locate the visual field in the anterior horn of the spinal cord, and each image was observed and analyzed using the same exposure settings. The number of positive cells in each visual field was quantified using ImageJ software.

H&E staining. The frozen sections were stained with hematoxylin solution (cat. no. G1120; Beijing Solarbio Science & Technology Co., Ltd.) for 2 min, followed by rinsing in running tap water. Subsequently, the sections were treated with differentiation solution for 30 sec and immersed in distilled water for 2 min. Eosin Y stain was applied for 1 min, and then the sections were washed with distilled water for 1 min. Finally, the sections were dehydrated sequentially in 30, 50, 75, 95 and 100% alcohol (35).

Detection of oxidative stress. Oxidative stress levels were assessed 7 days after SCI using assay kits for measuring superoxide dismutase (SOD) (cat. no. BC0170), glutathione (GSH) (cat. no. BC1175) and malondialdehyde (MDA) (cat. no. BC0025; all purchased from Beijing Solarbio Science & Technology Co., Ltd.). To perform the measurements, mice were first anesthetized and euthanized. Spinal cord tissue was collected and subjected to grinding, followed by centrifugation (8,000 x g for 10 min at 4˚C) to obtain the supernatant for subsequent experiments (33).

RNA isolation and RT-qPCR analysis. Total RNA was extracted from spinal cord tissue using TRIzol reagent (cat. no. CW0580; Jiangsu Cowin Biotech Co., Ltd.) following the provided instructions and a previous study (36). The extracted total RNA (1 μg) was reverse transcribed using the PrimeScript RT reagent kit (cat. no. RR047A; Takara Bio, Inc.), following the instructions provided in the manual. qPCR was performed on the transcribed cDNA using TB Green (cat. no. RR820A; Takara Bio, Inc.) and specific primers. The thermocycling conditions were as follows: 95˚C for 2 min, followed by denaturation at 95˚C for 15 sec and annealing/extension at 60˚C for 1 min, for a total of 40 cycles. Relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (37). The primer sequences used were as follows: Bcl-2 forward, 5'-GATGACTTCTCT CGTCGCTAC-3' and reverse, 5'-GAACTCAAAGAAGGC CACAATC-3'; Bax forward, 5'-TTGCCCTCTTCTACTTTG CTAG-3' and reverse, 5'-CCATGATGGTTCTGATCAGCT C-3'; IL-1β forward, 5'-TTCAGGCAGGCAGTATCACTC-3' and reverse, 5'-GAAGGGTCCACGGGAAAGACAC-3'; IL-4 forward, 5'-GGTCTCAACCCCCAGCTAGT-3' and reverse, 5'-GCCGATGATCTCTCTCAAGTGAT-3'; TNF-α forward, 5'-CACCACCATCAAGGACTCAA-3' and reverse, 5'-GAG ACAGAGGCAACCTGACC-3'; β-actin (reference gene) forward, 5'-TTGCTGACAGGATGCAGAAG-3' and reverse 5'-TTGCTGACAGGATGCAGAAG-3'; Nrf2 forward, 5'-CGG GACTATTGAAGGCTGTGA-3' and reverse, 5'-GGAGTG

