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Chitosan-Mediated Metformin Delivery Promotes Dose-Dependent Functional Recovery in a Rat Spinal Cord Injury Model

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ABSTRACT

Spinal cord injury (SCI) is a severe condition characterized by primary mechanical damage followed by secondary injury mechanisms, which worsen cellular dysfunction and death. Current treatment strategies remain insufficient in mitigating the full consequences of SCI. Metformin (Met) has demonstrated neuroprotective effects in the central nervous system (CNS), raising interest in its therapeutic potential for SCI. However, whether a chitosan (CH) hydrogel loaded with Met can enhance functional recovery after SCI remains unclear. Wistar rats were divided into five groups: a sham group, an SCI group (negative control, NC), and three groups receiving CH hydrogel containing 10, 50, and 100 mg/kg of Met. Behavioral assessments, including locomotor scoring and sensorimotor function tests, demonstrated that sustained delivery of the metformin-chitosan hydrogel significantly enhanced functional recovery in spinal cord-injured rats compared to untreated controls. Quantitative analysis revealed notable improvements in hindlimb coordination, weight-bearing capacity, and reflex responses, suggesting partial restoration of neural circuitry. Furthermore, the CH/Met hydrogel group exhibited accelerated recovery kinetics, with earlier onset of motor improvements relative to standard treatments. These findings collectively supported the therapeutic efficacy of CH/Met hydrogel in mitigating SCI-related deficits, potentially through its combined neuroprotective and regenerative mechanisms.

Introduction

Spinal cord injury (SCI) represents a major global health challenge, with over 200,000 new cases reported annually and millions living with its long-term consequences.[1] Epidemiologically, SCI disproportionately affects adults, with males experiencing twice the incidence rate of females (2:1 ratio).[2] The initial mechanical trauma is often compounded by secondary injury mechanisms - including oxidative stress, apoptotic cell death, and dysregulated autophagy- which critically impair neurological recovery.[3] These secondary processes not only exacerbate tissue damage but also significantly hinder the restoration of motor function, underscoring the need for therapies targeting both primary and secondary injury pathways.

SCI initiates with an acute primary injury phase characterized by immediate mechanical trauma, resulting in hemorrhage, vascular disruption, and rapid necrotic cell death at the lesion site. This is followed by a prolonged secondary injury phase that begins within minutes and may persist for months to years, driven by complex pathophysiological cascades. Key features of secondary damage include progressive glial scar formation, widespread demyelination of surviving axonal tracts, and degeneration of gray matter architecture. These pathological changes lead to severe and often permanent neurological deficits, including

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paraplegia/tetraplegia, neurogenic bladder and bowel dysfunction, and impaired sexual function.[4] The extended temporal window of secondary injury presents a critical therapeutic opportunity for neuroprotective interventions.

Despite significant advancements in the clinical management of SCI, patient recovery rates remain suboptimal, highlighting an urgent need for novel therapeutic interventions. The pathophysiology of SCI is critically mediated by oxidative stress and neuroinflammation, which perpetuate secondary tissue damage and impede neural repair. Mounting evidence suggests that targeted modulation of these pathological processes -particularly through antioxidant and anti-inflammatory strategies- represents a promising therapeutic avenue for SCI treatment.[5] This approach may not only mitigate secondary degeneration but also create a more permissive microenvironment for neural regeneration and functional recovery.

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.[6-8]

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.[9,10]

Metformin, a well-tolerated FDA-approved medication primarily prescribed for type 2 diabetes, demonstrates multiple neuroactive properties that extend beyond its metabolic effects.[11] Research indicates this drug exerts beneficial actions in the central nervous system by stimulating neural stem/progenitor cells (NSPCs) and oligodendrocyte precursor cells (OPCs) while suppressing microglial overactivation post-injury.[12-16] Its neurogenic effects occur through aPKC-CBP signaling pathways, enhancing both neurogenesis and oligodendrogenesis in patterns influenced by age and sex.[17] Additionally, metformin exhibits significant neuroprotective potential through its anti-inflammatory properties, as evidenced by reduced microglial activation in models of radiation injury and neonatal hypoxia-ischemia.[18] Preclinical studies further report improved cognitive function and motor recovery following cerebrovascular events, suggesting its therapeutic potential for various neurological conditions. These multifaceted mechanisms position metformin as a promising candidate for repurposing in neural repair strategies.[19]

This study investigated the neuroprotective and regenerative potential of a metformin-loaded chitosan hydrogel (CH/Met) in a rat model of SCI. We aimed to characterize the physicochemical properties of the CH/Met 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 metformin dosages (10, 50, and 100 mg/kg) delivered via chitosan hydrogel against control groups. The study design incorporated in vivo assessments of locomotor recovery to comprehensively evaluate the combinatorial therapeutic approach for SCI management.

