http://www.cjmb.org doi 10.34172/cjmb.2025.4567

Open Access Original Article

Crescent Journal of Medical and Biological Sciences
Vol. x, No. x, xx 2025, x–x
eISSN 2148-9696

The Effects of Chitosan Hydrogel Loaded With Metformin on Spinal Cord Injury Model in Rat

Erfan Sheikh-Akbarizadeh[1], Ahmad Asghari[1*], Gholamreza Abedi Cham-Heydari[1], Pejman Mortazavi[2]

Abstract

Objectives: Spinal cord injury (SCI) is recognized as a serious condition that leads to both primary and secondary complications. Secondary injuries exacerbate the initial phase of cell dysfunction and cell death. Current strategies remain limited in effectively addressing SCI consequences. However, there have been reports of metformin's (Met's) neuroprotective effects on the central nervous system (CNS). The potential of chitosan (CH) hydrogel containing Met to improve SCI remains unclear.

Materials and Methods: 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. We fabricated the CH/Met hydrogel and characterized it using scanning electron microscopy (SEM) and Fourier-transform infrared spectroscopy (FTIR). We then evaluated cell viability, Bax and Bcl2 gene expression, immunohistochemistry (IHC), and histopathological changes.

Results: The CH/Met hydrogel at a dose of 12.5 mg/mL significantly increased cell viability in the U87 cell line after 48 hours of exposure. The CH/Met hydrogel groups significantly modulated Bax and Bcl2 mRNA expression, particularly at doses of 50 and 100 mg/kg compared to the SCI group. Additionally, the upregulation of S100β protein in SCI was mitigated by the CH/Met hydrogel in a dose-dependent manner. The histopathological results showed that CH/Met hydrogel significantly improved SCI-induced alterations such as vacuolar degeneration, necrosis, severe cystic and axonal degeneration.

Conclusions: Our results suggest that the CH/Met hydrogel has neuroprotection effects in an SCI-induced model in rats. CH/Met hydrogel, significantly modulated histopathological changes and apoptosis-related gene expression in SCI.

Keywords: Spinal cord injury, Apoptosis, S100β, Metformin, Chitosan

Introduction

More than 200 000 spinal cord injuries (SCIs) occur globally every year, and millions of people are currently suffering from the repercussions of SCI (1). SCI occurs most frequently in adulthood and is more common in men than in women (2:1 ratio) (2). SCIs frequently led to secondary injuries in addition to the initial primary lesion to the spinal cord. The motor function recovery process is substantially influenced by secondary injuries, including oxidative stress, apoptosis, and cell autophagy (3).

When the spinal cord is damaged, the primary injury phase starts, leading to massive blood loss and cell death at the site of the injury. A secondary injury develops minutes to weeks after the main injury and can last for years, depending on the intricacy of the neurological cascade in which it happens. During the secondary damage phase, glial scarring, demyelinating of the white matter, and breakdown of the gray matter take place. Sexual dysfunction, incontinence, and paralysis of the limbs below the affected section are common outcomes of SCI (4).

Although the advances in the medical care of SCI have improved, the rate of recovery remains constrained, emphasizing the necessity for innovative therapeutic strategies aimed at enhancing outcomes. The progression of SCI is significantly influenced by oxidative stress and inflammatory mediators. Consequently, the suppression of oxidative stress and inflammatory reaction following SCI has emerged as an acknowledged approach for treating SCI (5).

S100β is primarily an astrocytic protein and is mostly located within the cytoplasm (6). The earliest experimental methods revealed a significant presence of S100β in cerebrospinal fluid and serum during the first 3-6 hours after SCI. However, in certain cases, an elevation in S100β levels was observed even after 3-7 days following SCI (7).

Metformin (Met), an FDA-approved medication, is widely employed for the management of type II diabetes mellitus. It can activate AMPK signaling and diminish the formation of reactive oxygen species (ROS) (8). Metformin may regulate oxidative stress, improve endoplasmic reticulum stress, and modulate AMPK and mTORC1 signaling, all of which would provide a sufficient level of neuroprotection in SCI rats (9). Met did not induce any significant impact on the blood sugar levels of healthy individuals when administered for illnesses or injuries unrelated to type 2 diabetes (10).

