Journal of Neurotrauma

DOI: 10.1089/neu.2018.6012

Melatonin treatment alleviates spinal cord injury-induced gut dysbiosis in mice

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Abstract

Spinal cord injury (SCI) disturbs the autonomic nervous system and induces dysfunction in multiple organs/tissues, such as the gastrointestinal (GI) system. The neuroprotective effects of melatonin in SCI models have been reported; however, it is unclear whether the beneficial effects of melatonin are associated with alleviation of gut dysbiosis. In this study, we showed that daily intraperitoneal injection with melatonin following spinal cord contusion at thoracic level 10 in mice improved intestinal barrier integrity and GI motility, reduced the expression levels of certain pro-inflammatory cytokines, improved animal weight gain and metabolic profiling, and promoted locomotor recovery. Analysis of gut microbiome revealed that melatonin treatment decreased the Shannon index and re-shaped the composition of intestinal microbiota. Melatonin-treated SCI animals showed decreased relative abundance of *Clostridiales* and increased relative abundance of *Lactobacillales* and *Lactobacillus*, which correlated with alteration of cytokine (monocyte chemotactic protein (MCP)-1) expression and GI barrier permeability, as well as with locomotor recovery. Experimental induction of gut dysbiosis in mice prior to SCI (i.e., via oral delivery of broad-spectrum antibiotics) exacerbates neurological impairment after SCI, and melatonin treatment improves the locomotor performance and intestinal integrity in antibiotic-treated SCI mice. The results suggest that melatonin treatment restores SCI-induced alteration in gut microbiota composition, which may underlie the ameliorated GI function and behavioral manifestations.

Keyword: intestinal microbiota, intestinal barrier integrity, gastrointestinal motility, spinal cord injury, melatonin

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Introduction

Spinal cord injury (SCI) disturbs the autonomic nervous system, and impairs its ability to coordinate organ functions throughout the body. Gastrointestinal (GI) physiology is influenced by signals arising both locally within the gut and from the central nervous system (CNS) [1]. Patients with SCI commonly have neurogenic bowel dysfunction due to the damaged control of the CNS over the GI system [2-4], leading to deficits in colonic motility, mucosal secretions, and vascular tone [5, 6]. GI dysfunction severely detracts from the quality of life of persons after SCI.

Human and rodent studies suggest that SCI causes gut dysbiosis, and changes in the composition of bacterial in the gut may affect systemic physiology and pathophysiology [7, 8]. The commensal microbiota has substantial effects on the host, contributing to digestion and nutrient absorption, maintenance of cellular metabolism, and immune system development [9-11]. Neurotransmitters, hormones and neuroactive metabolites produced in gut microbes also influence normal development and disease pathogenesis in the CNS [12-14]. Gut microbiome plays a vital role in the formation of blood-brain barrier, myelination, neurogenesis, and microglia maturation [15-17]. Accordingly, dysbiosis has been implicated in the onset or progression of neurological diseases including Parkinson's Disease, stroke, depression, anxiety, autism and so on [18-21].

Melatonin (N-acetyl-5-methoxytryptamine) is produced in the pineal gland as well as many other organs, including the enterochromaffin cells of the digestive mucosa [22]. Initially, melatonin was identified as a neurohormonal peptide, primarily involved in circadian rhythms, oxidative stress, neuroendocrine regulation and immune functions [23-26]. The concentration of melatonin in the GI tissues surpasses blood levels by 10-100 times [27] and is 400-fold higher than that in the pineal gland [28]. Melatonin has been reported for its capability of promoting neuro-repair and functional recovery after SCI [29, 30]. Accumulating evidence indicated that melatonin exerted various degrees of impact on oxidative stress, inflammation, BSCB leakage, apoptosis, and edema caused by SCI [31]. However, few studies have explored the influence of melatonin treatment on bowel dysfunction and gut dysbiosis in SCI.

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Several studies have shown a connection between melatonin and intestinal functions, pointing to a potential clinical intervention strategy to improve intestinal barrier functions. In the patient group with the alcohol use disorder, plasma concentration of melatonin was significantly lower, which was correlated with increased intestinal permeability [2932]. Melatonin administration markedly improved duodenal barrier functions [27, 30 33]. Also, melatonin is protective against inflammatory, oxidative stress and immune cell infiltration in jejunum after ischemia-reperfusion injury [23]. Interestingly, melatonin is capable of influencing intestinal microbiota. In an obesity study, it was demonstrated that melatonin treatment decreases the *Firmicutes*-to-*Bacteroidetes* ratio and increases the abundance of mucin-degrading bacteria *Akkermansia* [3134]. In another study, melatonin administration can re-shape the composition of intestinal microbiota to alleviate weanling-induced stress [3235]. Melatonin was demonstrated to increase the resistance to oxidative stress in mice with colitis and regulate the intestinal microbial flora, thus improving intestinal health [36]. Although the effect of melatonin on intestinal microbiota has been noticed, until recently there have been few investigations focusing on the beneficial effects of melatonin on the gut microbiota. In a study, Yin et al. indicated that melatonin alleviates the lipid accumulation and reverses gut microbiota dysbiosis. Antibiotics administration and microbiota transplantation were applied in this study, and the results suggested that the anti-dysmetabolism effect of melatonin may be associated with the reprogramming of gut microbiota [37].

As the importance of a balanced gut microbiota for health has attracted attention, an increasing number of studies have been conducted in the SCI area field. Clinical data demonstrated that SCI led to a significant change in the composition of the gut microbiome [7]. Other studies as well as our unpublished data suggested that induced gut dysbiosis exacerbates lesion pathology after SCI, while post-injury probiotic treatment improves locomotor recovery after SCI [8], indicating that intestinal microbiome may be closely associated with functional recovery after SCI.

In this study, we aim to explore the functions of melatonin on intestinal microbiota in SCI mice. We hypothesized that melatonin treatment may influence SCI-induced GI dysfunction through re-shaping the intestinal microbiota.

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Methods

Experimental animals

Adult female C57BL/6 (18-22 g) mice were obtained from the Center of Experimental Animals, Capital Medical University (Beijing, China). Mice were maintained in an air-conditioned room with a 12:12 light/dark cycle, where the temperature was (22 ± 2) °C and relative humidity was (55 ± 10) %. Food and water were available *ad libitum*. Animal protocols have been approved by the Animal Care and Use Committee of Capital Medical University.

Spinal cord injury

Mice were anesthetized with 2% isoflurane in a gas mixture of 30% oxygen and 70% nitrogen. A laminectomy was performed at the T10 level. Mice received a 70-kilodyne spinal contusion injury using the Infinite Horizons Impactor (Precision Systems & Instrumentation, Lexington, KY, USA). Afterwards, the muscle and the incision opening were sutured. During the surgical procedure and recovery from anesthesia, mice were placed in a warming chamber until they were completely awake. Postoperatively, animals were hydrated with 0.5 ml Ringer's solution (S. C.) for 5 days. Bladders were voided manually at least twice daily for the duration of the study. Surgical interventions and postoperative animal care were performed in accordance with the guidelines and policies for rodent survival surgery provided by the Experimental Animal Committee of Capital Medical University.

Experimental groups

Mice were randomly divided into four groups with 16 mice in each Mice were randomly divided into two groups (Sham group and SCI model group) with 32 mice in each. After surgery, mice in each group were further randomly assigned into two groups (Vehicle group and Melatonin treatment group) with 16 mice in each of the following groups: (1) mice in the Sham group underwent a T10 laminectomy without SCI and received vehicle (1% alcohol in 1 ml saline); (2) mice in the Sham + Mel group underwent a T10 laminectomy without SCI and received melatonin (10 mg/kg, twice a day, i.p.); (3) mice in the SCI group were subjected to moderate SCI and were given vehicle; and (4) mice in the

SCI + Mel group were subjected to SCI and were administered melatonin intraperitoneally at a dose of 10 mg/kg twice daily (07:00, 19:00) for 4 w.

Melatonin concentration measurement

The colonic tissue (approximately 2 cm) segments were removed at day 28 after injury, immediately put into liquid nitrogen and grinded. Then, melatonin was extracted with acetonitrile, which was concentrated with nitrogen and diluted and filtrated. The liquid chromatographic (LC) conditions and mass spectrometric (MS) conditions were performed as follows.

Liquid chromatographic conditions

Liquid chromatography was performed on a rapid resolution liquid chromatography system (ACQUITY UPLC I-Class, Waters, USA). An ACQUITY UPLC™ BEH C18 column (50× 2.1mm, 1.7μm) was used for LC separation .The auto-sampler was set at 10 ˚C , using gradient elution with 0.1% formic acid methanol as solvent A and 0.1% formic acid water as solvent B. The gradient program was as follows: 0-3 min 40% A, 3-5 min 40% A to 80 % A, 5-6 min80 % A-100% A, 6-7min 100%A,7-7.1 min 100% A to 40% A,7.1-10 min 40% A. The flow rate was set at 0.2 mL min-1, and the injection volume was 10 μL. The total run time was 10 min for each sample.

Mass spectrometric conditions

The detection was performed on an AB SCIEX Triple Quad™ 4500 (Applied Biosystems,Foster City, CA, USA) with an electrospray ionization source (Turbo Ionspray). The mass spectrometry detection was operated in positive electrospray ionization mode. The [M+H] of analyte was selected as the precursor ion. The quantification mode was multiple reaction monitoring (MRM) mode using the mass transitions (precursor ions/product ions).

The ESI ion source temperature was set at 500˚C. Other mass spectrometric parameters were: curtain gas flow, 15 psi; collisionally activated dissociation (CAD) gas setting, medium; ionspray voltage, 5000 V; ion gas 1 and 2, 50 psi. Data acquisition and processing were performed using AB SCIEX Analyst 1.6 Software (Applied Biosystems).

