




ORIGINAL ARTICLE

Functional Recovery in Spinal Cord-Injured Rats Treated with Melatonin-Chitosan Hydrogel: A Behavioral Study

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ABSTRACT

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Spinal cord injury (SCI) leads to the loss of neural and glial cells, along with significant neuroinflammation. Hydrogel formulation, designed for sustained melatonin release, has shown promising therapeutic potentials. In this study, SCI was induced in rats via T9 vertebral contusion, and the CH/Mel hydrogel was characterized using SEM and FTIR. SEM imaging confirmed the hydrogel porous structure. Twenty male Wistar rats were divided into five groups (n = 4 per group): SHAM group with no surgery. SCI group: SCI-induced rats were treated locally with 100 µl CH hydrogel as a control group. SCI/50 group: SCI-induced rats were treated locally with 100 µl CH hydrogel containing 50 mg/kg melatonin. SCI/100 group: SCI-induced rats were treated locally with 100 µl CH hydrogel containing 100 mg/kg melatonin. SCI/200 group: SCI-induced rats were treated locally with 100 µl CH hydrogel containing 200 mg/kg melatonin. *In vitro* tests revealed that 25 mg/ml CH/Mel hydrogel significantly boosted U87 cell viability after 24 hours. Behavioral assessments demonstrated that CH/Mel hydrogel treatment markedly improved functional recovery in SCI rats, supporting its therapeutic efficacy. The use of CH/Mel hydrogel was shown to effectively enhance the recovery of rats with SCI indicated by improving behavioral assessments.


Introduction

Restoring spinal cord function after traumatic injury represents one of the most critical challenges in modern regenerative medicine. Traumatic spinal cord injury (SCI) causes severe disability, with global incidence rates ranging from 10.4 to 83 cases per million people annually. The financial burden is staggering, with lifetime healthcare costs plus significant social impacts that are difficult to quantify.^{1,2}

Following SCI, the initial mechanical trauma triggers a cascade of secondary pathological events that exacerbate tissue damage. This secondary phase, characterized by inflammation, ischemia, vascular dysfunction, reactive oxygen species (ROS) production, and neural dysregulation, drives progressive cell death and creates a hostile microenvironment that significantly hinders functional recovery.^{3,4}

Oxidative stress, marked by free radical generation and lipid peroxidation (LPO), represents a hallmark of secondary SCI pathology. The oxidative stress cascade has become a major therapeutic target in SCI management, as its inhibition may prevent progressive tissue damage and improve outcomes.^{5,6}

SCI triggers the release of proinflammatory cytokines (IL-1 α / β , TNF- α , and IL-6) that mediate immune and vascular dysfunction. These cytokines activate the NF- κ B pathway, which amplifies inflammation through coordinated production of reactive oxygen species (ROS) and prostaglandins, exacerbating secondary tissue damage.⁷⁻⁹ SCI treatment remains challenging due to complex pathophysiology and limited therapeutic options. Current strategies focus on two complementary approaches: Neuroprotection to prevent secondary damage in acute

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phases, and neural regeneration to restore function during chronic stages. While neuroprotective interventions target early pathological cascades, regenerative therapies aim to repair damaged neural circuits in later phases.^{10,11}

Hydrogels are three-dimensional networks of hydrophilic polymers capable of retaining large amounts of water. These versatile biomaterials have gained prominence in biomedical applications due to their tunable physical-chemical properties and biocompatibility. Notably, hydrogels can reduce glial scar formation while serving as effective delivery systems for drugs, cells, and bioactive molecules to promote spinal cord repair. Among them, natural hydrogels such as chitosan offer distinct advantages, including biodegradability, low toxicity, cost-effectiveness, and sustainable sourcing, making them particularly attractive for clinical translation.¹²⁻¹⁴

Chitosan is a cationic polysaccharide copolymer derived from chitin deacetylation, consisting of N-acetyl-D-glucosamine and D-glucosamine units. Its exceptional biocompatibility and low toxicity make it an attractive biomaterial for diverse medical applications. These favorable properties have enabled the widespread use of chitosan and its derivatives in various formulations, including nanoparticles, fibers, films, and hydrogels.^{15,16}