Complex secondary injuries may be effectively managed

Received 14 January 2025, **Accepted** 7 March 2025, **Available online** xx xx xx

[1]Department of Clinical Science, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Pathobiology, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran.
*Corresponding Author: Ahmad Asghari, Email: dr.ahmad.asghari@srbiau.ac.ir

Key Messages

- The study demonstrates that CH hydrogel can serve as an effective carrier for Met, a drug traditionally used for diabetes, to target SCI in rats. CH-Met hydrogel significantly reduces apoptosis in SCI by downregulating the pro-apoptotic gene Bax and upregulating the anti-apoptotic gene Bcl2, particularly at higher doses (50 and 100 mg/kg). This indicates a dose-dependent protective mechanism against secondary neuronal damage.

with Met via its antioxidant, anti-inflammatory, and anti-apoptotic properties. Nevertheless, based on the one-compartment pharmacokinetic model's predictions, the probable toxicity and restricted distribution of Met in the entire body have hindered its continued utilization (11). Consequently, a novel approach is required to decrease the systemic distribution of Met while increasing its concentration in the damaged spinal cord. This will enhance the drug's efficacy, reduce its toxic and side effects, and improve the kinematic function of patients with SCI (12).

Chitosan (CH), a biopolymer derived from chitin, has demonstrated significant promise in the field of tissue regeneration. The CH has a multitude of attributes that render it appealing for utilization in biomedical contexts, including but not limited to biocompatibility, minimal toxicity, extensive antimicrobial efficacy, and various other properties (13). Significantly, it possesses the capability to be fabricated into a diverse range of structures, such as nanoparticles, scaffolds, membranes, and hydrogels, thereby enabling customization to achieve the desired result. It has also been demonstrated that CH-containing hydrogels enhance the regeneration of nerve tissue (4). Additionally, CH can enhance the sustained release of metformin, ensuring a prolonged therapeutic effect in the targeted area. Previously, it was indicated that the Met analog (Biguanide), is a hydrophilic cation, whereas CH is hydrophobic, so CH/Met may accumulate in the mitochondria more readily to influence mitochondrial function (14). This study aims to assess the effect of chitosan hydrogel loaded with Met on SCI in a rat model.

Material and Methods

Materials

Metformin, MTT, and CH were acquired from Sigma-Aldrich. The company Biowest provided the fasting blood sugar (FBS), antibiotics (penicillin-streptomycin), and DMEM cell culture medium.

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

CH was dissolved in a solution of acetic acid with a volumetric concentration of 2% (v/v) and a weight percentage of 4% (w/v), and the mixture underwent stirring overnight. The process involved dissolving Met in ethanol and subsequently combining it with the CH solution. We transferred the resultant mixture into 24-well plates, frozen it, and subjected it to lyophilization at -80 °C for 48 hours. Met and CH solutions were produced at a 1:100 ratio, respectively (14,15).

Investigation of Electrostatic Interactions

The electrostatic interactions between CH and Met in a formulation were examined using Fourier-transform infrared spectroscopy (FTIR) (Nexus Por Euro, Germany) spectroscopy. The test samples were subjected to an average of 32 scans within the wave range of 400 cm^{-1} to 4000 cm^{-1}, with a resolution of 4 cm^{-1}, resulting in the acquisition of FTIR spectra.

Morphological Study

The structure of the hydrogels was examined using scanning electron microscopy (SEM, KYKY-2800; China) with an electron acceleration voltage of 20 kV. The specimens underwent sputter coating. Afterwards, the test samples underwent SEM to get images.

Induction of SCI Model and Grouping

In this investigation, we used male Wister rats weighing between 250 and 280 g. All animal procedures were carried out in strict accordance with the requirements established by the Islamic Azad University Ethics Committee in Tehran, Iran (IR.IAU.SRB.REC.1402.014). 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 SCI rat model was constructed based on our previous study (16).