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FITC–dextran permeability assay

Four weeks after injury, the mice were fasted for14 h and gavaged with 60 mg per 100 g body weight of fluorescein isothiocyanate–dextran (FITC–dextran, 4 KD; Sigma-Aldrich, Madrid, Spain) in a volume of 0.2 ml. Four hour after FITC-dextran gavaging, blood were sampled by cardiac puncture and clotted for 30 min, followed by centrifugation for 90 s at 6,000 g. Serum was diluted with an equal volume of sterile PBS, and 100 µl of this dilution was added to individual wells of a 96-well plate. Fluorescence was measured on a plate reader (EnSpire; Perkin Elmer) at an excitation of 481 nm and an emission of 524 nm. The concentrations of FITC-dextran were calculated by referring to standard curve measurements run on the same plate.

Tight junction PCR array

Total RNA was extracted from the colon using Trizol (Takara, Dalian, China) and was reverse-transcribed into cDNA using Primescript RT Master Mix (Takara, Dalian, China). Real-time qPCR was performed on an Applied Biosystems 7500 Real-time PCR system using the SYBR Premix Ex Taq (Tli RnaseH Plus) (Takara, Dalian, China). Primer sequences were as follows.

ZO-1, forward (5′-AGGACACCAAAGCATGTGAG-3′) and reverse (5′-GGCATTCCTGCTGGTTACA-3′);

Occludin, forward (5′-TTGGCTACGGAGGTGGCTATGG-3′) and reverse (5′-ACTAAGGAAGCGATGAAGCAGAAGG -3′);

Claudin3, forward (5′-TACCGTCACCACTACCAGCAGTC-3′) and reverse (5′-CCAGCCTGTCTGTCCTCTTCCA-3′);

Claudin5, forward (5′-GCTCAGAACAGACTACAGGCACTT-3′) and reverse (5′-CCACTGGACATTAAGGCAGCATCT-3′).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control to normalize target gene transcript levels.

Immunohistochemistry

At 4 w after SCI, mice were perfused with 0.1 M PBS (pH 7.4, 37 °C), followed by 4% (w/v) paraformaldehyde in 0.1 M PBS. Colonic tissues were embedded in Optimal Cutting Temperature compound and cut serially into 20-µm sections on a cryostat microtome (Leica CM 3500, Wetzlar, Germany). All sections were thawed and mounted on gelatin-coated glass slides. Sections were equilibrated in 0.1 M Tris-buffered saline for 10 min, and finally permeabilized with 0.3% Triton X-100 for 30 min. Nonspecific antibody binding was then blocked with 10% normal goat serum in PBS for 1 h. Slides were incubated for 1 h with appropriate dilutions of primary antibodies, including rabbit polyclonal anti-ZO-1 (1:100, Abcam, Cambridge, MA, USA), or rabbit monoclonal anti-occludin (1:100, Abcam, Cambridge, MA, USA). Slides were rinsed after primary antibody incubation, then incubated with secondary antibody (goat anti-rabbit IgG H&L Alexa Fluor® 488, 1:500, Abcam, Cambridge, MA, USA) and counterstained with DAPI. The slides were coverslipped with glycerinum-mounting media and examined by fluorescence microscopy. The relative fluorescence intensity was calculated by using Image Pro Plus7.0. (Media Cybernetics, Silver Spring, MD, USA).

Gastrointestinal transit assessment

Alterations on GI motility were measured at 4 w following injury, using radiographic methods as previously described [33, 3438,39]. Intragastric gavage of barium (0.2ml, 2 g/ml) was performed. Plain facial radiographs of the GI tract were obtained using a digital X-ray apparatus (Siemens; 50 kV, 10 mA) and captured with NPG Real DVD Studio II software. Exposure time was adjusted to 0.06 s. Immobilization of the mice in a prone position was achieved by placing them inside adjustable hand-made transparent plastic tubes. To further reduce stress, mice were released immediately after each shot (immobilization lasted for 1-2 min). X-rays were recorded at different times (immediately and 0.5, 1, 2, 3, 4, 6 and 8 h) after administration of the barium. X-ray images were also morphometrically analyzed with the aid of an image analysis system (ImageJ 1.38, National Institute of Health, USA), and the alterations in size of the stomach and colorectum were quantified.

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Cytokine analysis

Cytokine analysis was conducted using a magnetic bead based multiplex assay system, with detection and quantification of multiple cytokines from a single sample of plasma (a mouse cytokine 23-plex assay, Bio-Plex Pro Mouse Cytokine 23-plex #M60009RDPD). In this test, 23 cytokines were simultaneously quantified that included eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF),interferon (IFN)-γ, interleukin (IL)-1β, IL-1α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, KC, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated on activation normal T cell expressed and secreted (RANTES), tumor necrosis factor (TNF)-α. All standards, reagents, and samples were prepared according to the manufacturer's instructions. Sections of colon were snap-frozen in liquid nitrogen. Tissues were stored at −80°C until homogenization in lysis buffer containing protease inhibitors (Beyotime, China). Cytokine levels in the supernatant (50 μL) were used for analysis, with a minimum of 50 beads acquired per analyte. The plates were analyzed using a multiplexing diagnostic instrument (Bio-Plex 200; Bio-Rad, Hercules, CA, USA), and fluorescent data for each cytokine was extracted using the manufacturer's software (Bio-Plex Manager 6.1; Bio-Rad, Hercules, CA, USA.). Standard curves for each analyte were generated using standards provided by the manufacturer.

DNA extraction and PCR amplification

Microbial DNA was extracted from stool samples using the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to manufacturer's protocols. The V3-V4 region of the bacteria 16S rRNA gene were amplified by PCR (95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min) using primers 338F 5'- ACTCCTACGGGAGGCAGCA-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3'. PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

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Illumina MiSeq sequencing

Amplicons were extracted from 2% agarose gels, purified by using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.), and quantified by using QuantiFluor™ -ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300 bp) on an Illumina MiSeq platform according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive database (Accession Number: SRP148843).

Processing of sequencing data

Raw fastq files were quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) The reads were truncated at any site receiving an average quality score < 20 over a 50 bp sliding window. (ii) Sequences whose overlap being longer than 10 bp were merged according to their overlap with mismatch no more than 2 bp. (iii) Sequences of each sample were separated according to barcodes (exactly matching) and Primers (allowing 2 nucleotide mismatching), and reads containing ambiguous bases were removed.

Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE (version 7.1) with a novel 'greedy' algorithm that performs chimera filtering and OTU clustering simultaneously. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm against the Silva (SSU128) 16S rRNA database using confidence threshold of 70% [35 40].

Metabolic parameters

At the start of the experiment, animals were housed individually in metabolic phenotyping chambers (Mouse Promethion Continuous caging system; Sable Systems™, Las Vegas, NV) and maintained on a standard 12 h light/dark cycle; both food and water were available *ad libitum*. Air within the cages was sampled through micro-perforated stainless steel sampling tubes located around the bottom of the cages, above the bedding. Gas sensors were calibrated before each run with 100% N_2 as zero reference .The incurrent flow rate was set at 2000 mL/min and gases were sampled continuously for each cage, from multiple points within the cage (250 ml/min). Oxygen consumption and carbon

dioxide (CO_2) production were recorded for each mouse. Respiratory exchange quotient (RQ) was calculated as the ratio of CO_2 production over O_2 consumption. Energy expenditure was calculated using the Weir equation: Kcal/h = 60 ∗ (0.003941 ∗ VO_2 + 0.001106 ∗ VCO_2) [36 41]. Values were calculated after application of algorithms using macros provided with the analysis software ExpeData [3742].

Behavioral analysis

The Basso Mouse Scale (BMS) was used to score hindlimb movements as previously described [3843]. Animals were assessed in an open field for 4 min pre surgically and at postoperative days 3, 7, 14, 21 and 28. The performance of left and right hindlimbs was rated separately and averaged to generate the BMS scores and subscores. Specific parameters of locomotion were quantified using the DigiGait Image Analysis System [39, 4044,45] Mice were pre-trained at a speed of 15 cm/s before SCI for 7 days, and then tested at 4 w at a speed of 9 cm/s. For each test, at least 5 complete step cycles were recorded. A high-speed digital camera captured the movement of each paw and then the footage was analyzed using the Digigait analysis software (Digigait 12.4). The behavioral evaluations i.e. BMS and Gait analysis were blinded to the experimenters.

Bacterial culture

From a separate set of animals, fecal samples were aseptically collected from mice at various time points after injury (n = 4 per time point) and homogenized in 1 ml of sterile saline （100 mg：1ml）. Samples were diluted to 10^4, and 100 µl of solution was plated onto Columbia Blood Agar Base Medium plates and incubated at 37°C for 48 hr.

Experimentally induced dysbiosis and melatonin treatment

To induce intestinal dysbiosis, mice started on were subjected to antibiotic treatment 14 d before SCI. An antibiotic cocktail (0.2 g/L ampicillin, neomycin, and metronidazole, and 0.1 g/L vancomycin) was given via drinking water to the mice until d 28 d after injury. The antibiotic-treated mice were further divided into 2 groups with 6 mice in each. Mice in the SCI+AB group were subjected to moderate SCI and were given vehicle (1% alcohol in 1 ml saline); and mice in SCI+AB+Mel group were subjected to SCI and administered with melatonin (10 mg/kg, twice a day, i.p.).

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Statistical analysis

Results are presented as the mean with the standard error of mean (SEM). The data were analyzed by SPSS, version 17.0 statistic software package (SPSS Inc., Chicago, Illinois, USA). Student's t-tests were used to determine significance between two groups. Multivariate analysis of variance (MANOVA) was conducted to test between-group differences on the dependent measures. One-way analysis of variance followed by post-hoc Tukey's analysis was performed to compare groups of three or more. In addition, relationships between significant microbiota changes and behavior scores (BMS), gut permeability and colonic cytokine were evaluated using the Pearson correlation method. Values of p less than 0.05 were considered statistically significant. All analyses were conducted with an alpha level of $p < 0.05$ using SPSS statistical software. Effect Size was reported as either a R^2 for the correlation analysis or Partial Eta^2 for MANOVA.