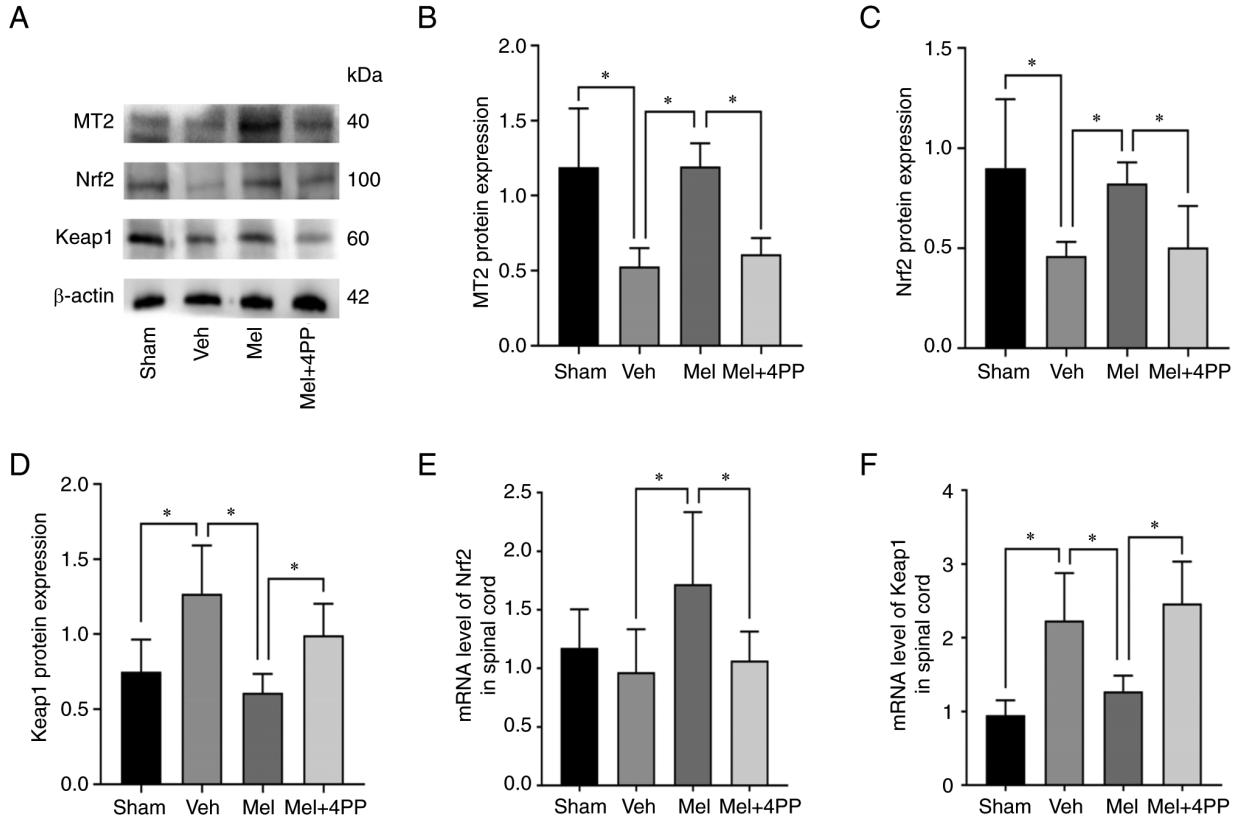

Figure 1. Melatonin can upregulate the expression of the MT2 receptor and activate the Nrf2 signaling pathway. (A-D) Representative blots and quantification of the protein expression levels of MT2, Nrf2 and Keap1 were assessed in each experimental group. (E and F) mRNA levels of Nrf2 and Keap1 were measured in the spinal cord tissue of each group at 7 days post-injury. *P≤0.05. Nrf2, nuclear factor-erythroid 2-related factor 2; Mel, Melatonin; Veh, Vehicle; 4PP, 4-phenyl-2-propionamidotetralin.

CTCTGGGGACGCT-3'; and Keap1 forward, 5'-GACTGG GTCAAATACGACTGC-3' and reverse, 5'-GAATATCTG CACCAGGTAGTCC-3'.

Western blot analysis. Protein was prepared from fresh spinal cord tissue and analyzed according to a previously described protocol (35). Total protein was extracted using RIPA Lysis Buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.), and the protein concentration was determined using the BCA kit (cat. no. PC0020; Beijing Solarbio Science & Technology Co., Ltd.). Equal amounts of protein (30 μg/lane) were separated on 8% gels by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes. The PVDF membranes containing proteins were incubated at 4˚C overnight in a blocking solution (cat. no. SW3012; Beijing Solarbio Science & Technology Co., Ltd.). The membranes were then probed with specific primary antibodies and incubated at 4˚C in a refrigerator for 2 h, including MT2 (1:3,000; cat. no. NLS932; Novus Biologicals, LLC), Nrf2 (1:3,000; cat. no. A0674; ABclonal Biotech Co., Ltd.), Keap1 (1:3,000; cat. no. A17061; ABclonal Biotech Co., Ltd.), iNOS (1:3,000; cat. no. AF0199; Affinity Biosciences, Ltd.) and Arg1 (1:3,000; cat. no. DF6657; Affinity Biosciences, Ltd.); followed by incubation with a secondary Goat Anti-Rabbit IgG (H+L) HRP-conjugated antibody (1:3,000; cat. no. S0001; Affinity Biosciences, Ltd.) for 2 h. Protein bands were visualized using enhanced chemiluminescence (ECL) (cat. no. PE0010; Beijing Solarbio Science & Technology Co., Ltd.), and the intensity of the bands was quantified using ImageJ 1.54 software (National Institutes of Health).

Statistical analysis. Data analysis was performed using SPSS 21.0 software (IBM Corp.). The results are presented as the mean ± standard deviation. Prior to analysis, normal distribution and homogeneity of variance assumptions were checked. For data that followed a normal distribution and met the assumption of homogeneity of variance, one-way analysis of variance (ANOVA) was used to compare multiple groups, and the unpaired Student's t-test was used to compare two groups. If the data did not meet the assumptions of normal distribution and homogeneity of variance, the Kruskal-Wallis nonparametric test was employed, and post-hoc analysis was conducted using the Bonferroni correction to assess differences between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Melatonin activates the Nrf2/Keap1 signal pathway through the MT2 receptor. Initially, the protein expression levels of the MT2 receptor and the Nrf2/Keap1 signaling pathway were assessed (Fig. 1A). Western blot analysis revealed that the MT2 receptor protein expression in the Mel group was significantly higher compared with the Veh group (1.19±0.16 vs. 0.53±0.12, N=5/group, P<0.001). However, the MT2 receptor blocker, 4P-PDOT, reduced the MT2 receptor protein content

in the Mel + 4PP group (1.19±0.16 vs. 0.61±0.11, N=5/group, P=0.029) compared with the Mel group (Fig. 1B). The Nrf2 protein expression levels in the Veh group and the Mel + 4PP group were comparable (0.46±0.07 vs. 0.50±0.21, N=5/group, P=0.760) (Fig. 1C). By contrast, the Mel group exhibited significantly increased Nrf2 protein expression compared with both the Veh and Mel + 4PP groups (0.46±0.07 vs. 0.82±0.21 and 0.50±0.21 vs. 0.82±0.21, N=5/group, P=0.015 and P=0.029). Additionally, the expression of Keap1 protein decreased in the Mel group compared with the Veh and Mel + 4PP groups (1.27±0.32 vs. 0.61±0.13 and 0.99 ± 0.21 vs. 0.61±0.13, N=5/group, P<0.001 and P=0.018) (Fig. 1D).

In addition to protein expression analysis, the transcription levels of Nrf2 and Keap1 mRNA were investigated using RT-qPCR. The results revealed that Nrf2 mRNA expression was significantly increased in the Mel group compared with the Veh group (0.97±0.38 vs. 1.72±0.62, N=6/group, P=0.005) (Fig. 1E). Conversely, Keap1 mRNA expression was decreased in the Mel group compared with the Veh group (2.23±0.65 vs. 1.27±0.21, N=6/group, P=0.002) (Fig. 1F). However, in the Mel + 4PP group, these effects were not observed (0.97±0.38 vs. 1.07±0.25, N=6/group, P=0.683) (2.23±0.65 vs. 2.47±0.57, N=6/group, P=0.381), indicating that Melatonin could activate the Nrf2/Keap1 signaling pathway through the MT2 receptor after SCI (Fig. 1B).