Materials and Methods

Metformin, MTT, and CH were obtained from Sigma-Aldrich, while fetal bovine serum (FBS), antibiotics (penicillin-streptomycin), and DMEM cell culture medium were supplied by Biowest.

Fabrication of Metformin-Containing Chitosan (Met/ CH) Hydrogel

CH was dissolved in a 2% (v/v) acetic acid solution with a 4% (w/v) concentration and stirred overnight. Metformin (Met) was dissolved in ethanol and then mixed with the CH solution. The resulting mixture was transferred into 24-well plates, frozen, and lyophilized at -80 °C for 48 hours. The Met and CH solutions were prepared at a 1:100 ratio, as referenced in previous studies.[20,21]

Investigation of Electrostatic Interactions

The electrostatic interactions between CH and Met in the formulation were analyzed using Fourier-transform infrared spectroscopy (FTIR) (Nexus Pro, Euro, Germany). The samples were scanned 32 times on average across a wavenumber range of 400/cm to 4000/cm, with a resolution of 4/cm, to obtain the FTIR spectra.

Morphological Study

The morphology of the hydrogels was analyzed using scanning electron microscopy (SEM) (KYKY-2800, China) at an accelerating voltage of 20 kV. Prior to imaging, the samples were sputter-coated with a conductive layer to enhance electron conductivity. Subsequently, SEM was

performed to capture high-resolution images of the hydrogel structure.

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.).

The animals were randomly placed to five groups (n = 8), including the Sham group, SCI-induced group (negative control, NC), and groups 3, 4, and 5 were given CH hydrogel containing Met at doses of 10, 50, and 100 mg/kg, respectively, immediately after SCI induction. The dose of metformin was chosen based on the previous study of Tao *et al.*[15] Before surgery, the rats were anesthetized with an intraperitoneal injection of 2% xylazine hydrochloride (5 mg/kg) and 10% ketamine hydrochloride (80 mg/kg) (both supplied by Alfasan International, Woerden, Holland). A contusive SCI was created using an NYU impactor device. The surgical site was prepared by shaving and disinfecting the skin over the 9th thoracic vertebra (T9). Following a midline incision, the connective tissue was carefully dissected, and the T9 lamina was removed using a dental drill to expose the spinal cord. The rats were positioned prone, with the T8 and T10 vertebrae clamped for spinal stabilization. A standardized contusion injury was induced by dropping a 10 g weight from a height of 25 cm onto the exposed spinal cord. Immediate tail flutter reflexes and hind limb retraction confirmed successful model induction.

Cell Viability Assay

The biocompatibility of CH/Met hydrogels was evaluated indirectly using the MTT assay. U87 glioblastoma cells (obtained from the Pasteur Institute, Tehran, Iran) were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C in a 5% CO_2 humidified incubator. Cells were seeded in a 96-well plate at a density of 10^4 cells per well and incubated for 24 hours. The medium was then replaced with fresh medium containing varying concentrations of Met (2.5, 12.5, and 25 mg/ml) that was chosen based on the study of Yuan *et al.*[22] After incubation periods of 24, 48, and 72 hours, the medium was removed, and an MTT solution (5 mg/mL in PBS) was added for 4 hours. The resulting formazan crystals were dissolved in DMSO, and the optical density (OD) was measured at 570 nm using an ELISA reader to assess cell viability.