Qiime software package (Version 1.7.0) was used to analyze alpha diversity and beta diversity. Rarefaction curves and alpha diversity indices referring to community diversity (Shannon), community richness (Ace) were calculated based on the OTUs information of Sham, Sham+Mel, SCI and SCI+Mel groups. The beta diversities, including principal component analysis (PCA) analysis and principal co-ordinate analysis (PCoA), were evaluated with Bray-Curtis distance created by Qiime 1.7.0. Differential abundance of genera was tested by Wilcoxon rank sum test, and P values were corrected for multiple testing with Benjamin and false discovery rate method.

Results

Melatonin treatment maintains intestinal barrier integrity in SCI mice

We observed that SCI led to a decreased concentration of melatonin in the colonic tissue when compared to that from Sham group (Sham: 2.41 ± 0.24 pg/mg, n = 5; SCI: 1.24 ± 0.20 pg/mg, n = 5; $p < 0.01$). And melatonin treatment by intraperitoneal delivery could cause a rapid elevation of melatonin in the gut lumen which peaked at 0.5 h, and maintained at an elevated level in the colonic tissues for up to 4 hr after administration. (Fig. S1).

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To test whether melatonin treatment had any effect on intestinal barrier permeability after SCI, mice were gavaged with FITC-labeled dextran (4 KD) at 4 w following injury and then FITC levels in blood were measured. Gut permeability significantly increased after SCI. Remarkably, melatonin treatment protected against injury-induced gut leakiness (Fig. 1a). Interepithelial tight junctions influence intestinal epithelial leakage and are critically important for maintaining the barrier integrity [41, 4246,47]. We assessed the expression of tight-junction proteins in colonic tissues. Consistent with the leaky gut phenotype, colons from SCI mice contain decreased gene expression of *ZO-1*, *OCLN, CLDN3* and *CLDN5*. However, melatonin treatment remarkably restored the expression of *ZO-1* and *OCLN*, but not *CLDN3* and *CLDN5* (Fig. 1b). Similar changes were observed in immunoreactivity of ZO-1 and occludin in the colon, which was restored by melatonin treatment (Fig. 1c-e). Collectively, the results demonstrated that melatonin improved intestinal barrier integrity and upregulated the expression of tight junction components after SCI.

Melatonin treatment accelerates GI transit in SCI mice

GI transit, as an overall measure of GI motility, was assessed in the mice. Representative images from different groups were shown in Fig. 2a at different time points (0, 2, 3 and 8 h). Overall, mice subjected to injury displayed a higher level of filling of GI tract, indicative of a slower GI transit, compared to mice in sham and sham+Mel groups. Treatment with melatonin increased GI transit in mice with SCI. The morphometric analysis of the images showed that gastric emptying was almost completed 2 hours after barium administration (Fig.2b). Three hours after administration, barium had arrived at colons (Fig. 2a and c). Morphometric analysis showed that injury increased the filling of colorectum areas, which was reversed by melatonin treatment (Fig. 2a and c). The results showed that melatonin treatment accelerated GI transit in SCI mice.

Melatonin treatment down-regulates expression of pro-inflammatory cytokines in SCI mice

Levels of 23 cytokines were analyzed using a multiplex enzyme immunosorbent assay in colonic tissues 4 w following injury. SCI induced up-regulation of IL-1β, IL-17, IFN-γ, and MCP-1 in SCI vs. sham group (Fig. 3a, b). No significant changes were found in IL-1α, IL-2, IL-3, , IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, Eotaxin, G-CSF, GM-CSF, KC,

MIP-1α, MIP-1β, RANTES or IFN-α following SCI. Melatonin treatment significantly reversed injury-induced increase in IL-17, IFN-γ, and MCP-1 levels (Fig. 3c-e), but not in expression of IL-1β (Fig. 3f). The results suggest an anti-inflammatory effect of melatonin on SCI-associated gut inflammation.

Melatonin treatment re-shapes gut bacterial composition in SCI mice

To examine the effects of melatonin treatment on gut microbiota composition, we performed 16S rRNA (V3+V4 regions) gene sequencing on microbiota in mouse feces. After removing unqualified sequences, a total of 679981 qualified reads were generated for the following analysis. Based on a 97% similarity threshold, the effective reads were clustered into 433 operational taxonomic units (OTUs), which included 186 species, 119 genera, 59 families, 39 orders, 25 classes and 14 phyla. The curve of rarefaction curves and the indices of shannon and ace were calculated. As shown in Fig. 4a, the curves in each group are near smooth, indicating that the sequencing depth has covered rare new phylotypes and most of the diversity. As shown in Fig.4b, c, there were significant differences in the shannon and ace indices between the sham group and SCI group. Melatonin treatment significantly reduced the diversity and the richness of the intestinal microbiota in SCI mice. To measure the degree of similarity between microbial communities, the intestinal microbiota structural changes were then analyzed using principal component analysis (PCA) and principal coordinate analysis (PCoA). SCI group clustered distinctly from sham group by Bray Curtis PCoA and PCA analysis, indicating robust differences in the membership of gut bacteria between SCI and Sham groups. However, melatonin intervention shifted the overall structure of the SCI-disrupted gut microbiota towards that of the Sham group (Fig. 4d, e). Sham+Mel group clustered closely to Sham group, suggesting that melatonin treatment did not influence gut microbiota in normal animals without SCI. Compared to Sham group, SCI mice showed distinct microbiota up to the phylum level. There was an increase in the abundance of *Bacteroidetes* and a decrease in the abundances of *Firmicutes* after injury, but the difference was not statistically significant (data not shown). As shown in Fig. 4f, the order level analysis demonstrated that SCI significantly decreased the relative abundance of *Lactobacillales* and *Bifidobacteriales*, and increased the relative abundance of *Clostridiales*. Melatonin treatment markedly decreased the relative

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abundance of *Clostridiales* and significantly increased that of *Lactobacillales*, while it had little effect on that of *Bifidobacteriales* (Fig. 4g). To further exhibit the widespread changes in the gut microbial community structure among groups, the genus level analysis was performed. As shown in Fig. 4h and i, melatonin treatment significantly increased the relative abundance of *Lactobacillus* belonging to the *Lactobacillale* order in SCI mice. Significant changes in the taxa were accompanied by lesser but opposite change in minor taxa including *Lachnospiraceae_NK4A136_group*, and *unclassified_f___Lachnospiraceae.* Compared to Sham group, Sham+Mel group did not show significant changes in microbiota at order and genus levels. In short, melatonin treatment remarkably re-shaped the fecal microbiota in SCI mice rather than in sham mice; the changes of microbiota lied in the composition of the microbial community, particularly the relative abundance of *Clostridiales, Lactobacillales* and *Lactobacillus*.

Melatonin treatment increases weight gain and metabolic profiling in SCI mice

The body weights were measured and compared in all experimental groups. The Sham and Sham+Mel groups showed stable and comparable body weights over the 4 w of observation. In contrast, SCI and SCI+Mel groups lost body weight during the first 3 days after surgery, and gradually gained back in the following days. Compared with SCI group, the rate of body weight gain was higher in SCI+Mel group, which showed significantly difference at 14, 21 and 28 days post-injury (Fig. 5a). In addition, the metabolic parameters were assessed over a 24 h period at 4 w post-injury. Circadian energy expenditure and food consumption were determined by using metabolic cages. SCI+Mel vs. SCI group showed increased food intake in agreement with the improved weight gain (Fig. 5b). Indirect calorimetry revealed that the average energy expenditure was significantly elevated after melatonin administration in SCI animals (Fig. 5c). As shown in Fig. 5d and e, the average respiratory exchange quotient (RQ) values were restored in SCI+Mel group. These data demonstrated that melatonin administration after SCI led to more food intake, higher energy expenditure, and improved body weight gain.

Melatonin treatment improves locomotor recovery in SCI mice

In this study, BMS and digigait were applied as complementary measures to evaluate changes in locomotion that result from SCI and in the subsequent recovery. Compared to injured mice, mice treated with melatonin exhibited a relatively more rapid and greater improvement in measures of locomotion as assessed using BMS and BMS subscores. The improvement in BMS and BMS subscores was first observed at 14 days post-injury and persisted till the end of the experiment (4 w post-injury) (Fig. 6a and b). DigiGait is a test sensitive enough to trace even subtle changes in gait patterns in mice subjected to injury at a thoracic level [4348]. The percentage of stride duration spent in the swing phase was decreased in SCI mice vs. sham control (data not shown), and this parameter was significantly improved in melatonin treatment group (Fig. 6c). Accordingly, the percentage of the stride duration spent in the stance phase was significantly reduced following melatonin treatment compared to SCI group (Fig. 6c). Melatonin treatment resulted in a significant increased swing to stance ratio in SCI mice (Fig. 6c). No significant difference was detected in stride length and stride frequency in SCI vs. SCI+Mel group (Fig. 6d). Additionally, the paw area at peak stance was decreased in SCI group, which was reversed after melatonin treatment (Fig. 6e). To summarize, melatonin treatment can at least partly correct SCI-induced gait abnormalities.

Correlations between bacterial taxa and locomotor recovery/ intestinal barrier permeability/cytokines

We assessed whether the abundances of *Clostridiales, Lactobacillales,* and *Lactobacillus* were linked to locomotor recovery, intestinal integrity or cytokine expressions. A multivariate analysis of variance (MANOVA) was performed with each group as an independent variable, and motor outcomes (BMS scores), gut function (gut microbiota: Clostridiales, Lactobacillales and Lactobacillus; intestinal permeability: FITC-dextran) and cytokine profiles (MCP-1, IL-17, IFN-γ) as dependent variables. Hotelling's Trace revealed a significant multivariate effect of group on corresponding index [$F(24,32) = 14.16$; $p < 0.001$]. Univariate ANOVAs revealed significant effects among different groups on BMS scores [$F(3,19) = 31.79$; $p < 0.001$], Clostridiales [$F(3,19) = 7.59$; $p = 0.002$],

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Lactobacillales [F(3,19) = 8.65; p = 0.001], Lactobacillus [F(3,19) = 8.73; p = 0.001], FITC-dextran [F(3,19) = 5.14; p = 0.009], MCP-1 [F(3,19) = 6.38; p = 0.004], IL-17 [F(3,19) = 3.7; p = 0.03] and IFN-γ [F(3,19) = 3.19; p = 0.047] (Table 1).