Melatonin attenuates inflammation and oxidative stress through the MT2 receptor. On day 7 after SCI, immunofluorescence staining of GFAP on frozen sections of the injured spinal cord tissue was performed (Fig. 2A and B). The immunofluorescence results demonstrated that melatonin treatment significantly reduced the number of astrocytes compared with the Veh group (161.33±27.23 vs. 217.00±39.34, N=3/group, P=0.048). However, when the 4P-PDOT MT2 receptor blocker was used, the effect of melatonin on astrocytes was completely abolished (225.67±28.04 vs. 161.33±27.23, N=3/group, P=0.029). The number of astrocytes in the Mel + 4PP group was similar to that in the Veh group but significantly less than that in the Mel group.

To assess the impact of melatonin on inflammation following SCI, the levels of cytokines, including IL-1β, IL-4 and TNF-α, in the SCI site were examined. As expected, the proinflammatory factors IL-1β and TNF-α exhibited higher expression after SCI compared with the baseline levels (0.96±0.33 vs. 9.56±2.74, N=6/group, P<0.001) (1.07±0.23 vs. 5.40±1.59, N=6/group, P<0.001) (Fig. 2C and D). Similar results were observed in the Mel + 4PP group. However, in comparison to the Veh group and the Mel + 4PP group, the Mel group showed a significant decrease in the expression of these two proinflammatory factors (1.52±0.52 vs. 9.56±2.74 and 1.52±0.52 vs. 7.93±1.37, N=6/group, both P<0.001) (0.70±0.20 vs. 5.40±1.59 and 0.70±0.20 vs. 4.30±0.89, N=6/group, both P<0.001). Conversely, the levels of the anti-inflammatory factor IL-4 showed an opposite trend (Fig. 2E). In the Mel group, IL-4, which serves as a marker of favorable prognosis, exhibited an increase compared with the Veh group (0.76±0.15 vs. 0.25±0.08, N=6/group, P<0.001) and the Mel + 4PP group (0.76±0.15 vs. 0.30±0.09, N=6/group, P<0.001). Moreover, the IL-4 level in the Mel + 4PP group did not differ significantly from that of the Veh group (0.30±0.09 vs. 0.25±0.08, N=6/group, P=0.477), indicating that 4P-PDOT could block the therapeutic effect of the MT2 receptor.

To assess the antioxidant effect of melatonin, the levels of three indicators, SOD, MDA and GSH were examined. The levels of SOD (275.31±60.08 vs. 249.65±50.94, N=6/group, P=0.455) (Fig. 2F) and GSH (3.49±0.86 vs. 3.13±0.82, N=6/group, P<0.001) (Fig. 2H) in the Veh group and Mel + 4PP group were similar but lower than those in the Mel group (499.61±70.09 vs. 275.31±60.08 and 499.61±70.09 vs. 249.65±50.94, N=6/group, both P<0.001) (9.22±1.18 vs. 3.49±0.86 and 9.22±1.18 vs. 3.13±0.82, N=6/group, both P<0.001). Melatonin treatment significantly reduced the MDA content (8.99±2.06 vs. 28.38±4.49, N=6/group, P<0.001) (Fig. 2G). However, when the MT2 receptor blocker 4P-PDOT was used, the MDA content in the Mel + 4PP group was higher than that in the Mel group (20.58±3.87 vs. 8.99±2.06, N=6/group, P<0.001), although it remained lower than that in the Veh group (20.58±3.87 vs. 28.38±4.49, N=6/group, P<0.001). These findings suggested that the MT2 receptor plays a crucial role in the antioxidant effect observed.

Melatonin regulates the direction of polarization of microglia through the MT2 receptor. Microglia polarization into M1 and M2 types is a crucial phenomenon in the pathological process of SCI. The activation of microglia by inflammatory factors such as IL-1β and TNF-α can lead to their polarization into M1 type microglia (38). M1 type microglia, in turn, exacerbate the inflammatory response and cause damage to spinal cord cells. Conversely, during the progression of the inflammatory response, the upregulation of IL-4 receptors on microglia can induce the conversion of M1 type microglia to M2 type microglia (39). The M2 type microglia possess anti-inflammatory properties and contribute to tissue repair and regeneration in the spinal cord.

To investigate the effect of melatonin treatment on microglial polarization, the protein expression of iNOS and Arg1was examined using western blot analysis (Fig. 3A-C). Treatment with melatonin significantly increased the expression of Arg1 protein (0.12±0.06 vs. 1.14±0.10, N=5/group, P=0.001) and decreased the expression of iNOS protein (1.24±0.30 vs. 0.55±0.21, N=5/group, P=0.001). However, when the MT2 receptor inhibitor 4P-PDOT was used, the effect of melatonin on microglial polarization was reversed. The expression of Arg1 protein (0.12±0.06 vs. 0.24±0.16, N=5/group, P=0.126) and iNOS protein (1.24±0.30 vs. 1.08±0.20, N=5/group, P=0.293) in the Mel + 4PP group showed no significant difference compared with the Veh group. These results suggested that melatonin can induce the polarization of microglia towards the M2 type by stimulating the MT2 receptor.