Melatonin (N-acetyl-5-methoxytryptamine) is primarily synthesized in the pineal gland through tryptophan metabolism, with secondary production occurring in extra-pineal sites including the retina, Harderian gland, gut, and immune cells. This pleiotropic molecule exhibits potent neuroprotective effects, functioning as both a direct free radical scavenger and an indirect antioxidant through enzyme activation. Its multimodal action includes neutralization of reactive oxygen (ROS) and nitrogen species (RNS), coupled with significant anti-inflammatory properties.^{17,18}

This study investigated the neuroprotective and regenerative potential of a melatonin-loaded chitosan hydrogel (CH/Mel) in a rat model of spinal cord injury (SCI). We aimed to characterize the physicochemical properties of the CH/Mel composite, evaluate its therapeutic effects on functional recovery through behavioral assessments. Using a controlled contusion model at the T9 vertebral level, we systematically compared multiple melatonin dosages (50, 100, and 200 mg/kg) delivered via chitosan hydrogel against control groups. The study design incorporated both *in vitro* analyses of cellular protection and *in vivo* assessments of locomotor recovery to comprehensively evaluate this combinatorial therapeutic approach for SCI management.

Materials and Methods

All chemicals and reagents were obtained from commercial suppliers and used without further

purification unless specified. Chitosan with a deacetylation degree of around 75-85%, melatonin and MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] were purchased from Sigma-Aldrich. The DMEM cell culture media, antibiotics (penicillin-streptomycin) and FBS were obtained from Biowest, company.

Fabrication of Melatonin-Containing Chitosan (M/CH) Hydrogel

Chitosan (CH) was dissolved in a 2% (v/v) acetic acid solution with a concentration of 4% (w/v) while being stirred overnight. Melatonin was dissolved in ethanol (EtOH) and then mixed with the chitosan solution. The resulting mixture was placed into 24-well plates, frozen and lyophilized at -80 °C for 48 hours. Solutions were prepared to achieve a ratio of 1:100 (w/w) for melatonin and chitosan, respectively.^{19,20}

Investigation of Electrostatic Interactions

The molecular interactions between chitosan (CH) and melatonin within the hydrogel formulation were characterized using Fourier transform infrared spectroscopy. Spectral analysis was performed across the 400-4000/cm range with 4/cm resolution. This analytical approach enabled precise identification of characteristic functional groups and detection of potential electrostatic interactions between the amino groups of chitosan and the indole moiety of melatonin through comparative analysis of peak shifts and intensity variations in the infrared spectra. The FTIR measurements were conducted under controlled ambient conditions (25 °C, 45% relative humidity) with background subtraction performed prior to sample analysis to eliminate atmospheric interference.

Morphological study

The structure of the synthesized hydrogels was examined using scanning electron microscopy (SEM, KYKY-2800; China) with an electron acceleration voltage of 20 kV. Prior to imaging, the samples were prepared via sputter coating, where a thin gold layer was deposited using an automated fine coater. The coated specimens were then analyzed under SEM to obtain high-resolution micrographs.

Induction of SCI Model and Grouping

In this study, we used male Wistar rats (250–280 g), and all animal procedures strictly followed the guidelines approved by the Ethics Committee of Islamic Azad University, Tehran, Iran (Ethics Code: IR.IAU.SRB.REC.). Twenty rats were randomly assigned to five groups (n = 4 per group): (1) SHAM group (no surgery), (2) SCI group (SCI-induced rats treated locally with 100 µl CH hydrogel

as control), (3) SCI/50 group (SCI-induced rats treated with 100 μ l CH hydrogel containing 50 mg/kg melatonin), (4) SCI/100 group (SCI-induced rats treated with 100 μ l CH hydrogel containing 100 mg/kg melatonin), and (5) SCI/200 group (SCI-induced rats treated with 100 μ l CH hydrogel containing 200 mg/kg melatonin).