Cell Viability Assay

The indirect assessment of the biocompatibility of CH/Met hydrogels was conducted using the MTT test. The U87 glioblastoma cell line (obtained from Pasture Institute in Tehran, Iran) was cultured in DMEM medium supplemented with 10% FBS and antibiotics (penicillin/streptomycin 1%) in an incubator set at a temperature of 37 °C and a CO2 concentration of 5%. The cells were seeded in a 96-well plate at a density of 104 cells per well and allowed to incubate for 24 hours. Subsequently, the media was removed and changed with new media containing different concentrations of Met (2.5, 12.5, and 25 mg/mL) in the wells. After the exposure period of 24, 48, and 72 hours, the media was replaced with MTT solution (5 mg/mL PBS) and allowed to incubate for 4 hours. The formazan crystals were then dissolved in DMSO, and an ELISA reader was used to determine the optical density (OD) at 570 nm.

Histopathology and Immunohistochemistry (IHC)

After the 30-day experimental period, the rats were

anesthetized using CO_2, and their entire spinal cords were extracted for further investigation. The spinal cord of each rat was dissected at the site of damage and then divided into two separate segments. One segment was stored in an RNAlater solution for real-time PCR, while the other segment was fixed in 10% formaldehyde for histological analysis. Once the samples were fixed, they were submerged in paraffin for embedding. Following that, slices with a thickness of 5 mm were generated in both transverse and longitudinal orientations and employed for either hematoxylin or eosin (H&E) or immunohistochemical (IHC) staining. Ultimately, an experienced pathologist examined and evaluated the slides thoroughly under a light microscope (Olympus BX51, Japan), blindly documenting any changes that were seen. We stained the slices for IHC using a rabbit polyclonal antibody against S100β (dilution 1:400, Dako) (16).

Bax and Bcl2 mRNA Expression Quantification by Real-time PCR

The experimental procedure involved extracting total RNA from spinal cord samples using Trizol reagent (Bio basic, Canada) and then synthesizing cDNA using the cDNA synthesis kit (TAKARA, Japan). We performed a quantitative real-time polymerase chain reaction with SYBR green master mix (TAKARA, Japan) using the StepOnePlus equipment (Applied Biosystems, USA). The relative expression levels of Bax and Bcl2 were determined using the ΔΔCt technique, with β-actin as the housekeeping gene and the following primers in Table 1.

Statistical Analysis

The statistical analysis was conducted using GraphPad Prism (version 8.0), and the results were displayed as the mean ± standard deviation (SD). To analyze and evaluate the difference between the groups, a one-way analysis of variance (ANOVA) following the Tukey post hoc test was used. If a *P* value was less than 0.05, it was deemed statistically significant.

Results

Characterization of CH/Met Hydrogel

Figure 1 illustrates the FTIR spectra of chitosan hydrogel, metformin drug, and metformin drug loaded in chitosan hydrogel. According to the FTIR spectra of chitosan hydrogel, the characteristic peaks are as follows: A broad spectrum appears in the region between 3200-3400 cm^{-1} wavelengths related to the stretching vibration of O-H and N-H groups as well as intramolecular hydrogen

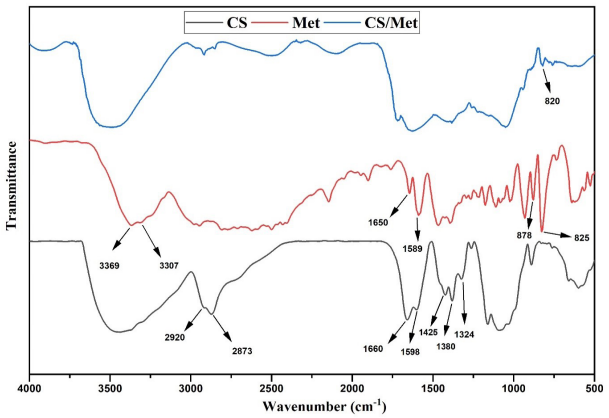

Figure 1. FTIR Spectrum of Chitosan Hydrogel (CS), Metformin Drug (Met) and Metformin Drug Loaded in Chitosan Hydrogel (CS/Met)