Immunofluorescence staining was further employed in spinal cord sections using markers for M1 type (iNOS) and M2 type (Arg1) microglia to assess the effect of melatonin treatment on microglial polarization. Consistent with the previous immunofluorescence results, the quantitative analysis demonstrated significant differences among the groups. In comparison to the Veh group and the Mel + 4PP group, the Mel group exhibited a notable reduction in the number of iNOS-positive cells (32.00±5.57 vs. 17.67±6.43 and 35.67±7.37 vs. 17.67±6.43, N=3/group, P=0.036 and P=0.015) (Fig. 3D and E). Conversely, the number of Arg1-positive cells increased in the Mel group,

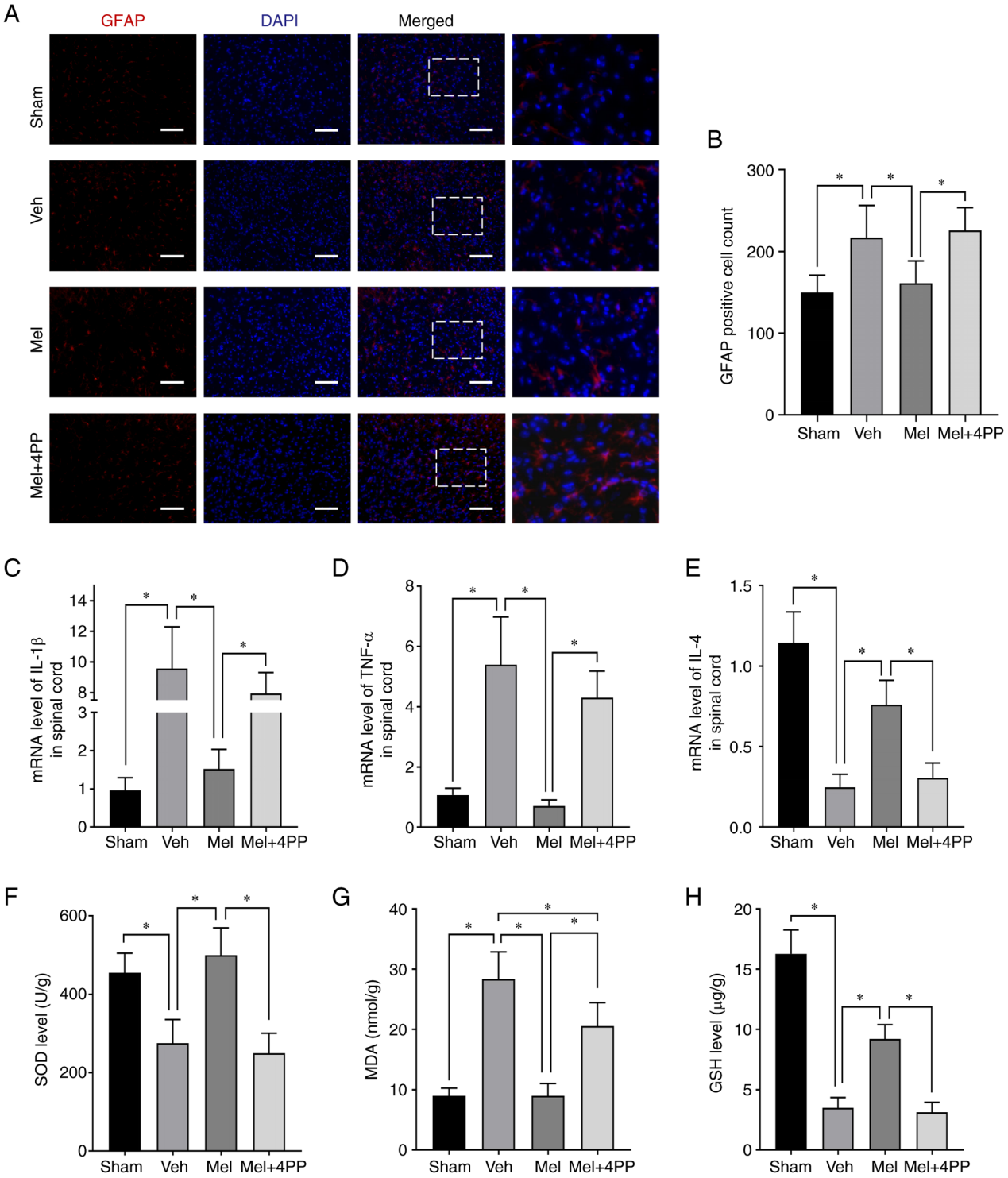

Figure 2. Melatonin exerts its effects by reducing inflammation and attenuating the oxidative stress response. (A and B) Representative images of immunofluorescence staining were obtained to assess the levels of GFAP in the anterior horn of the spinal cord at the epicenter of the lesion. The scale bar represents 200 μm. (C-E) mRNA levels of pro-inflammatory factors IL-1β and TNF-α, as well as the anti-inflammatory factor TNF-α, were assessed in spinal cord tissue at 7 dpi. (F-H) The oxidative stress index was evaluated at 7 dpi to assess the levels of oxidative stress in each group. *P≤0.05. GFAP, glial fibrillary acidic protein; dpi, days post-injury; SOD, superoxide dismutase; MDA, malondialdehyde; GSH, reduced glutathione; Mel, Melatonin; Veh, Vehicle; 4PP, 4-phenyl-2-propionamidotetralin.

indicating an enhanced M2-type polarization (23.67±5.86 vs. 55.00±21.28 and 19.97±3.51 vs. 55.00±21.28, N=3/group, P=0.025 and P=0.015) (Fig. 3F and G).

Melatonin increases the number of nerve cells surviving through the MT2 receptor. The NeuN antibody was utilized to label neurons. The immunofluorescence analysis revealed that treatment with melatonin resulted in an increased number of viable neurons. In comparison to the Mel group, the number of neuron-positive cells was significantly lower in the Veh group (163.67±34.02 vs. 65.67±30.92, N=3/group, P=0.004) and the Mel + 4PP group (163.67±34.02 vs. 68.00±7.21, N=3/group,