Prior to surgery, the rats were anesthetized via intraperitoneal injection of xylazine hydrochloride 2% (5 mg/kg) and ketamine hydrochloride 10% (80 mg/kg) (both from Alfasan International, Woerden, Holland). A contusive spinal cord injury (SCI) model was induced using an NYU impactor apparatus. After shaving and disinfecting the skin over the 9th thoracic vertebra (T9), the underlying connective tissue was carefully dissected, and the T9 lamina was removed with a dental drill to expose the spinal cord. The rats were secured in a prone position, with the T8 and T10 vertebrae clamped to stabilize the spine. A 10 g weight was then dropped from a 25 cm height onto the exposed spinal cord to induce contusion.¹⁸ Successful model induction was confirmed by tail flutter reflexes and hind limb retraction immediately after impact.

Cell Viability Assay

The biocompatibility of CH/Mel hydrogels was evaluated indirectly using the MTT assay. The U87 glioblastoma cell line, obtained from the Pasteur Institute (Tehran, Iran), was cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a 5% CO₂ incubator. Cells were seeded in a 96-well plate (10⁴ cells/well) and allowed to adhere for 24 hours. After incubation, the media was replaced with fresh media containing different concentrations of melatonin (12.5, 25, and 50 mg/ml). Following 24, 48, and 72 hours of exposure, the media was removed, and MTT solution (5 mg/ml in PBS) was added to each well for 4 hours. Subsequently, the resulting formazan crystals were dissolved in DMSO, and the optical density (OD) was measured at 570 nm using an ELISA reader.

Behavioral Assessments

To evaluate functional recovery following SCI, open-field locomotor performance was assessed using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale, a well-validated measure of hind limb motor function in rodents. Testing was conducted once per week for the first four weeks post-injury (Figure 1).²¹ To ensure objective scoring, all animals were randomly coded, and behavioral evaluations were performed independently by two trained investigators who were blinded to treatment group assignments throughout the study period. Each testing session involved placing rats in an open-field arena and systematically scoring their hind limb movements, weight support, coordination, and stepping patterns according to the standardized BBB scale criteria

(0 = no observable hind limb movement to 21 = normal locomotion). Following each assessment, BBB scores were averaged across investigators for each animal, then group means were calculated for statistical analysis. The resulting data were plotted to visualize temporal recovery trajectories, with mean BBB scores presented as a function of time post-injury to compare therapeutic outcomes between experimental groups. This rigorous longitudinal assessment protocol allowed for sensitive detection of both early-phase spontaneous recovery and late-phase treatment effects on locomotor function.²¹ At the end of the study, the rats were euthanized using carbon dioxide (CO₂), transcardially perfused with a fixative containing 2% paraformaldehyde and 1% glutaraldehyde buffer (pH = 7.4).

Statistical Analysis

Statistical analyses were performed using PASW 24.0 (SPSS Inc., Chicago, IL, USA). Longitudinal changes in BBB locomotor scores across the four experimental groups were statistically analyzed using a two-way repeated measures ANOVA, with treatment group as the between-subjects factor and time as the within-subjects repeated measure. This analysis allowed simultaneous evaluation of both between-group differences (treatment effects) and within-group changes over time (recovery patterns). Following significant ANOVA results ($p < 0.05$), post hoc comparisons were conducted using Tukey's honestly significant difference (HSD) tests to control for multiple comparisons. These tests specifically examined: (1) inter-group differences at each timepoint (8 post-injury weeks), and (2) intra-group temporal changes across the recovery period. All statistical tests maintained a pre-determined significance threshold of $\alpha = 0.05$, with exact p-values reported for transparency. Effect sizes (η^2 for ANOVA, Cohen's d for pairwise comparisons) were calculated to assess the magnitude of observed differences beyond mere statistical significance.

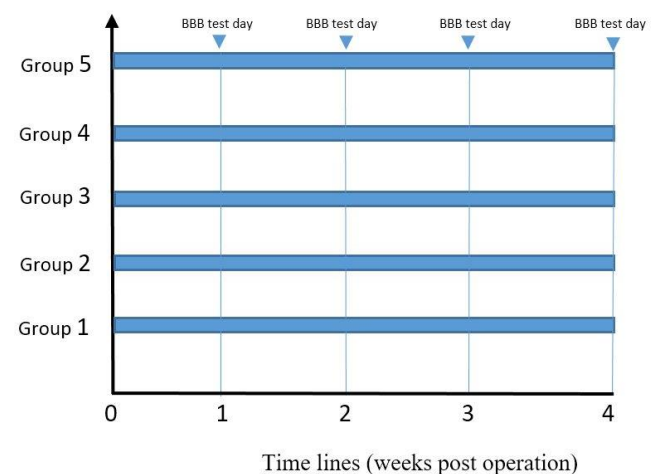


Figure 1. The time line of the BBB test performed on four time points post operation.