Behavioral Assessments

Functional recovery after SCI was systematically evaluated using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale, a standardized method for assessing hind limb motor function in rodents.[23] Weekly testing sessions were conducted during the first four weeks post-injury to track recovery progression. To maintain objectivity, animals were randomly coded, and two trained, blinded investigators independently performed all behavioral assessments. During testing, rats were placed in an open-field arena where their hind limb movements, weight-bearing capacity, coordination, and stepping patterns were meticulously scored according to the 21-point BBB scale (ranging from 0 = complete paralysis to 21 = normal locomotion). The investigators' scores were averaged for each animal, and group means were subsequently computed for statistical comparisons. The longitudinal data were graphically represented to illustrate recovery trajectories, with weekly BBB scores plotted against time post-injury to facilitate intergroup comparisons of therapeutic efficacy. This comprehensive assessment protocol enabled sensitive detection of both initial spontaneous recovery patterns and later treatment-mediated improvements in locomotor function, providing robust quantitative measures of functional restoration.[23]

Statistical Analysis

The temporal progression of BBB locomotor scores across the four experimental groups was analyzed using two-way repeated measures ANOVA, with treatment condition as the between-subjects factor and post-injury time points as the within-subjects repeated measure. This analytical approach enabled simultaneous assessment of both treatment-related group differences and temporal recovery trajectories. When significant main or interaction effects were detected ($p < 0.05$), Tukey's HSD post hoc tests were applied for pairwise comparisons, evaluating: (1) inter-group differences at each weekly assessment (8 time points total), and (2) intra-group score changes across the observation period. The analysis maintained a significance threshold of $\alpha = 0.05$, with exact p-values reported to enhance reproducibility. To complement significance testing, effect size measures were computed (η^2 for ANOVA effects; Cohen's d for pairwise comparisons) to quantify the practical magnitude of observed differences. This comprehensive statistical approach ensured rigorous evaluation of both the statistical and biological significance of treatment outcomes.

Results

Characterization of Metformin-Containing Chitosan Hydrogel

Figure 1 (up) presents the FTIR spectral analysis of chitosan hydrogel, pure metformin, and metformin-

loaded chitosan hydrogel. The chitosan spectrum exhibited characteristic absorption bands, including a broad peak at 3200-3400/cm corresponding to O-H and N-H stretching vibrations and hydrogen bonding interactions. Distinct peaks at 2873 and 2920/cm represented C-H symmetric and asymmetric stretching vibrations, while peaks at 1660/cm (amide I C=O stretch) and 1323/cm (amide III C-N stretch) confirmed the presence of residual acetyl groups. Additional characteristic bands appeared at 1598/cm (N-H bending of primary amines), 1425/cm (CH_2 deformation), and 1380/cm (CH_3 deformation). The metformin spectrum showed prominent peaks at 3369 and 3307/cm (N-H stretching), 1650/cm (C=N stretching), 1589/cm (N-H bending), and 825-878/cm (C-H stretching). The metformin-loaded hydrogel spectrum demonstrated significant peak broadening between 900-1800/cm, indicating molecular interactions between chitosan and metformin. Notably, the metformin C-H stretching peak shifted from 825/cm to 820/cm, confirming successful drug incorporation through hydrogen bonding or electrostatic interactions. Figure 1 (down) displays SEM micrographs of the freeze-dried CH/Met hydrogel, revealing a highly porous, interconnected three-dimensional network structure. This unique architecture, characterized by uniform pore distribution and high surface area, facilitated efficient nutrient and metabolite transport while providing an optimal scaffold for cell adhesion and proliferation, making it particularly suitable for tissue engineering applications.