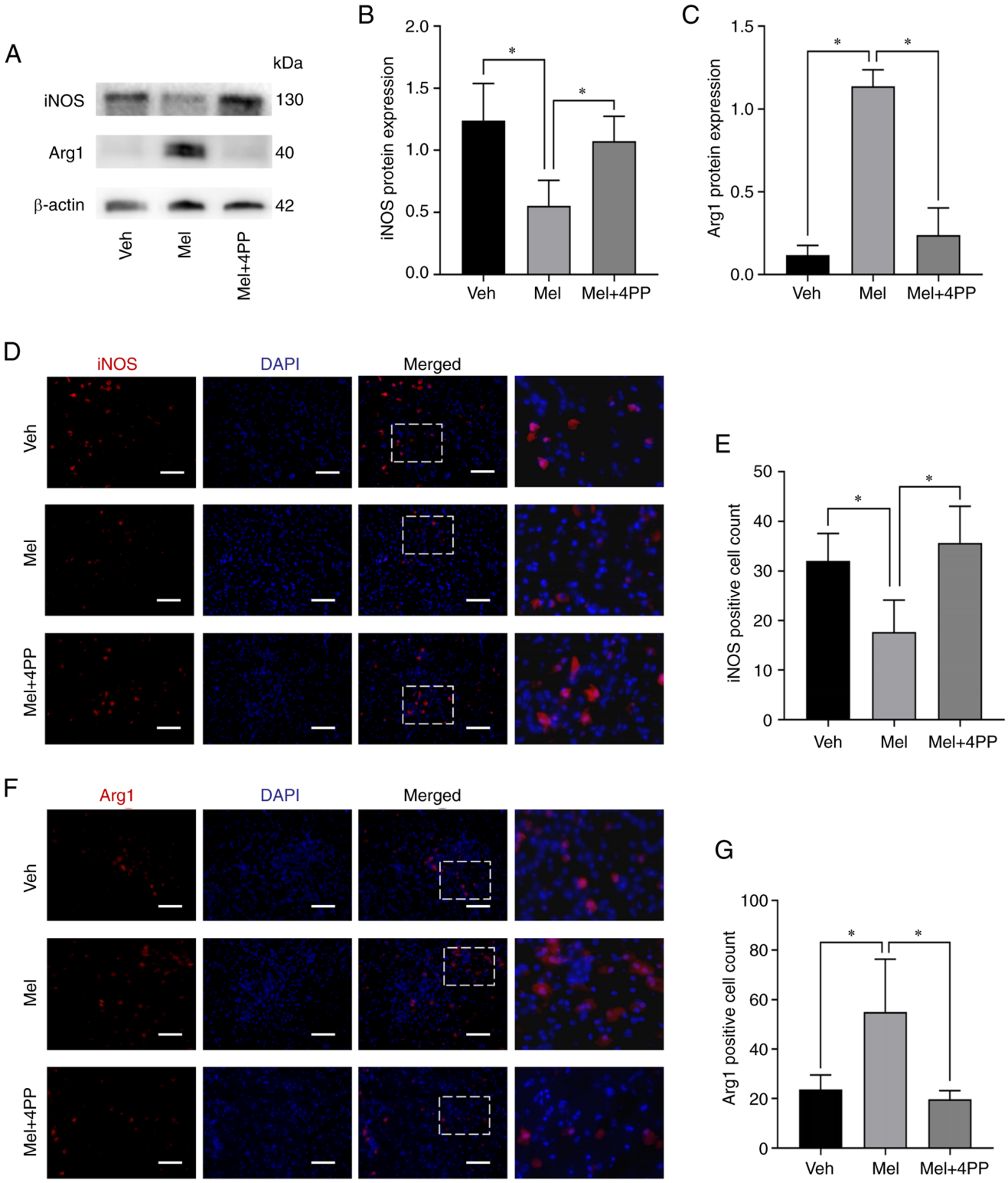

Figure 3. Melatonin plays a role in regulating the polarization of microglia. (A-C) The expression levels of iNOS and Arg1 proteins were assessed using representative blots and quantification. (D and E) Immunofluorescence staining was performed to visualize and quantify the number of iNOS-positive cells in the anterior horn of the spinal cord at the lesion epicenter. The scale bar represents 200 μm. (F and G) Immunofluorescence staining was also conducted to assess the number of Arg1-positive cells in the anterior horn of the spinal cord at the lesion epicenter. The scale bar represents 200 μm. *P≤0.05. iNOS, inducible nitric oxide synthase; Arg1, arginase 1; Mel, Melatonin; Veh, Vehicle; 4PP, 4-phenyl-2-propionamidotetralin.

P=0.005) following SCI (Fig. 4A and B). These findings collectively indicated that melatonin exerts a pronounced neuroprotective effect on the damaged spinal cord through the involvement of the MT2 receptor.

Furthermore, the expression of apoptosis-related genes, Bcl2 and Bax was assessed. The PCR results demonstrated a significant decrease in Bax expression (a pro-apoptotic gene) (4.75±0.32 vs. 1.07±0.21, N=6/group, P<0.001) and an increase