As shown in Fig. 7, open field locomotor (BMS) scores were positively correlated with the relative abundance of *Lactobacillales* and *Lactobacillus*; similar results were observed in the relative abundance of *Clostridiales* with FITC-dextran permeability and MCP-1 expression levels. In contrast, inverse relationships were found between BMS scores and the relative abundance of *Clostridiales.* The relative abundances of *Lactobacillus* and *Lactobacillales* negatively correlated with FITC-dextran permeability. In addition, the relative abundances of *Lactobacillales* negatively correlated with MCP-1 expression levels. And, the association between MCP-1 expression levels and the relative abundance of *Lactobacillus* was not statistically significant. It seems the abundances of certain bacteria could predict the functional recovery, barrier integrity and MCP-1 expression after SCI.

Gut dysbiosis impairs locomotor recovery after SCI

Our statistical data showed that intestinal microbiota was closely correlated with functional recovery. To further examine the relationship between the two, antibiotics treatment was used to establish gut dysbiosis in mice (Fig. 8a). Gut dysbiosis was induced prior to SCI by mixing a cocktail of broad-spectrum antibiotics into the drinking water. To confirm the gut bacteria depletion and intestinal microbial disturbance, fecal samples from each group were collected and cultured. The plate culture results indicated that the induced gut dysbiosis persisted for at least 28 dpi. It was also apparent that bacteria were notably absent in feces from mice receiving antibiotics 14 days (Fig. 8b). Although antibiotics were treatment was maintained throughout the experiment, there was an overgrowth of bacteria in antibiotic-treated group after SCI (Fig. 8b), indicating an induced intestinal microbial disturbance which probably due to a proliferation of antibiotic-resistant bacteria. When compared to SCI mice, the decrease in BMS score was observed but the difference was not statistically significant in SCI mice with antibiotic-induced dysbiosis (SCI+AB group) from 14 dpi to 28 dpi (Fig. 8c). When compared to SCI group, the

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BMS subscores were markedly lower at 21 and 28 dpi in SCI+AB group, and the difference mainly lies lied in stepping coordination and trunk instability (Fig. 48 d). These data suggested that gut dysbiosis impaired locomotor recovery after SCI which was consistent with the results of Kigerl et al.[7]. To further examine the effect of antibiotics on intestinal function, weight gain and intestinal barrier integrity were quantified. At 0 dpi, mice in the SCI+AB group had already been dosed with antibiotics for 14 days. Preinjury weights in these mice were not significantly different from SCI mice. Weight gain and intestinal permeability were measured and were indistinguishable between the two groups (Fig. 8e and f).

Microbiota-depletion by antibiotics (gut dysbiosis) partly negated the neuroprotective effect of melatonin in SCI-induced mice.

Data in Figs 1-6 demonstrated that melatonin treatment rescued outcomes locomotor functions and improved gut microbiota in SCI mice. Therefore, we hypothesized that gut microbiota might have mediated the neuroprotective effect of melatonin. Compared to SCI+AB group, melatonin treatment significantly improved BMS and BMS subscores at 14 dpi, and the improvement persisted for at least 28 dpi. And at 21 and 28 dpi, we observed a notable difference between SCI+AB+Mel and SCI+Mel group (Fig. 9a and b). The gait results of SCI+AB+Mel and SCI+Mel mice demonstrated that gut dysbiosis had a marked impact on the swing to stride ratio and paw area (Fig. 9c). The SCI+AB group was omitted for the gait analysis due to the failure to complete the gait test on the running belt (9 m/s). Additionally, the gut permeability was further examined among the three groups. Compared to SCI+AB group, melatonin treatment significantly ameliorated gut dysbiosis-induced hyperpermeability of intestinal barrier after SCI (Fig. 9d). And intestinal permeability was indistinguishable between SCI+AB+Mel and SCI+Mel groups (Fig. 9d). Immunostaining of ZO-1 and occludin showed that in SCI+AB+Mel mice, melatonin treatment significantly upregulated the expression of ZO-1, but not the expression of occludin. Similarly, compare to SCI+AB+Mel mice, both ZO-1 ($p=0.05$) and occludin ($p<0.05$) were significantly increased in SCI+Mel group (Fig. 9e and f). Taken together, melatonin treatment improved locomotor function and intestinal barrier integrity in SCI

mice, but microbiota-depletion by treatment with antibiotics partly negated the protective effect of melatonin.

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Discussion

Gut microbiota is closely correlated with numerous biological functions of CNS and is linked to the pathogenesis of various neurological diseases [15, 4449] Although growing evidences showed that melatonin could re-shape intestinal microbiota in different animal models [31, 3234,35], whether melatonin modulates pathogenesis of SCI through exerting an effect on intestinal microbiota has yet to be investigated. In this study, we examined the effect of melatonin treatment on the gastrointestinal functions following SCI in mice. Melatonin showed a protective effect against injury-induced alterations in intestinal barrier function, gastrointestinal transit, and gut inflammation. In addition, melatonin treatment corrected the gut microbiota disturbance, which was correlated with functional recovery in SCI mice.

Melatonin is an ancient molecule whose origin can be traced back to photosynthetic bacteria and other primitive unicellular organisms [4550]. The initial function of melatonin is to defend oxidative stress as a free radical scavenger, and with evolution, other functions have been adopted [4550]. Melatonin can be produced not only by the pineal gland, but also by gastrointestinal tract at even a much higher level [4651]. In the current study, we found that melatonin treatment significantly ameliorated gastrointestinal dysfunction in SCI models. Improved intestinal barrier integrity was exemplified by reduced gut leaking and restored expression of tight-junction proteins in melatonin-treated mice, consistent with previous studies showing that melatonin can decrease duodenal epithelial paracellular permeability in several animal models [27, 3033]. Other beneficial effects of melatonin, such as improved intestinal peristalsis, and dampened levels of pro-inflammatory cytokines, were also observed, in agreement with the enhanced locomotor function and metabolic rate. In light of the pleiotropic roles of melatonin, we cannot exclude the possibility that melatonin may have direct interaction with intestine cells, and improve the status through classical functions such as the anti-oxidant ones [47-4952-54]. However, an increasingly important

element in regulation of gut functions - intestine microbiota, has been recognized and attracted more interest of researchers.

SCI can alter the composition of gut microbiota, as we have seen in the current study and others [7, 8], which creates a state of “dysbiosis” where the balance between helpful bacteria and pathogenic bacteria is disturbed. Prolonged stress and gastrointestinal dysfunction are common causes of gut bysbiosis [10, 5055]. Emerging data suggested that gut dysbiosis might further affect the intestinal immune function, motility and barrier function [51, 5256,57]. As one of the "oldest" molecules on earth (estimated to exist 2.5-3.5 billion years ago [4550]), melatonin plays critical roles in unicellular and multicellular organisms; yet its functions in gut microbiome have not been explored until recently. Paulose et al. reported that melatonin induces a swarming activity of *Enterobacter aerogenes,* a commensal bacterium from the human gastrointestinal system; and the activity of this bacterium can be synchronized by melatonin in a circadian rhythm [5358]. In another study, Zhu et al. found that melatonin treatment alters the intestinal microbiota in a mouse colitis model [5459]. In our study, administration of melatonin reduced the relative abundance of *Clostridiales* and enhanced those of *Lactobacillales* and *Lactobacillus*.

Clostridiales expresses genes with sequence similarity to the melatonin binding sites in human genome [5358], suggesting a possibly direct regulatory effect of melatonin on this strain of bacteria. Also, gacias et al reported that mice with gut microbiota enriched for the taxa *Clostridiales* show increased anxiety-like behavior, and impaired oligodendrocyte differentiation and myelin gene transcription, highlighting a potential molecular mechanism by which gut microbiota impact CNS homeostasis [5560].

Lactobacillales and *Lactobacillus* belong to the group of lactic acid bacteria that can exhibit health promoting effects on the host. Ren et al [3134] reported that melatonin increases the relative abundance of *Lactobacillus*, and reduces the relative abundance of *Prevotellaceae* in weanling mice. In Ren's study, melatonin treatment also increases the body weight of weanling mice, similar to the finding in this study, indicating that melatonin may promote weight gain possibly through intestinal microbiota, especially *Lactobacillus*. Colonizing of intestinal microbiota to germ-free rats showed that *Lactobacillus* and

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Bifidobacterium can reduce the migrating myoelectric complex period and accelerate small intestinal transit [5661]. In another study, *Lactobacillus* was shown to possess an anti-cancer effect, and this effect is augmented by co-administration of melatonin [5762]. Recently, using a mouse model of SCI, Kigerl et al. reported that sustained delivery of VSL#3, a medical-grade probiotic including *Lactobacillus*, can increase the relative abundance of lactic acid-producing bacteria in the gut, improve immune functions and promote recovery of locomotor function following SCI [58637]. *Lactobacillus* produce neuroactive metabolities and neurotransmitters [59-6164-663-65], which can influence the immune function and CNS function [61-6366-6865-67]. Nevertheless, the exact mechanisms underlying the effect of *Lactobacillales* and *Lactobacillus* still require further study.