Results

Characterization of Melatonin-Containing Chitosan Hydrogel

The FTIR spectrum of chitosan (Figure 2) displayed characteristic absorption bands confirming its molecular structure. A broad hydroxyl (-OH) stretching vibration appeared at 3439/cm, while the free amino (-NH₂) group was identified at 1160/cm. The spectrum revealed C-H stretching vibrations appearing as a broad band between 3400-2500/cm, with specific asymmetric stretching observed at 2872/cm. Characteristic peaks at 1658/cm (amide I, C=O stretch) and 1323/cm (amide III, C-N stretch) confirmed the presence of residual N-acetyl groups. The polysaccharide backbone was evidenced by strong absorptions between 1200-800/cm, corresponding to glycosidic bond vibrations and C-O/C-O-C stretching, with specific peaks at 1020/cm (C-O) and 1150/cm (asymmetric C-O-C bridge stretching). Melatonin's FTIR spectrum showed distinctive peaks at 3325/cm (N-H bending), 3160/cm (C-N stretching), 1611/cm (C=O), and aromatic C=C stretches at 1563/cm and 1439/cm. The CSNPs spectrum exhibited a broadened -OH stretch around 3400/cm, suggesting nanoparticle formation, while spectral differences between Mel-CSNPs and CSNPs confirmed successful melatonin encapsulation.

SEM analysis of the freeze-dried CH/Mel hydrogel cross-section (Figure 1) revealed a highly porous, interconnected network structure. This three-dimensional architecture, characterized by uniform pore distribution and optimal pore size, appeared ideal for facilitating nutrient transport and metabolite exchange. The observed morphology suggests the hydrogel provides an excellent scaffold for cell adhesion, proliferation, and extracellular matrix deposition, making it particularly suitable for tissue engineering applications. The porous structure's integrity and interconnectivity were maintained throughout the sample, indicating good structural stability of the CH/Mel composite.

Cell Viability

The MTT assay results demonstrated time- and dose-dependent effects of CH/Mel hydrogels on cell viability. After 24 hours of exposure, only the 25 mg/ml CH/Mel treatment showed a statistically significant increase in cell viability compared to controls ($p < 0.01$, Figure 3A). While Group 5 (200 mg/ml) exhibited the highest numerical cell survival rates at both 48 and 72 hours, these differences did not achieve statistical significance relative to the control group (Figure 3B and 3C). Interestingly, at the 72-hour time point, the 100 mg/ml dose group showed significantly greater cell survival compared to the 200 mg/ml group ($p < 0.05$, Figure 3C),

suggesting a potential biphasic response where higher concentrations may become less beneficial or even slightly cytotoxic over extended exposure periods.

Findings of Behavioral Assessments Using BBB Scores

The functional recovery analysis demonstrated that Group 5 (SCI/200) exhibited significantly greater improvement ($p < 0.01$) in locomotor function compared to all other treatment groups, as assessed by the BBB locomotor rating scale over the 9-week study period. While Groups 3 (SCI/50) and 4 (SCI/100) showed moderate recovery, only the highest melatonin dose (200 mg/kg) in Group 5 resulted in statistically superior outcomes. This dose-dependent therapeutic effect was further supported by earlier recovery onset in Group 5, with significant improvement versus controls first observed at Week 3 ($p < 0.05$). In contrast, the SHAM group maintained normal locomotor function throughout, while the SCI control group showed limited spontaneous recovery (Figure 4). These findings indicated that 200 mg/kg melatonin in CH hydrogel produced optimal neurorestorative effects after SCI, with significantly better outcomes than lower doses.