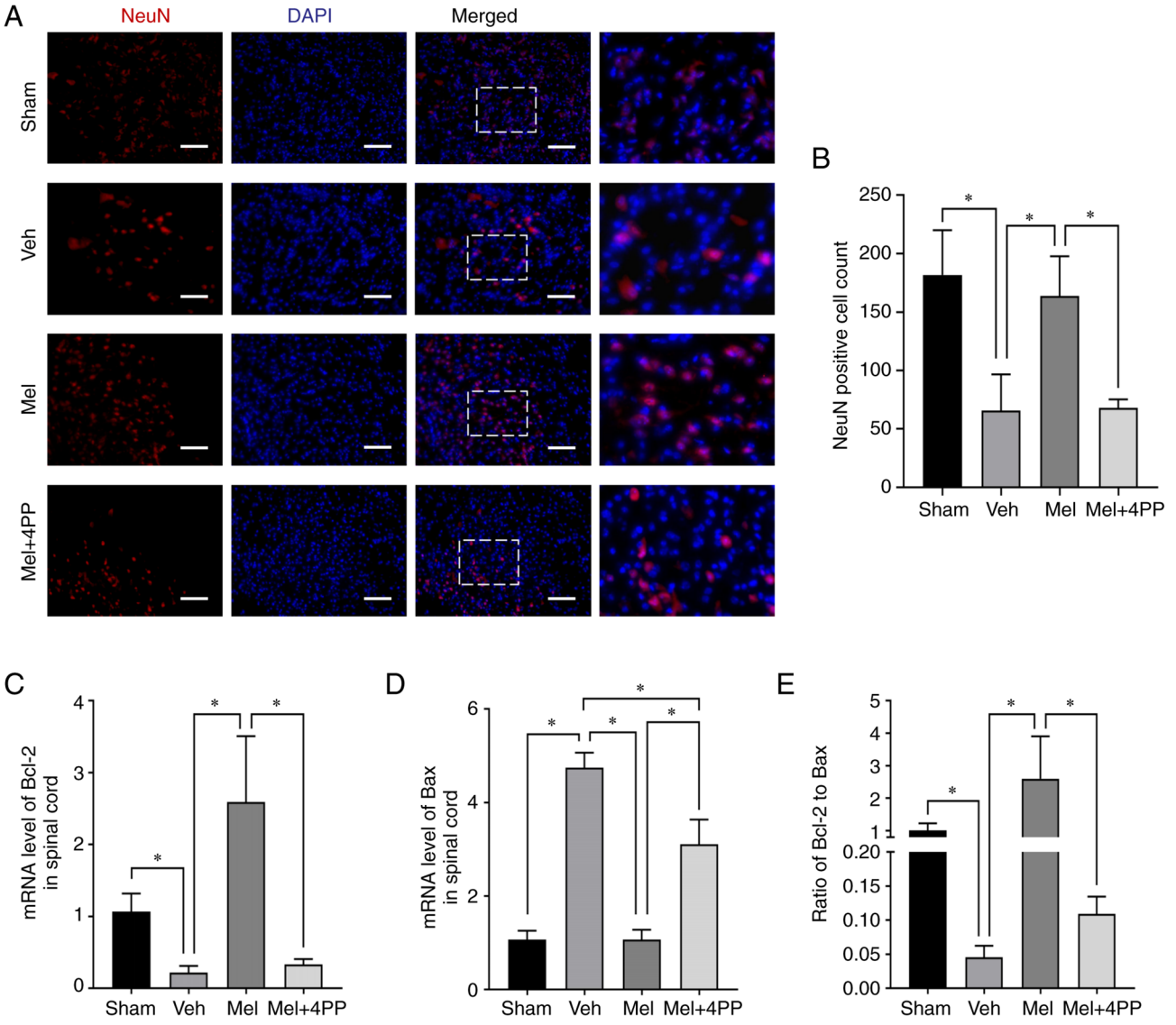

Figure 4. Melatonin enhances cell viability in the spinal cord. (A and B) Immunofluorescence staining was performed to visualize and quantify the number of NeuN-positive cells in the anterior horn of the spinal cord at the lesion epicenter. The scale bar represents 200 μm. (C-E) mRNA levels of Bcl-2 and Bax, as well as the ratio of Bcl2/Bax, were evaluated in spinal cord tissue at 7 days post-injury. *P≤0.05. Mel, Melatonin; Veh, Vehicle; 4PP, 4-phenyl-2-propionamidotetralin.

in Bcl2 expression (an anti-apoptotic gene) (0.22±0.09 vs. 2.59±0.92, N=6/group, P<0.001) in the spinal cord tissues of mice treated with melatonin compared with the Veh group. The ratio of Bcl2 to Bax (Bcl2/Bax) was significantly different between the Veh and Mel groups (0.05±0.02 vs. 2.60±1.30, N=6/group, P<0.001), while these data were similar between the Veh group and Mel + 4PP group (0.05±0.02 vs. 0.11±0.03, N=6/group, P=0.869). These findings indicated that melatonin can effectively inhibit cell apoptosis, as reflected by the alteration in the expression of Bcl2 and Bax genes (Fig. 4C-E).

Melatonin promotes functional recovery after SCI through the MT2 receptor. The BMS scores were used to assess the effect of melatonin and 4P-PDOT on locomotor function after SCI. The evaluation was conducted on days 1, 3, 5, 7, 14, 21, and 28 following the injury. During the first week after SCI, there were no significant differences in the BMS scores among all groups. However, at 14 days post-injury, mice treated with melatonin exhibited improved functional ability compared with the Veh group (2.29±0.76 vs. 1.50±0.84, N=6/group, P=0.039) and the Mel + 4PP group (2.29±0.76 vs. 1.40±0.55, N=6/group, P=0.028). Meanwhile, there was no significant difference between the Veh group and the Mel + 4PP group (1.50±0.84 vs. 1.40±0.55, N=6/group, P=0.798). Although the BMS score of the Mel + 4PP group was slightly higher than that of the Veh group on day 21, the difference between the two groups was not statistically significant (2.17±0.75 vs. 2.20±0.84, N=6/group, P=0.945) (Fig. 5A). These findings indicated that melatonin could enhance functional recovery following SCI, while the MT2 receptor blocker 4P-PDOT could impede this process.