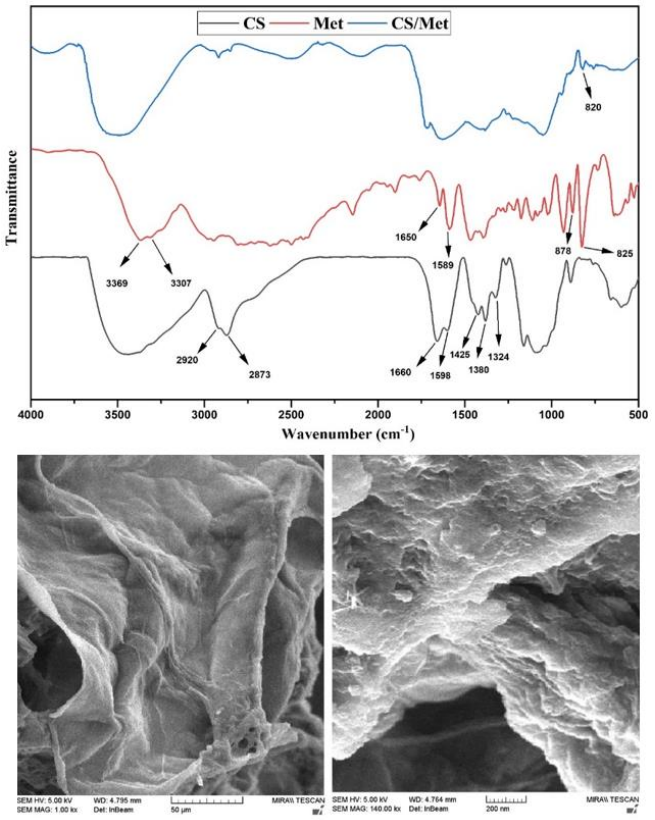

Figure 1. (Up) FTIR spectrum of chitosan hydrogel (CS), Metformin drug (Met) and metformin drug loaded in chitosan hydrogel (CS/Met). (Down) Scanning electron microscopy micrograph CH/met hydrogel.

Cell Viability

The MTT assay revealed distinct temporal and concentration-dependent patterns in U87 cell viability. After 24-hour exposure, no significant difference was observed between the control and treatment groups, though CH/Met at 25 mg/mL showed increased viability compared to the 2.5 mg/mL dose ($p < 0.05$, Figure 3A). At 48 hours, the 12.5 mg/mL CH/Met dose demonstrated both a significant viability increases versus 24-hour timepoint ($p < 0.05$, Figure 3B) and superior performance compared to both pure chitosan and the 25 mg/mL CH/Met group ($p < 0.01$). Notably, by 72 hours, all treatment groups exhibited non-significantly comparable viability levels ($p > 0.05$, Figure 3C), suggesting either metabolic adaptation or saturation of treatment effects. These results indicate that 12.5 mg/ml CH/Met represents an optimal concentration-time combination for enhancing glioblastoma cell viability under these experimental conditions.

Findings of Behavioral Assessments Using BBB Scores

Functional recovery analysis using the BBB locomotor rating scale revealed that Group 5 (SCI/100), receiving the highest metformin dose (100 mg/kg in CH hydrogel), showed significantly greater improvement in locomotor function compared to all other treatment groups ($p < 0.01$) throughout the study. While Groups 3 (SCI/10) and 4 (SCI/50) demonstrated moderate recovery, group 5 achieved statistically superior outcomes. The SHAM group maintained normal locomotor function, whereas the SCI control group exhibited minimal spontaneous recovery (Figure 3). These results clearly demonstrated that 200 mg/kg metformin delivered via CH hydrogel provided optimal neurorestorative effects following spinal cord injury, yielding significantly better functional outcomes than lower doses.

Discussion

Spinal cord injury is a catastrophic neurological condition resulting from traumatic damage to the central nervous system, typically caused by accidents, falls, or sports-related impacts. This injury triggers a complex pathological cascade involving both immediate mechanical destruction and progressive secondary damage mechanisms, leading to neuronal death, axonal disruption, demyelination, and permanent neurological deficits including motor paralysis, sensory loss, and

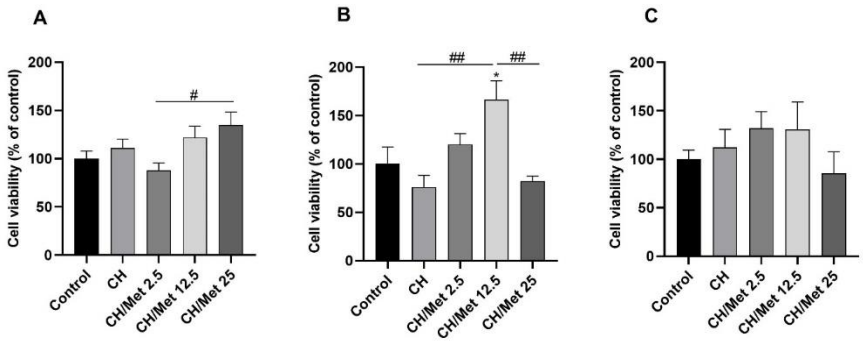

Figure 2. The effect of CH/Met hydrogel on the viability of U87 cells. The cells were cultured at a density of 104 cells per well in 96-well plates. Cells were then exposed to CH and different concentrations of CH/Met hydrogel (2.5, 12.5, and 25 mg/ml Met) for 24 (A), 48 (B), and 72 (C) hours of exposure. The MTT test was used to evaluate cell viability, following the procedure described in the Materials and Methods section (* $p < 0.05$ versus control group; # $p < 0.05$ versus CH/ Met 25; ## $p < 0.01$ versus CH/Met 12.5 mg/ml).