Posttraumatic inflammation is a complicated and crucial progress which plays a major role in mediating secondary damages after SCI. Yana and colleges recently reported changes in cytokine profiles up to 14 days post injury both in the serum samples and in the injured spinal cord using a rat contusion model [6968]. Their results echo a previous study conducted by Stammers et al., which showed a similar change of several inflammation cytokines (IL-6, IL-1a, IL-1b, IL-13, MCP-1, MIP1a, RANTES, and TNFa) in the acute SCI model within 24 hr [7069]. The changes in the expression of cytokines in both blood and spinal cord tissues suggested a local and systematic inflammatory reaction. Infiltration of inflammatory cells into secondary organs and persistence of an inflammatory microenvironment may contribute to organ dysfunction[7170]. Considering the increase of inflammatory factors combined with the defected GI barrier throughout the 4w time course, as observed in our study, the inflammation may be considered a persistent effect of the systematic inflammation. However, due to the complexity of this matter, one cannot rule out the possibility that a separate mechanism may underlie the intestinal inflammation, such as neurogenic bowel dysfunction and the imbalance of microbiota [2,7,8].

Melatonin treatment also improved the immune microenvironment after injury. In this study, colons from SCI mice displayed increased levels of IL-1 β , IL-17, IFN- γ , and MCP-1, which represented a pro-inflammatory microenvironment caused by the progression of SCI. With treatment of melatonin, levels of IL-17, IFN- γ , and MCP-1 were significantly reduced, shifting the niche towards an anti-inflammatory one in the colon.

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Previous work showed that MCP-1 level in cerebrospinal fluid (CSF) is significantly increased in SCI patients [647271]. Similar elevation of MCP-1 at protein and mRNA levels in the spinal cord tissues has been observed in rodents with experimental spinal cord contusion [65, 6673,7472,73] and MCP-1 was found negatively associated with behavioral measure of long-term outcome after SCI [647271]. In this study, we found that colonic MCP-1 expression was positively correlated with the relative abundance of *Clostridiales /* intestinal permeability (data not shown) and negatively correlated with those of *Lactobacillales*. There was evidence indicating that the gut microbiota plays an important role in modulating host immune responses [677574]. The data from this study suggested that melatonin might affect immune microenvironment through intestinal microbiota, and the relative abundances of certain bacteria in gut may be used to predict the magnitude of functional recovery following SCI.

Previous studies have shown that gut microbiota are sensitive to melatonin exposure and melatonin treatment markedly reprograms gut microbiota in animal models [34,35,37,7675]. In this study, we demonstrated that melatonin treatment ameliorated improved locomotor recovery and reshaped gut microbiota in SCI models. To investigate whether gut microbiota may have directly contributes contributed to melatonin-induced beneficial potentials, antibiotics were introduced to interfere with gut microbiota. Our data indicated that antibiotic-induced gut dybiosis exacerbated functional recovery. Furthermore, the effect of melatonin treatment on SCI mice was partly abrogated by antibiotics intervention administration. We also observed that SCI led to a decreased concentration of melatonin in intestine, and melatonin treatment by intraperitoneal delivery could directly elevate the melatonin concentration in the GI tract (data not shown). These results suggest that melatonin might have a direct influence on gut microbiota.

However, we could not exclude the other possibility, especially for a compound with such multifaceted properties, that the observed GI improvement may be a result of the neuroprotective effects on spinal cord. one point worth emphasizing is that even though antibiotic disruption of the gut microbiota did reduce the efficacy of the melatonin treatment, melatonin still improved recovery compared to SCI alone. These pieces of evidences indicated that even though some effects of melatonin might be related to gut

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microbiota, melatonin also seemed to be acting independent of the gut microbiota. This puzzle could be further tackled by a relatively more local delivery of melatonin, such as oral gavage or water intake. It is also worth notice that the distribution of melatonin in blood, intestine and the spinal cord after oral administration will provide vital information to help explain the beneficial effect of melatonin. Oral gavage combined with direct interventions such as antibiotics, probiotics as well as fecal microbiota transplantation may be helpful to elucidate the mechanisms of melatonin on GI functions and intestinal microbiota.

Although our study showed that melatonin treatment improved recovery and GI function, there is a lack of evidence to confirm that melatonin acts directly on the GI tract. Melatonin absorbed into the blood influences host metabolism and systemic physiological activity through respective receptors in different tissues and organs. Although melatonin membrane receptors MT1, MT2 and cytosolic MT3 receptor are widely expressed throughout GI tract[76-78], yet we did not detect the binding of melatonin to the receptors in various parts of GI tract. Therefore, we could not exclude the possibility, that the observed GI improvement may be a result of the neuroprotective effects on spinal cord. Our data showed that melatonin might affect gut microbiota. Microbiota metabolisms and neurotransmitters influence the pathological and physiological functions of the CNS through brain-gut axis, which may further shape GI functions. Future studies, metagenome and metabolic profilings in particular, are needed to fully explicate the mechanisms of melatonin on SCI.

Conclusions

Melatonin treatment significantly increased the body weight gain, improved functional recovery, alleviated GI motility and improved intestinal barrier integrity, as well as decreased the pro-inflammatory cytokine expression following injury. Additionally, melatonin administration significantly altered the composition of intestinal microbiota (e.g., increase in the abundance of *Lactobacillus* and *Lactobacillales*, and reduction in the abundance of *Clostriddiales*). Gut dysbiosis induced by antibiotics treatment partly negated the neuroprotective effect of melatonin on SCI. Changes in gut microbiota may be

one of the mechanisms underlying the beneficial effect of melatonin on GI and neurological functions.

Acknowledgements

We thank Prof. Yi Ding and Wei Hua Wang from Center of Pharmaceutical Technology Tsinghua University for the measurement of melatonin concentration by LC-MS technology.

This work was supported by the Special Fund for Basic Scientific Research of Central Public Research Institutes, grant number: 2016cz-1, 2018cz-8, and Beijing Municipal Science and Technology Commission (BSTC, No.Z151100001615055).

Author Disclosure Statement

No competing interests exist.

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Table 1 Univariate comparisons for variables in the MANOVA.

Variable	group						
	Sham	Sham+Mel	SCI	SCI+Mel	F(3,19)	Significance	Partial Eta^2
BMS	9 ± 0	9 ± 0	3.58+0.79	6.58+0.38	31.79	<0.001	0.834
Clostridiales	0.10 ± 0.04	0.11 ± 0.04	0.30 ± 0.04	0.09 ± 0.03	7.59	0.002	0.545
Lactobacillales	0.46 ± 0.07	0.60 ± 0.09	0.10 ± 0.04	0.40 ± 0.08	8.65	0.001	0.578
Lactobacillus	0.46 ± 0.07	0.60 ± 0.09	0.10 ± 0.04	0.40 ± 0.08	8.73	0.001	0.58
FITC-dextran	4.26 ± 0.83	4.99 ± 0.84	9.88 ± 1.79	5.35 ± 0.54	5.14	0.009	0.448
MCP-1	53.24 ± 1.26	57.73 ± 1.61	63.39 ± 2.05	57.02 ± 1.36	6.38	0.004	0.502
IL-17	4.21 ± 0.20	4.59 ± 0.14	5.09 ± 0.10	4.31 ± 0.31	3.7	0.03	0.369
IFN-γ	8.94 ± 0.31	9.15 ± 0.49	10.61 ± 0.30	9.18 ± 0.54	3.19	0.047	0.336

MANOVA (Hotelling's Trace): [$F(24,32) = 14.16$; $p < 0.001$]

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Figure legends

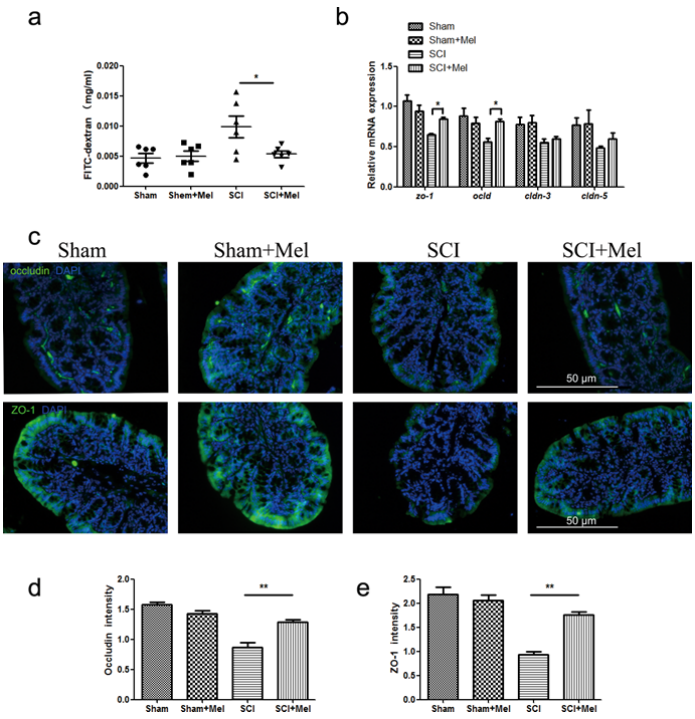

FIG. 1. melatonin treatment alleviates GI Barrier Defects and Abnormal Expression of Tight Junction Components. **a** Intestinal permeability was assessed 4 weeks following injury by measuring FITC intensity in serum after oral gavage of FITC-dextran. **b** The expression levels of ZO-1, occludin, claudin3 and claudin5 were examined in colon tissues by quantitative PCR. **c** Quantification of ZO-1 immunoreactivity (green) with representative immunofluorescence microscope images of murine colon. **d, e** Quantification of occludin immunoreactivity (green) with representative immunofluorescence microscope images of murine colon. DAPI: blue. (Ocld, occludin; ZO-1, Zonula Occludins; Cldn-3, claudin 3; Cldn-5, claudin 5; DAPI, 4', 6-diamidino-2-phenylindole) *p<0.05 compared to SCI group; **p<0.01 compared to SCI group.