H&E staining was conducted on the spinal cord sections from each experimental group (Fig. 5B). The results revealed a substantial disruption of spinal cord tissue integrity in all three groups. Vacuoles were observed at the site of injury, and local bleeding was evident. Furthermore, the boundary between the grey matter and white matter in the center of the injury appeared indistinct. However, compared with the Veh group

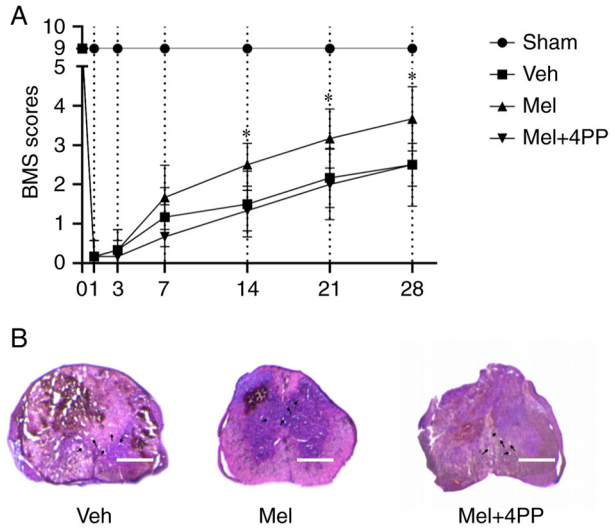

Figure 5. Melatonin facilitates functional recovery following spinal cord injury in mice. (A) Hindlimb movements were assessed using the BMS to evaluate the extent of functional recovery. (B) Representative images of H&E-stained sections at different time points post-spinal cord injury were obtained. The scale bar represents 200 μm. *P≤0.05. BMS, Basso Mouse Scale; Mel, Melatonin; Veh, Vehicle; 4PP, 4-phenyl-2-propionamidotetralin.

and the Mel + 4PP group, the Mel group exhibited relatively preserved spinal cord tissue.

Discussion

SCI is known to cause significant damage to the nervous, motor and sensory functions of the human body, leading to a severe decline in patients' quality of life and placing a substantial burden on society. Long-term statistical analysis of SCI patients over a decade has revealed a persistently high mortality rate among the elderly, particularly those over 75 years of age, which is ~five times higher than that of younger individuals (40).

In the context of SCI, the initial injury triggers the rapid death of local neurons and glial cells at the injury site within min to h. Subsequently, the nervous system undergoes an inflammatory response that leads to secondary damage (3). Hypoxia, ischemia-reperfusion injury and microenvironmental imbalances following SCI contribute to the generation and release of a significant number of free radicals and reactive oxygen species, further disrupting the local microvascular system and causing dysfunction in nerve cells (4,5). Therefore, achieving a swift equilibrium between oxidative stress and the inflammatory response plays a critical role in safeguarding the spinal cord and preserving the functionality of spinal cord nerve cells.

Although the exact mechanism is not fully understood, Melatonin has gained significant attention in recent years due to its recognized anti-inflammatory and antioxidant effects. Melatonin is a neurokinin peptide primarily secreted by the pineal gland and is widely distributed throughout various parts of the body, including the bone marrow, lymphocytes, gastrointestinal tract and skin (41,42). The discovery of melatonin receptors initially occurred in the African clawed toad in 1994 and were initially named Mel1a and Mel1b (43,44). However, they were later renamed MT1 and MT2 according to the official IUPHAR nomenclature. The human MT1 receptor consists of 350 amino acids, while MT2 receptor consists of 362 amino acids. These receptors share an overall amino acid homology of 55% and exhibit a 70% homology within the structural transmembrane domain (45). Recent studies suggest that MT1 receptors are primarily associated with biological circadian rhythms. For instance, Giannoni-Guzmán *et al* (46) found that a prolonged photoperiod resulted in decreased co-expression levels of Tph2 and Pet-1 in 5-HT neurons, and this modulatory effect of the photoperiod on TREK-1 was lost in MT1 knockout mice. Li reported that melatonin reduces the expression of Nox2 and Nox4, thereby decreasing ROS levels and attenuating the inflammatory response in mice with ischemic stroke through the activation of MT2 receptors (47).

In the present study, a simultaneous administration of melatonin and the MT2 receptor blocker 4P-PDOT occurred, to treat SCI mice. A significant increase in the expression of the MT2 receptor in mice treated with melatonin was observed, while the administration of 4P-PDOT successfully reduced the expression of the MT2 receptor. The findings of the present study in SCI mice demonstrated the following: i) Melatonin increased the expression of the MT2 receptor and activated the Nrf2/Keap1 signal pathway; ii) Melatonin reduced the inflammatory response and oxidative stress after SCI through MT2 receptor activation; iii) Melatonin regulated the inflammatory microenvironment through MT2 receptor modulation, thereby improving the polarization direction of microglia; iv) Melatonin reduced neuronal death and increased neuronal survival through MT2 receptor activation; and v) Melatonin promoted the recovery of hindlimb motor function in SCI mice through MT2 receptor activation.

Tissue destruction and extensive cell necrosis can result in hypoxia, ischemia-reperfusion injury and microenvironment imbalance (48). These conditions can trigger the production and release of a large number of oxygen free radicals and inflammatory factors, leading to local microvascular system disorder and dysfunction of nerve cells (4,5). Therefore, it is crucial to rapidly restore the balance of oxidative stress and stabilize the inflammatory microenvironment as a means of treating SCI. The Nrf2/Keap1 pathway plays a key role in regulating the expression of antioxidant genes. Keap1, a substrate adaptor protein, is responsible for degrading Nrf2 through the ubiquitin-26S proteasome pathway in the cytoplasm (49). The Nrf2/Keap1 pathway has been shown to play an important role in hippocampal neurogenesis (50), but its specific role in how melatonin improves the prognosis of SCI through the activation of the Nrf2/ARE pathway remains unclear.

In the present study, the expression of Nrf2 and Keap1 was simultaneously detected through western blotting and RT-qPCR analyses. The results revealed an increase in Nrf2 expression, while the expression of Keap1, which inhibits Nrf2, decreased. However, when the MT2 receptor was blocked using 4P-PDOT, the activation of the Nrf2/Keap1 signaling pathway was hindered. These findings suggested that melatonin can activate the Nrf2 signaling pathway through the MT2 receptor after SCI, indicating its potential role in promoting antioxidant responses and reducing oxidative stress.