Discussion

Spinal cord injury (SCI) represents a devastating neurological disorder characterized by traumatic damage to the central nervous system (CNS), most commonly caused by external physical trauma such as vehicular accidents, falls, or sports injuries. This condition leads to severe and often permanent neurological deficits, including motor paralysis, sensory loss, and autonomic dysfunction below the level of injury. The pathophysiology involves immediate mechanical damage

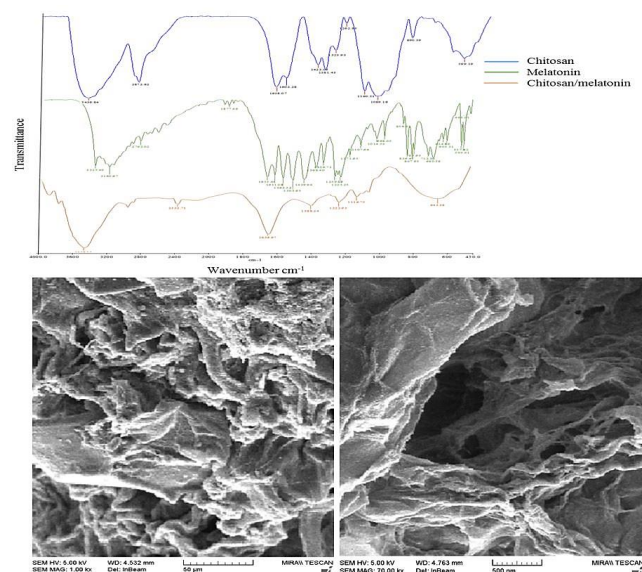


Figure 2. (Up) FTIR spectra of the melatonin, chitosan, and the final formulation (CH/Mel hydrogel). (Down) scanning electron microscopy (SEM) micrograph of CH/Mel hydrogel.

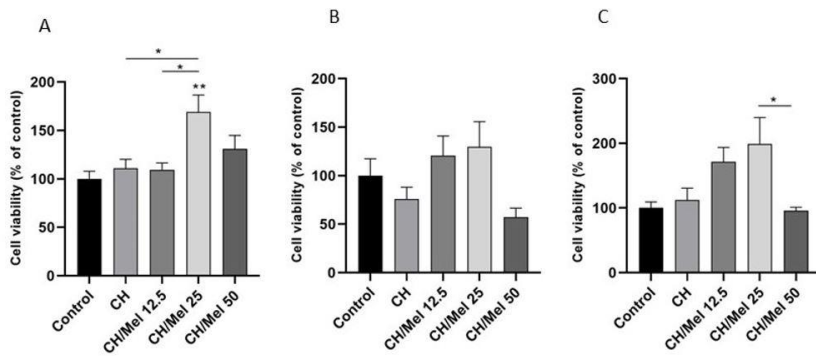


Figure 3. The CH/Mel hydrogel effect on U87 cell viability. Cells were seeded at 10^4 /well in 96-well plates in DMEM media and treated with Chitosan (CH) and increasing concentrations of CH/Mel hydrogel (12.5,25, 50 mg/ml Melatonin) for 24 (A), 48 (B) and 72 h (C). Cell viability was assessed using the MTT test following the protocol outlined in the Materials and Methods section. The percentage of viable cells was determined in relation to the control group. Statistical significance was indicated by * $p < 0.01$ and ** $p < 0.01$ compared to the control group. The values are expressed ad Mean \pm SEM.