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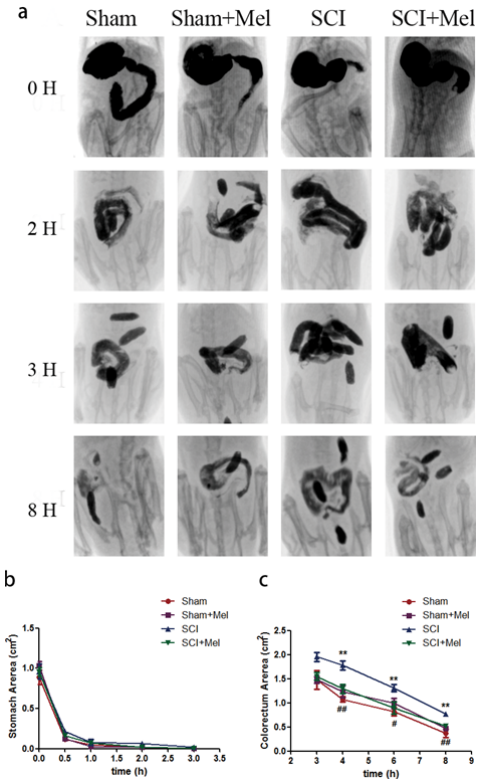

FIG. 2. Melatonin treatment modulates gastrointestinal motility in SCI mice. **a** Representative images of Sham, Sham+Mel, SCI, and SCI+Mel groups at 0, 2, 3 and 8 h after administration of the barium. **b, c** Stomach size (**b**) and colorectum size (**c**) were determined with Image J. **p<0.01 compared to Sham group. #p<0.05 compared to SCI group; ##p<0.01 compared to SCI group.

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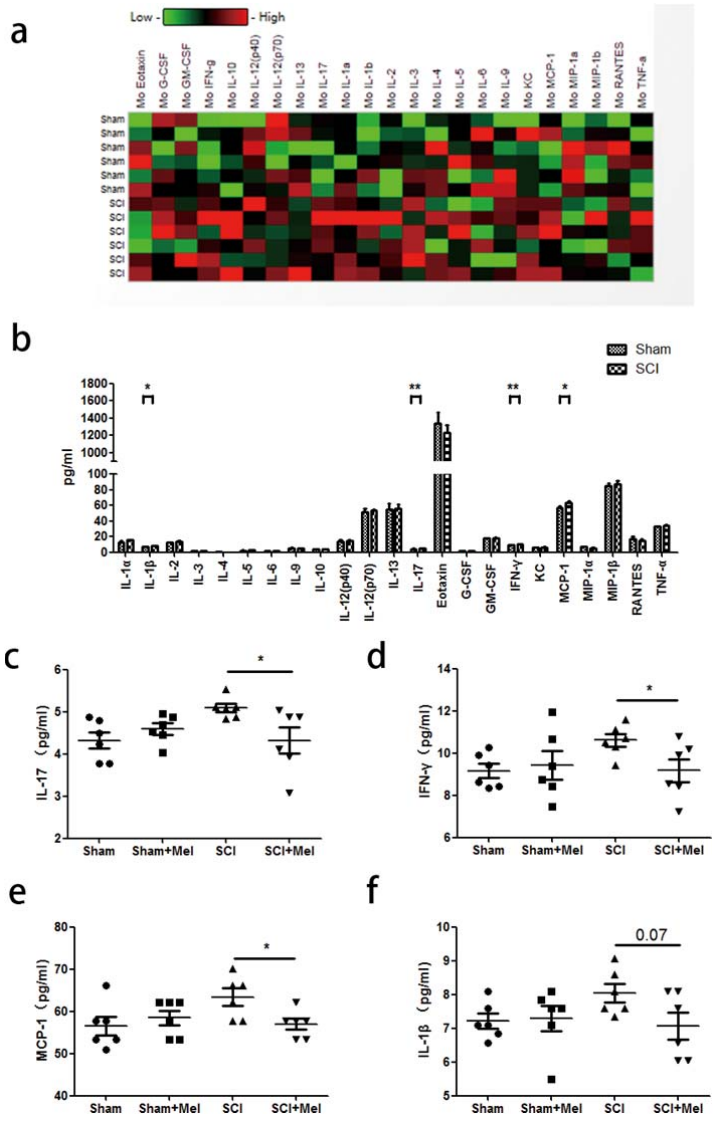

FIG. 3. Melatonin treatment inhibits the expression of pro-inflammatory cytokines. **a** The heatmap showing the colonic protein levels of cytokines between sham and SCI groups. **b** Quantitative analyses of 23 cytokines in the colons were performed by multiplex enzyme immunosorbent assay. **c-f** Quantitative analyses of IL-17 (**c**), IFN-γ (**d**), MCP-1 (e) and IL-1β (**f**) levels among different groups. *p<0.05 compared to SCI group; **p<0.01 compared to SCI group.

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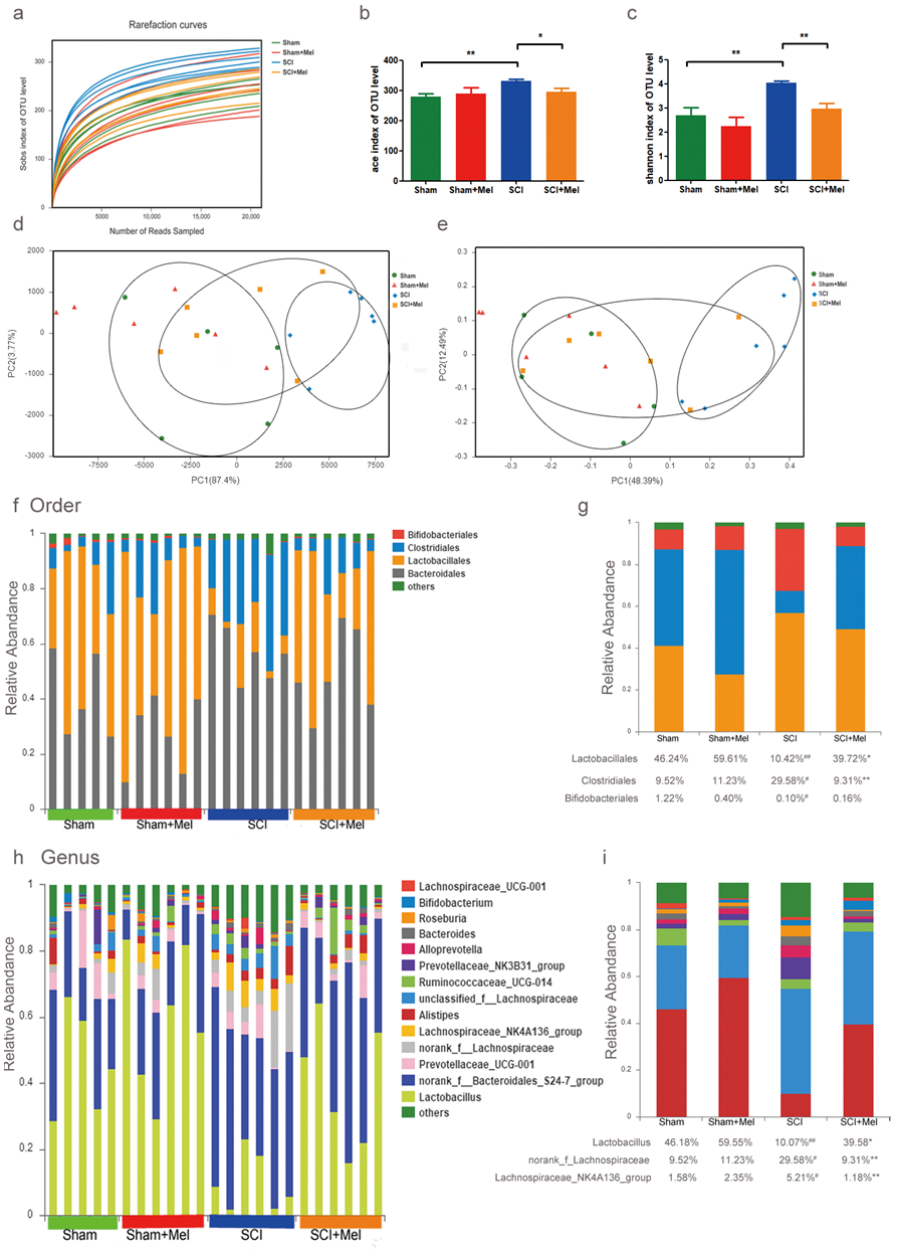

FIG. 4. Melatonin treatment reverses alterations in gut bacterial composition after SCI. **a** Rarefaction curves for OTU levels in the four groups. **b, c** Comparison of the diversity (assessed by shannon index) and the richness (assessed by ace index) based on the OUT levels in the four groups. **d, e** Scatter plots of principal component analysis (PCA) and principal coordinate analysis (PCoA) scores showing the similarity of the bacterial communities based on the Bray Curtis distance. **f** Bacterial composition of the different communities at the order level. **g** Quantitative analyses of the relative abundances of

Lactobacillales, Clostridiales and *Bifidobacteriales* among different groups. **h** Bacterial composition of the different communities at genus level. **i** Quantitative analyses of the relative abundances of *Lactobacillus*, *Lachnospiraceae_NK4A136_group*, and *unclassified_f__Lachnospiracea* among different groups.

#p<0.05 compared to Sham group; ##p<0.01 compared to Sham group; *p<0.05 compared to SCI group; **p<0.01 compared to SCI group.