The Nrf2/Keap1 signaling pathway was important in the regulation of the inflammatory response (51). Based on

this knowledge, it was hypothesized that melatonin could also mitigate the inflammatory response. Considering that significant changes in gene expression occur within 28 days after SCI, with the most prominent alterations observed on day 7 (32), it was chosen to examine the injured tissue on this particular day for the improved comprehension of the microenvironmental changes within the tissue. The pathophysiology of SCI is multifaceted, involving processes such as apoptosis, inflammation, vascular injury, electrolyte imbalance and mitochondrial dysfunction (52). Injured spinal cord tissue is characterized by the presence of myelin debris and glial scars, as well as various factors that restrict neuronal regeneration and axonal growth (53). The microenvironment of injured spinal cord tissue poses challenges for neural tissue regeneration. Following tissue damage, astrocytes release pro-inflammatory factors (54) and proliferate to form glial scars, which impede nerve tissue regeneration (55). Moreover, through the astrocyte-microglial co-regulatory network, astrocytes can influence the polarization of microglia (56).

Consistent with the findings of the present study on the Nrf2/Keap1 signaling pathway, it was observed that melatonin treatment effectively suppressed the release of inflammatory factors and promoted the release of anti-inflammatory factors. Additionally, it was also observed an increased expression of SOD and GSH in the Mel group, while the expression of MDA, a marker of oxidative stress, decreased. These findings suggested that melatonin has the potential to attenuate the inflammatory response and enhance antioxidant defenses, leading to a more favorable microenvironment for tissue repair and regeneration in the injured spinal cord.

The inflammatory microenvironment of the injured spinal cord is closely associated with the polarization of microglia (57,58). Pro-inflammatory factors such as IL-1β and TNF-α can induce microglia to polarize into the M1 phenotype (38), which in turn release large amounts of pro-inflammatory factors, thereby exacerbating the inflammatory response (59). Interestingly, IL-4 receptors on the surface of M1 microglia have the ability to convert them into M2 phenotype (39), which release neurotrophic factors including anti-inflammatory substances, transforming growth factor-β, and vascular endothelial growth factor. This M2 phenotype plays a crucial role in restoring *in vivo* homeostasis. The concept of M1/M2 macrophages was first proposed by Mills *et al (*60). It was also observed that M1 macrophages could inhibit cell proliferation and induce cell death, while M2 macrophages could promote cell proliferation and tissue growth (61). The microenvironment at the site of injury may determine the polarization of microglia. Following SCI, most microglia tend to polarize toward the M1 phenotype rather than the M2 phenotype. Therefore, modulating the microenvironment after injury may regulate the polarization of microglia toward the M2 phenotype.

In the present study, the effects of melatonin on the inflammatory response after SCI by examining the cytokine profiles of spinal cord tissue were investigated. Based on the results of the present study, it was hypothesized that melatonin, acting through the MT2 receptor, can influence the local microenvironment, inhibit inflammatory factors, and modulate the polarization of microglia toward the M2 anti-inflammatory phenotype. By promoting the shift from M1 to M2 microglia, melatonin may contribute to the resolution of inflammation and create a more favorable environment for tissue repair and recovery after SCI.

The spinal cord coordinates various motor reflexes in the body, as it contains numerous motor neurons that innervate skeletal muscles. However, the regenerative capacity of neurons in the spinal cord is limited, highlighting the importance of protecting neurons from injury following SCI. The results of the NeuN staining in the present study demonstrated that melatonin treatment leads to an increased number of surviving neurons. Based on the observed differences between the treatment groups, it can be hypothesized that this neuroprotective effect is mediated through the MT2 receptor. Furthermore, the present study's analysis of the apoptotic factors Bcl2 and Bax revealed that melatonin may partially inhibit neuronal apoptosis. This finding suggested that melatonin may have a role in preventing cell death and promoting neuronal survival after SCI.

The assessment of motor function using the BMS score indicated that melatonin treatment has a significant and sustained positive impact on the recovery of motor ability in mice over the medium and long term. Given that motor neurons responsible for innervating skeletal muscles are located in the anterior horn of the spinal cord, the observed improvement in motor ability strongly suggests that melatonin exerts a neuroprotective effect after SCI by effectively reducing neuronal loss.

Overall, the findings of the present study suggested that melatonin has the potential to enhance neuronal survival, inhibit apoptosis, and improve motor function following SCI. These effects are likely mediated through the MT2 receptor, highlighting the therapeutic potential of melatonin for neuroprotection in the context of SCI.

In conclusion, the findings of the present study provided evidence that melatonin exerts its beneficial effects in SCI by reducing oxidative stress and suppressing local inflammation through the activation of the Nrf2/Keap1 signaling pathway via MT2 receptors. This mechanism leads to improvements in the local microenvironment, reduction in nerve cell apoptosis within the anterior horn of the spinal cord, increased neuronal survival, modulation of microglial differentiation, and ultimately, enhanced long-term prognosis in mice with SCI. These findings suggested that melatonin holds promise as a potential therapeutic agent for clinical treatment of SCI.

However, it is important to acknowledge certain limitations to the present study. Firstly, there was no investigation into the effects of MT2 receptor agonists, which could provide additional insights into the mechanisms of action and further validate the role of the MT2 receptor in mediating the effects of melatonin. Future studies incorporating MT2 receptor agonists could provide a more comprehensive understanding of the therapeutic potential of melatonin in SCI.

Secondly, there was no assessment in the expression of the MT2 receptor in different tissues of melatonin-treated mice. Examining the expression of MT2 receptors in various tissues would provide valuable information regarding the specificity and distribution of the receptor, and further elucidate its involvement in the observed neuroprotective effects of melatonin.

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Availability of data and materials

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

Authors' contributions

LY made significant contributions to conceptualization. XH and MZ conducted the investigation and validated the data. HK interpreted the data and drafted the manuscript. HL and TC designed and conceptualized the study, and revised the essential content of the manuscript. All authors read and approved the final version of the manuscript. LY and XH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved (approval no. ZZUIRB 2022-145) by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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