interactions. The symmetric and asymmetric stretching vibrations of the C-H group correspond to the peaks appearing at wavelengths 2873 and 2920. The presence of the remaining acetyl groups is proved by the wavelengths of 1660 cm^{-1} (C=O stretching of amide group type one) and 1323 cm^{-1} (C-N stretching of the amide group of the third type). The band appearing in the 1598 cm^{-1}area is related to the N-H amine group of the first type. The stretching vibrations of the CH2 and CH3 groups have been observed at 1425 cm^{-1} and 1380 cm^{-1} wavelengths, respectively. According to the FTIR spectra of the metformin drug, the characteristic peaks, as seen, were the symmetric and asymmetric N-H stretching peaks at 3369 cm-1 and 3307 cm^{-1} wavelengths, respectively. Also, the peaks related to C=N stretching at the wavelength of 1650 cm^{-1} and N-H bending at the wavelength of 1589 cm^{-1} have appeared. Also, the stretching vibration of the C-H group has appeared at the wavelengths of 825 cm^{-1} and 878 cm^{-1}. In the spectrum of the chitosan hydrogel containing the drug metformin, there are many interactions between the drug and hydrogel. There are also many peaks in the FTIR spectrum of both the hydrogel and the drug, which means that all of their peaks overlap. This overlap has led to the emergence of broad peaks in various regions, particularly in the range from 900 cm^{-1} to 1800 cm^{-1}. In the spectrum related to hydrogel loaded with metformin drug, the peak appearing in the area of 820 cm^{-1} can be related to the stretching of the C-H group of metformin drug, which has shifted to lower wavelengths due to the interaction with chitosan hydrogel.

SEM was employed to analyze the morphology of the cross-section of the freeze-dried CH/Met hydrogel. SEM

Table 1. List of Primers Used in This Study

Gene Name	Forward Primer (5′>3′)	Reverse Primer 5′>3′
β-actin	CAACCTTCTTGCAGCTCCTC	TTCTGACCCATACCCACCAT
Bax	GCCTCCTTTCCTACTTCGGG	CTTTCCCCGTTCCCCATTCA
Bcl2	TCGCGACTTTGCAGAGATGT	CAATCCTCCCCCAGTTCACC

images of CH/Met hydrogel were presented in Figure 2, and demonstrate, that the freeze-dried CH/Met hydrogel had a porous and interconnected pore structure. This particular shape was well-suited for the transfer of nutrients and metabolites. Therefore, CH/Met hydrogels offered a suitable framework for cellular adhesion and growth.

Cell Viability

Our results show that there is no significant difference between the control group and the other group after 24-hour exposure. But cell viability increased in CH/Met at the dose of 25 mg/mL compared to CH/Met at the dose of 2.5 mg/mL (P<0.05, Figure 3A). However, after 48-hour exposure, cell viability increased in CH/Met at 12.5 mg/mL (P<0.05, Figure 3B). Additionally, in this dose, U87 cell viability was significantly enhanced compared to CH and CH/Met 25 mg/mL (P<0.01, Fig 3B). In addition, we did not show any significant alteration between different groups after 72 hours of exposure time (P>0.05, Figure 3C).

Bax and Bcl2 Gene Expression Pattern After SCI and Effects of CH/Met Hydrogel

The Bax gene expression significantly increased in the NC and various CH/Met hydrogel groups compared to the sham group. However, the use of CH/Met hydrogel reduced Bax gene upregulation at doses of 50 and 100 mg/kg (P<0.001, Figure 4A) compared to the NC. Also, the expression of Bcl2, an anti-apoptotic gene, significantly decreased in the NC group compared to the sham group, and this decline was modulated by CH/Met hydrogel, especially at 50 and 100 mg/kg (P<0.05, Figure 4B).