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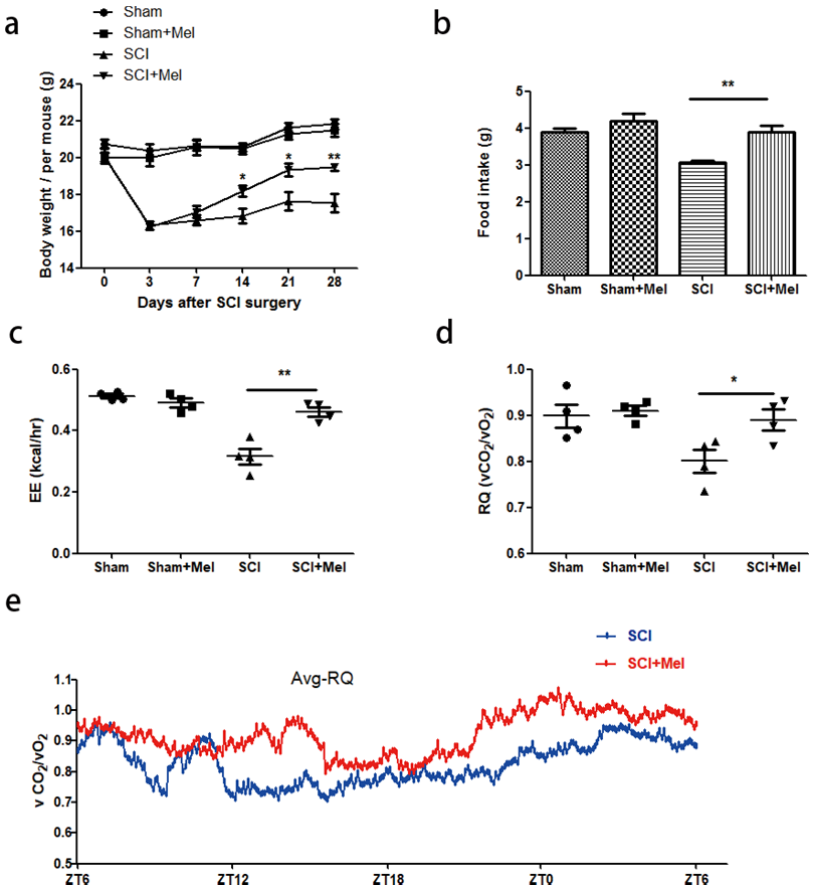

FIG. 5. Body weight, food intake and metabolism in the four groups. **a** Changes in body weight during the 4 weeks in Sham, Sham+Mel, SCI, and SCI+Mel groups. b-e Cumulative food intake for 24 hours (**b**), energy expenditure (EE) (**c**) and respiratory quotient (RQ) (**d**) in all groups were measured at 4 w. (**e**) The mean respiratory quotient (Avg_RQ) every 5 min during 24 h for SCI group and SCI+Mel group. *p<0.05 compared to SCI group; **p<0.01 compared to SCI group.

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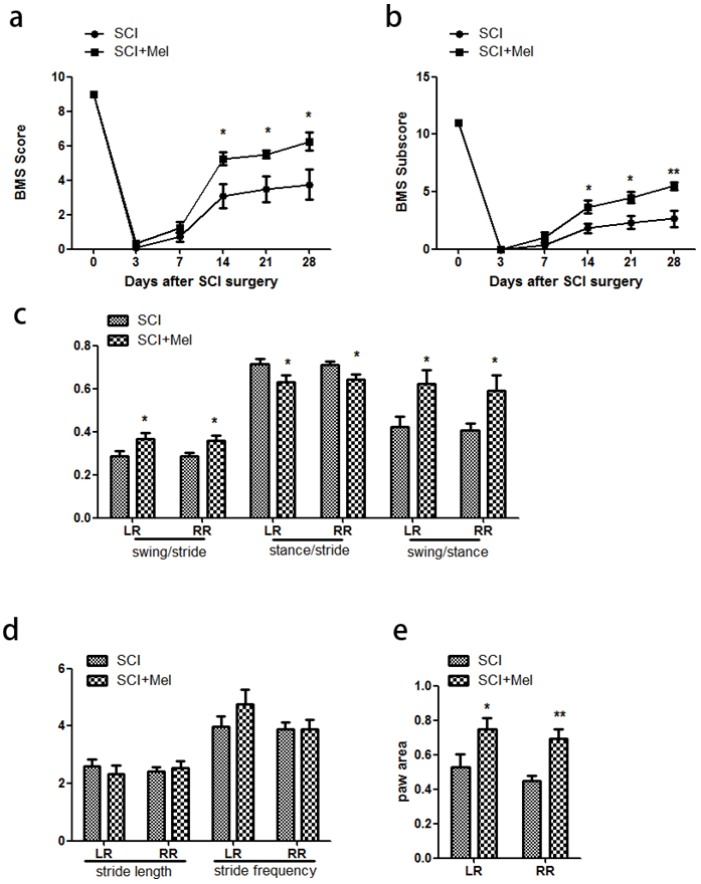

FIG. 6. Locomotor recovery is improved with melatonin treatment. **a, b** Time course of locomotor functional recovery assessed by BMS (**a**) and BMS subscore (**b**). **c** Gait analyzed using an automated treadmill (DigiGait). **d** swing to stride ratio, stance to stride ratio, and swing to stance ratio. **e** Stride length and stride frequency. **f** Paw areas. *p<0.05 compared to SCI group, **p<0.01 compared to SCI group. (LR: left rear; RR: right rear)

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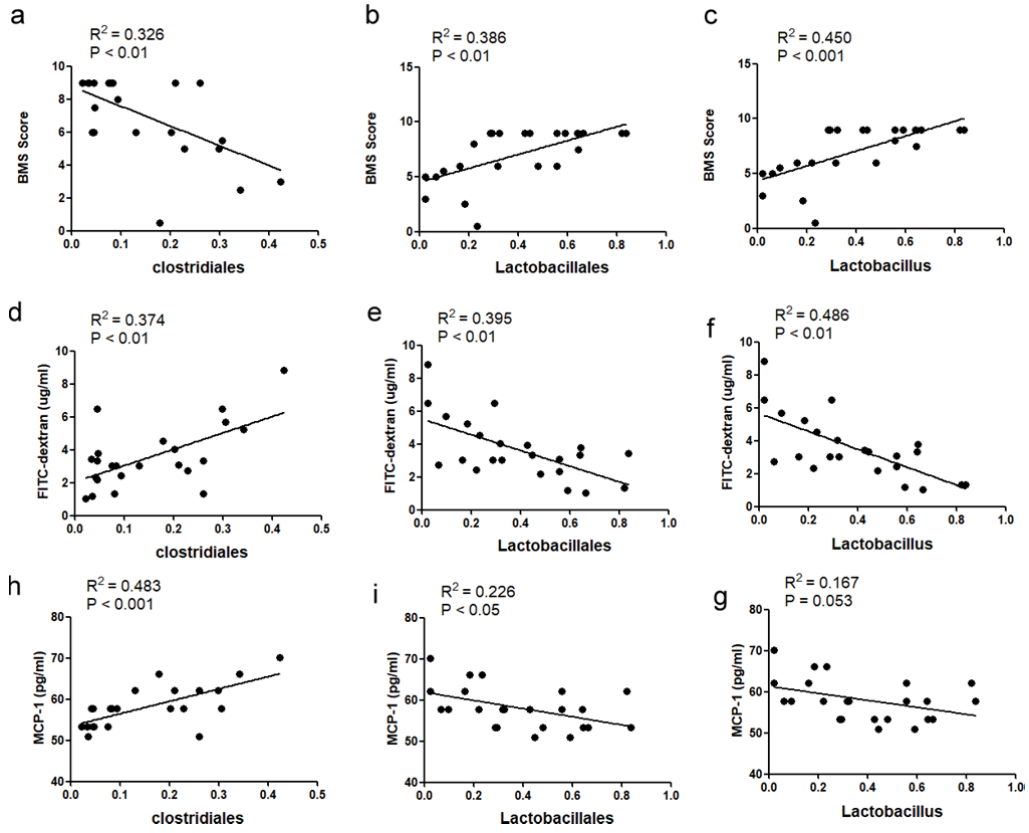

FIG. 7. Correlations between bacterial taxa and BMS scores/FITC-dextran permeability/MCP-1 expression. **a-c** Correlations between BMS scores and the relative abundances of *Clostridiales* (**a**), *Lactobacillales* (**b**) and *Lactobacillus* (**c**). **d-f** Correlations between FITC-dextran permeability and the relative abundances of *Clostridiales* (**d**), *Lactobacillales* (**e**) and *Lactobacillus* (**f**). **h-g** Correlations between MCP-1 expression levels and the relative abundances of *Clostridiales* (**h**), *Lactobacillales* (**i**) and *Lactobacillus* (**g**).