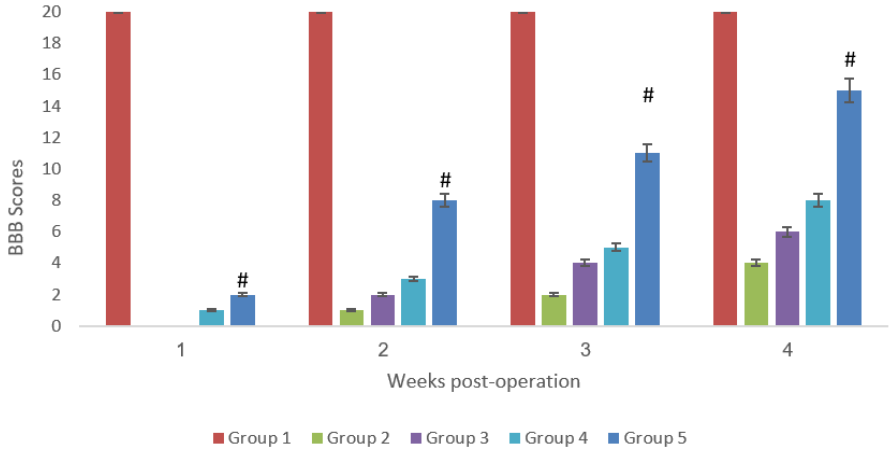

Figure 3. The BBB locomotor scale assessment revealed significant functional recovery differences among treatment groups during the 4-week postoperative period. #, Group 5 (treated with hydrogel containing 200 mg/kg metformin) demonstrated markedly superior motor function improvement ($p < 0.01$) compared to all other experimental groups.

autonomic dysfunction. The adult mammalian spinal cord's limited regenerative capacity often renders these impairments irreversible, causing lifelong disability and dramatically reduced quality of life. Current clinical management focuses on acute-phase interventions: surgical decompression to alleviate cord compression, vertebral stabilization, and controversial high-dose methylprednisolone administration within an 8-hour window to potentially mitigate inflammation. While vasopressor therapy maintains spinal perfusion pressure (85-90 mmHg for 5-7 days), these standard treatments primarily address secondary damage without promoting meaningful neural repair. The persistent lack of regenerative therapies underscores the urgent need for innovative neuroprotective and neuroregenerative approaches to restore neurological function after SCI. Recent advances in biomaterial scaffolds (like the metformin-loaded hydrogel in this study), neurotrophic factors, and stem cell therapies represent promising avenues to overcome the spinal cord's regenerative limitations and improve functional outcomes.[24]

Despite these clinical interventions, current therapeutic strategies remain fundamentally limited in their ability to promote neural regeneration following spinal cord injury. While existing approaches like surgical decompression and pharmacological management can stabilize the injury site and reduce secondary damage, they fail to reconstruct disrupted neural pathways or stimulate meaningful axonal regeneration across the lesion site. This therapeutic gap primarily results from two key biological challenges: the intrinsic regenerative limitations of adult CNS neurons and the hostile post-injury microenvironment characterized by inhibitory glial scarring and myelin-derived growth inhibitors. The resulting regeneration failure typically leads to permanent neurological impairment, highlighting the critical need for innovative treatment paradigms that can simultaneously address multiple barriers, including modulating the inhibitory extracellular environment, enhancing neuronal intrinsic growth capacity, and providing structural guidance for axonal regrowth. Emerging regenerative approaches combining biomaterial scaffolds, growth factors, and cell-based therapies show particular promise in creating permissive conditions for neural repair and functional reconnection, potentially offering the first genuine opportunities for structural and functional restoration after spinal cord injury.[24]

Chitosan-based hydrogels represent a transformative approach to SCI treatment by simultaneously addressing multiple pathophysiological barriers. These biomaterials combine exceptional biocompatibility with precisely tunable physical properties, including adjustable mechanical strength (matching native neural tissue stiffness), controllable degradation rates (from weeks to months), and an optimally porous architecture (50-200 μm pore size) that promotes vascularization and cellular migration. Unlike conventional therapies, chitosan hydrogels function as bioactive, three-dimensional matrices that actively participate in the repair process through several mechanisms: (1) providing structural support to bridge lesion cavities and maintain tissue continuity, (2) serving as sustained-release depots for neuroprotective compounds (e.g., metformin) and neurotrophic factors, and (3) creating a permissive microenvironment that guides axonal extension while modulating inhibitory glial responses. Their inherent biological properties, including anti-inflammatory effects, reduction of reactive astrogliosis, and suppression of chondroitin sulfate proteoglycan deposition - enable chitosan hydrogels to not only physically support regeneration but also chemically reprogram the injury microenvironment. This multifunctional capacity to deliver structural, pharmacological, and biological interventions through a single implantable platform positions chitosan-based hydrogels as a next-generation therapeutic strategy capable of overcoming the complex, multifactorial barriers to spinal cord regeneration that current clinical approaches fail to address.[23-25]