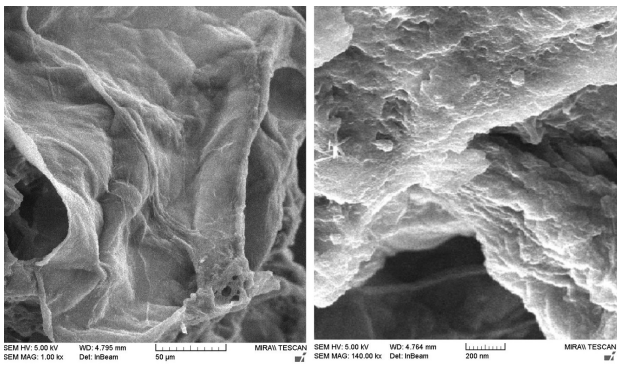

Figure 2. Scanning Electron Microscopy Micrograph CH/Met Hydrogel.

CH/Met Hydrogel Effects on S100β Protein Expression and Histopathological Alteration in SCI Model in Rat

The histopathological analysis was conducted for 30 days following the SCI model at the T9 segment of the Wistar rat. The results of the H&E staining revealed the presence of inflammatory cell infiltration, vacuolar degeneration, and significant cystic and axonal degeneration in the anterior funiculus region within the SCI group. Furthermore, the line that separates gray and white matter within the spinal cord demonstrated a lack of clarity. Furthermore, this group exhibited notable neuronal necrosis in the posterior horn, as depicted in Figures 5A and B. The application of the CH/Met hydrogel resulted in a decrease in gliosis and severe cystic and axonal degeneration at the site of SCI. However, it is important to note that this reduction is more pronounced when using large doses of CH/Met hydrogel, as depicted in Figure 5C-H. The group with 100 CH/Met had a substantial decrease in the quantity of necrotic nerve and inflammatory cells compared to the SCI group. Also, the IHC results indicated a substantial decrease in the S100β protein expression in the CH/Met hydrogel compared to the NC group. Collectively, our IHC and histological results indicate that the CH/Met hydrogel has the ability to enhance the recovery of SCI in rats.

Discussion

SCI is a severe neurological condition that can cause irreversible harm and neurological disability. The main causes of disability are attributed to a sequence of secondary damages, such as neuronal cell death (apoptosis

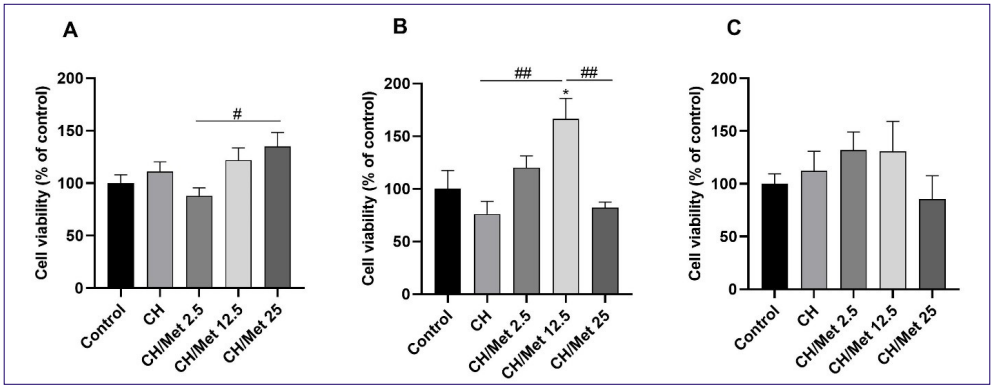

Figure 3. The effect of CH/Met Hydrogel on the Viability of U87 Cells. The cells were cultured at a density of 10^4 cells per well in 96-well plates. Cells were then exposed to CH and different concentrations of CH/Met hydrogel (2.5, 12.5, and 25 mg/mL Met) for 24 (A), 48 (B), and 72 (C) hours of exposure. 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).