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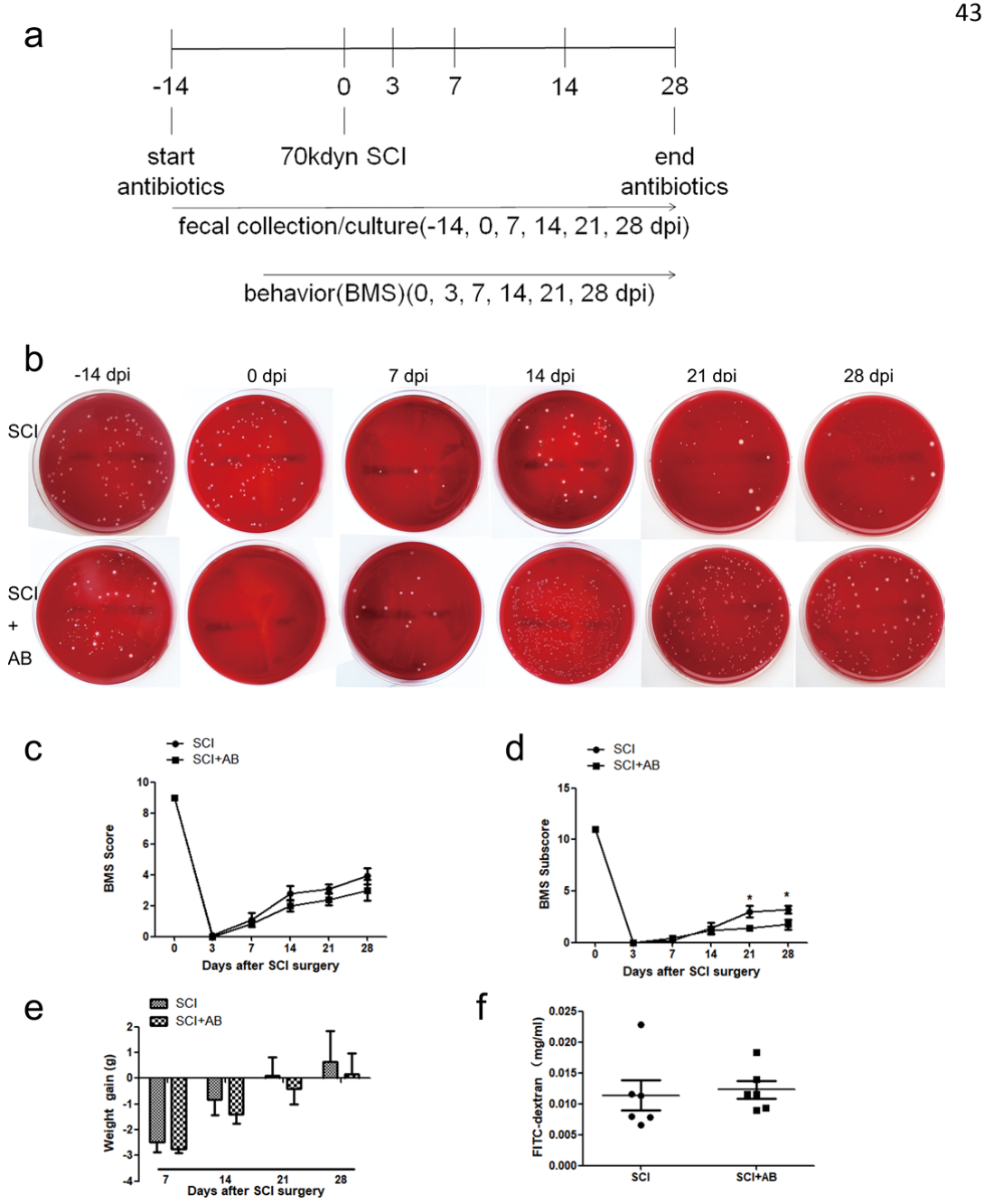

FIG. 8. Gut dysbiosis has a negative effect on locomotor recovery after SCI. **a** Experimental layout. **b** Fecal cultures from SCI mice with or without antibiotics show altered gut microbiota. Time course of locomotor functional recovery assessed by BMS (**c**) and BMS subscore (**d**). **e** Weight gain of SCI mice with or without antibiotics treatment at different time point. **f** Intestinal permeability was assessed 4 weeks following injury by measuring FITC intensity in serum after oral gavage of FITC-dextran.*p<0.05 compared to SCI group. (SCI+AB: SCI mice with antibiotics treatment)

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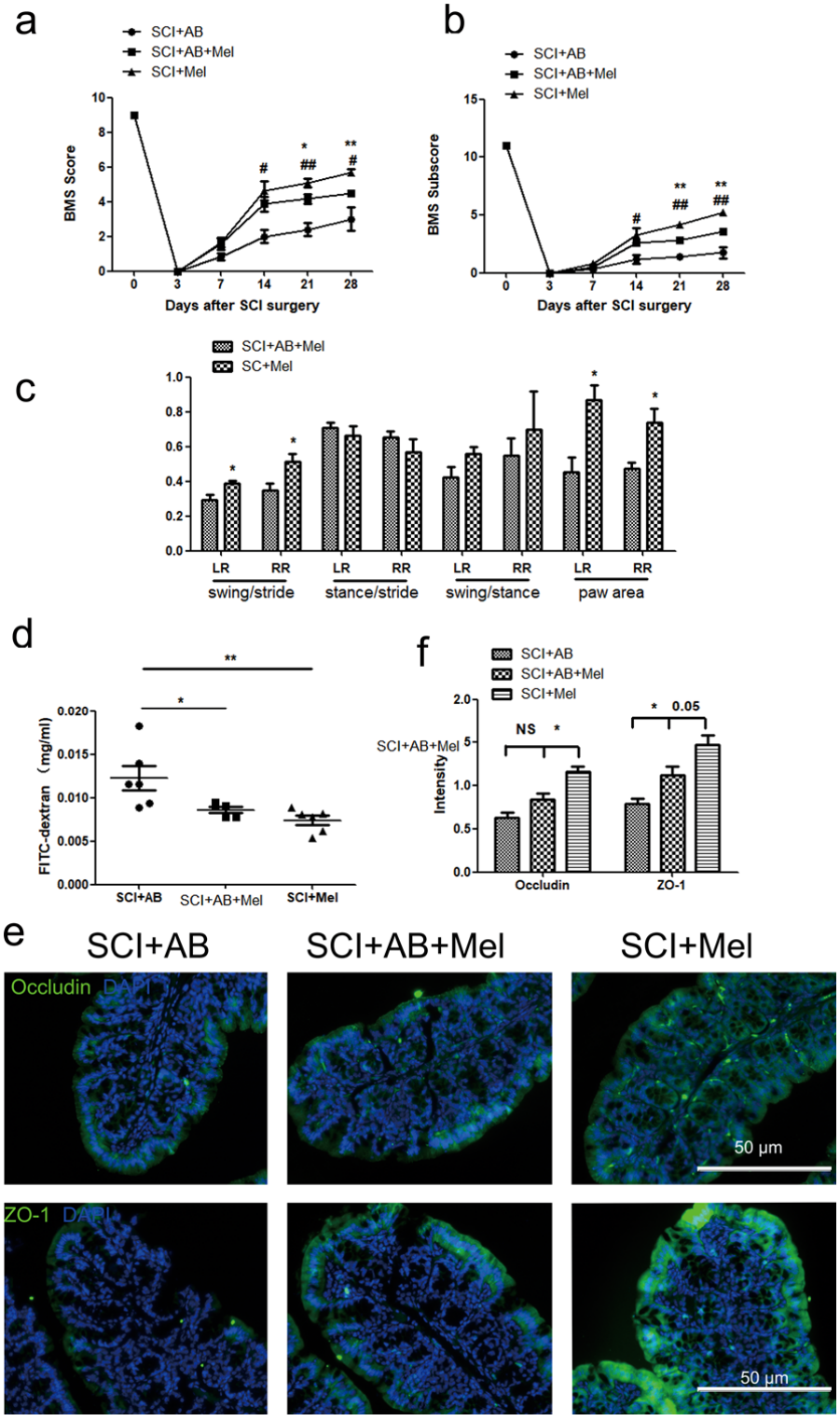

FIG. 9. Melatonin ameliorated dysfunction exacerbated by gut dysbiosis. Time course of locomotor functional recovery assessed by BMS (**a**) and BMS subsore (**b**). #p<0.05 SCI+AB VS SCI+AB+Mel; ##p<0.01 SCI+AB VS SCI+AB+Mel; *p<0.05 SCI+AB+Mel VS SCI+Mel;

p<0.01 SCI+AB+Mel VS SCI+Mel. **c Gait analysis using an automated treadmill (DigiGait). (Indexes: swing to stride ratio, stance to stride ratio, swing to stance ratio and paw area). *p<0.05 SCI+AB+Mel VS SCI+Mel. **d** Intestinal permeability was assessed 4 weeks following injury by measuring FITC intensity in serum after oral gavage of FITC-dextran. **e**, **f** Quantification of Occludin and ZO-1 immunoreactivity (green) with representative immunofluorescence microscope images of murine colon.

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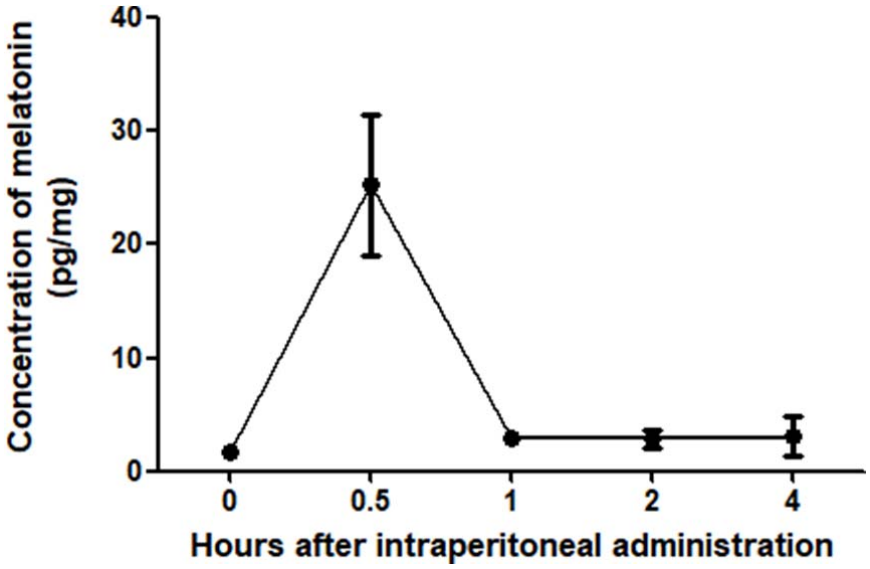

FIG. S1. melatonin concentration in the colonic tissue was measured at different time point namely 0 h, 0.5 h, 1 h, 2 h, and 4 h after intraperitoneal injection of melatonin. Concentration of melatonin in colonic tissue at 0, 0.5, 1, 2 and 4 h after melatonin administration intraperitoneally (10 mg/kg) (n = 4) was 1.76 ± 0.62; 25.28 ± 6.20; 2.85 ± 0.63; 2.88 ± 0.81; 3.10 ± 1.71 pg/mg, respectively.