The FTIR spectroscopic analysis verified successful molecular interactions between chitosan and metformin, while SEM imaging confirmed the hydrogel porous architecture, both critical features for drug delivery applications in SCI treatment. Our experimental data revealed a time-dependent and concentration-specific therapeutic window, where only the 12.5 mg/ml CH/Met formulation significantly enhanced U87 cell viability after 48 hours of exposure, with no comparable effects observed at earlier (24-hour) or later (72-hour) time points. This temporal pattern suggests: (1) sustained drug release kinetics that reach therapeutic threshold concentrations by 48 hours, and (2) potential differential activation timelines of metformin downstream signaling pathways.

In the injured spinal cord, metformin demonstrated multiple neuroprotective mechanisms that contributed to functional recovery. The drug alleviated neuropathic pain by modulating AMPK and STAT3 phosphorylation in dorsal horn neurons, while exhibiting sex-specific effects on microglial activation. In SCI models, metformin treatment improved motor function through enhanced autophagy and reduced apoptosis, as evidenced by decreased caspase-3 activation. The compound effectively mitigated neuroinflammation by suppressing NF-κB signaling and promoting a phenotypic shift in microglia from pro-inflammatory to anti-inflammatory states, while enhancing their capacity for myelin debris clearance. Recent findings also revealed metformin pro-angiogenic effects in the injured spinal cord, where it stimulated endothelial proliferation and increases vascular density. While these mechanisms have been well-characterized in brain pathology, their effects on spinal cord precursor cells remain unclear. [26-31]

In a study on therapeutic effect of metformin on inflammation and apoptosis after spinal cord injury in rats through the Wnt/β-catenin signaling pathway, it was reported that metformin could promote functional recovery of SCI rats through activating Wnt/β-catenin signaling pathway.[32] In other studies, BBBs scores significantly improved in the metformin group, compared with the SCI control group suggesting that the administration of metformin contributed to the recovery of motor function in SCI rats through the antioxidative mechanism.[33,34] Others showed that metformin had potent effects on neural precursor cells (NSPCs and OPCs) in the spinal cord and also demonstrated that the timing of administration had a significant impact on the functional outcomes.[35]

In the present study, in agreement with the aforementioned findings, the application of metformin-incorporated hydrogel at varying concentrations led to significant motor improvement in BBB assessments. Remarkably, metformin at a concentration of 200 mg/kg demonstrated the most pronounced therapeutic effects from the second week onward.

The BBB locomotor scale evaluation revealed that chitosan hydrogel loaded with metformin (CH/Met) significantly improved functional recovery after spinal cord injury in a concentration-dependent fashion. The highest tested dose of 200 mg/kg (Group 5) produced the most pronounced therapeutic effects, demonstrating markedly better recovery trajectories compared to both lower-dose groups (50 and 100 mg/kg) and untreated controls. Notably, animals receiving the 200 mg/kg formulation achieved near-complete restoration of hindlimb coordination by the study endpoint at 9 weeks post-injury. These results indicate that while metformin demonstrates some neurorestorative capacity across all tested doses, only the 200 mg/kg concentration delivered via chitosan hydrogel generated both statistically robust and functionally meaningful recovery. The superior performance of this high-dose formulation suggests it may optimally activate multiple repair mechanisms, including neuroprotection and axonal regeneration pathways. These promising findings warrant further investigation into the long-term durability of recovery

and the specific molecular mechanisms driving the observed dose-response relationship, which could inform clinical translation of this combinatorial therapy for spinal cord injury treatment.

In conclusion, the findings suggested that high-dose metformin (100 mg/kg) optimally promoted neuroprotection and axonal regeneration**,** while lower doses showed modest benefits, only the 100 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 Interest

There are no conflicts of interests to be declared.

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