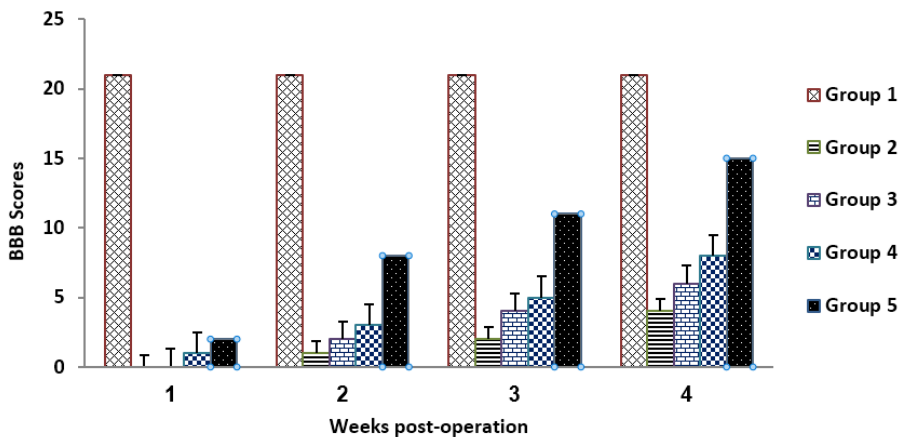


Figure 4. BBB scores in the experimental groups within 4 weeks after surgery. * the hydrogel containing 200 mg/kg melatonin (Group 5) showed a significant improvement in the motor behavior of mice ($p < 0.01$) compared to the other treatment groups. The values are expressed ad Mean \pm SEM.

followed by complex secondary injury cascades, resulting in neuronal cell death, axonal degeneration, demyelination, and disruption of neural pathways. These pathological changes frequently lead to irreversible functional impairments, with patients experiencing varying degrees of disability depending on the injury severity and location. The lack of effective regenerative capacity in the adult mammalian spinal cord makes SCI particularly challenging to treat, often resulting in lifelong disability and significant reduction in quality of life.²² Current clinical management of SCI primarily involves acute interventions focused on minimizing secondary damage and stabilizing patients. The standard therapeutic approach combines surgical decompression to relieve pressure on the spinal cord with mechanical stabilization of vertebral fractures. Pharmacologically, high-dose methylprednisolone (a corticosteroid) may be administered within 8 hours post-injury to potentially reduce inflammation, though its efficacy remains controversial due to limited clinical benefits and significant side effects. Vasopressor therapy, typically using norepinephrine or phenylephrine, is employed to maintain mean arterial pressure (85-90 mmHg for 5-7 days) and optimize spinal cord perfusion. Despite these

interventions, existing treatments demonstrate limited effectiveness in promoting neural regeneration, highlighting the critical need for novel neuroprotective and neuroregenerative strategies to improve functional recovery.²³

Despite these clinical interventions, current therapeutic approaches fail to address the fundamental challenge of neural regeneration in spinal cord injury. While surgical decompression and pharmacological management can stabilize the injury site and mitigate secondary damage, they are incapable of restoring disrupted neural circuits or promoting axonal regrowth across the lesion. This critical limitation stems from the CNS's inherently poor regenerative capacity, compounded by the inhibitory microenvironment created by glial scarring and myelin-associated inhibitors. Consequently, most patients experience permanent neurological deficits, underscoring the urgent need for regenerative therapies that can overcome these biological barriers and facilitate true structural and functional recovery.²³

Chitosan-based hydrogels offer several advantageous properties that address key limitations of current SCI treatments. Their unique combination of

biocompatibility, tunable mechanical strength, and controllable degradation kinetics makes them particularly suitable for neural tissue applications. The material interconnected porous structure (typically ranging 50-200 μm in diameter) facilitates nutrient diffusion and cellular infiltration while providing optimal surface area for cell-matrix interactions. Unlike conventional surgical or pharmacological approaches, these hydrogels can serve as: 3D scaffolds to bridge lesion cavities, delivery vehicles for therapeutic agents and permissive microenvironments for axonal regrowth. Their ability to modulate inflammation and reduce glial scar formation further positions chitosan hydrogels as a promising multimodal platform that may overcome the regenerative barriers inherent to spinal cord repair.²¹⁻²⁴

Preclinical studies have demonstrated melatonin's significant neuroprotective potential in spinal cord injury models, showing promising effects on both structural preservation and functional recovery. The indoleamine exerts its therapeutic benefits through multiple synergistic mechanisms: Potent antioxidant activity via direct free radical scavenging and upregulation of endogenous antioxidant systems, suppression of pro-inflammatory cytokines (TNF- α , IL-6, IL-1 β) through NF- κB pathway inhibition; anti-apoptotic effects mediated by reduced caspase-3 activation and mitochondrial protection; and modulation of autophagy flux to prevent excessive neuronal self-degradation. Notably, melatonin's amphiphilic nature enables effective penetration of the blood-brain and blood-spinal cord barriers, allowing systemic administration while maintaining therapeutic CNS concentrations. These multimodal actions collectively preserve neural tissue, reduce secondary damage, and create a more permissive microenvironment for recovery, positioning melatonin as a compelling candidate for SCI treatment translation.¹⁷

Based on the FTIR analysis, chitosan effectively interacted with melatonin. The SEM images revealed the presence of porosity in the CH/Mel hydrogel. These features were thought to enhance the ability of melatonin to reduce the SCI secondary injury like oxidative stress, inflammation and apoptosis. Our finding indicated that CH/Mel hydrogel in dose of 25 mg/ml significantly increased cell viability in U87 cell line after 24 hours exposure. However, in 48 and 72 hours after exposure we did not observe the same results. The impaired microenvironment after SCI triggers secondary damages such as neuroinflammation and cellular apoptosis.²⁵

The significant improvement in BBB scores observed with 200 mg/kg melatonin-loaded chitosan hydrogel (CH/Mel) aligns with emerging evidence supporting melatonin neurorestorative potential in SCI.

In a study by Chen *et al.* (2023), a dual-responsive injectable collagen hydrogel, sensitive to both pH and

photothermal stimuli, was designed for the treatment of spinal cord injury (SCI). This system consisted of a ligand compound formed by the self-assembly of 2-methylimidazole and a zinc-based complex. The degradation of the hydrogel elevated the pH of the microenvironment, enhanced the proliferation of neural stem cells, and provided neuroprotection. The results demonstrated that the dual-responsive injectable collagen hydrogel significantly improved BBB scores in SCI-induced mice compared to other groups.²⁶ Liu *et al.* (2023) developed an injectable, self-healing, electrically conductive hydrogel combined with neural stem cells to enhance the localized therapeutic effects of spinal cord injury in rats. Their study showed that rats treated with the injectable, self-healing conductive hydrogel loaded with neural stem cells achieved significantly higher BBB scores than other groups.²⁷ Luo *et al.* (2021) designed an injectable, self-healing extracellular matrix-based hydrogel to promote tissue repair following traumatic spinal cord injury. Behavioral assessments revealed that mice treated with the injectable, self-healing extracellular matrix hydrogel exhibited significantly higher BBB scores compared to control groups.²⁸ Li *et al.* (2019) demonstrated that melatonin improved motor recovery after spinal cord injury by enhancing autophagy and reducing apoptosis. Mice administered melatonin achieved significantly higher BBB scores than those in other groups.²⁹ Similarly, Gao *et al.* (2020) investigated melatonin for treating induced spinal cord lesions in rats and concluded that melatonin facilitated motor recovery via the SIRT1/AMPK signaling pathway by upregulating autophagy and suppressing apoptosis. Consistent with previous findings, melatonin-treated rats exhibited significantly higher BBB scores than control groups.³⁰ In the present study, in agreement with the aforementioned findings, the application of melatonin-incorporated hydrogel at varying concentrations led to significant motor improvement in BBB assessments. Notably, melatonin at a concentration of 200 mg/kg demonstrated the most pronounced therapeutic effects from the second week onward.

The BBB locomotor assessment demonstrated that melatonin-loaded chitosan hydrogel (CH/Mel) significantly enhanced functional recovery following SCI in a dose-dependent manner, with the 200 mg/kg dose (Group 5) showing the most substantial improvement. Compared to lower doses (50 and 100 mg/kg) and the control group, Group 5 exhibited superior recovery kinetics, achieving near-normal hindlimb coordination by Week 9. These findings suggested that high-dose melatonin (200 mg/kg) optimally promoted neuroprotection and axonal regeneration, while lower doses showed modest benefits, only the 200 mg/kg treatment yielded statistically significant and clinically

relevant recovery, highlighting its potential as a translational therapy for SCI. Future studies should explore long-term outcomes and molecular mechanisms underlying this dose-dependent efficacy.

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Conflict of interests

There are no conflicts of interests to be declared.

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