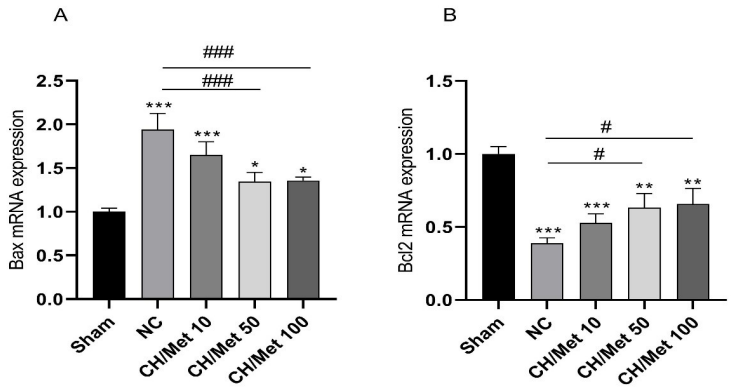

Figure 4. The Effects of CH/Met Hydrogel on the Bax and Bcl2 mRNA Expression in SCI Model in Rat. Total RNA was extracted and cDNA synthesized then gene expression was analyzed by qPCR using specific primers. To normalize the gene expressions, we used β-actin as a housekeeping gene (** P<0.01; *** P<0.001 versus control group, ($^{\#}$ P<0.05; $^{\#\#\#}$ P<0.001 versus NC group).

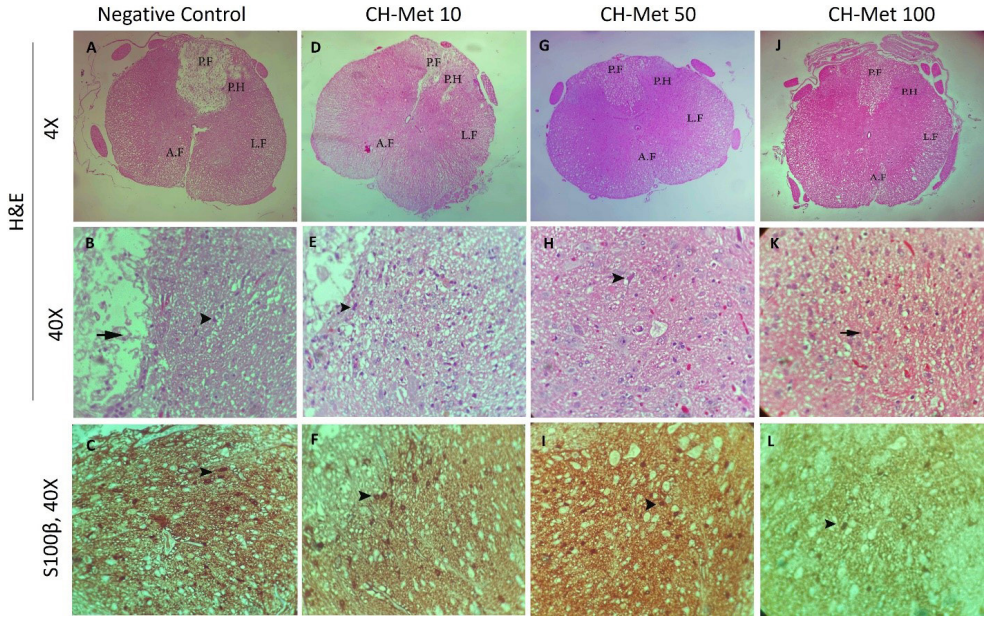

Figure 5. Light Micrographs of the Spinal Cord in Different Experimental Groups in Rat Spinal Cord After 4 Weeks Injury. H&E: A, B (Negative Control), D, E (CH/Met 10 mg/kg), G, H (CH/Met 50 mg/kg), J, K (CH/Met 100 mg/kg). IHC for S100β protein expression: C (Negative Control), F (CH/Met 10 mg/kg), I (CH/Met 50 mg/kg), L (CH/Met 100 mg/kg). posterior funiculus (PF), lateral funiculus (LF), posterior horn (PH), and anterior funiculus (AF).

and necrosis), mitochondrial dysfunction, and oxidative stress (17). Therefore, there is an urgent need for novel and efficacious therapeutic interventions aimed at mitigating neurological diseases and tissue damage resulting from SCI.

The basic issue with these patients' nerve regeneration has not been resolved, despite the availability of current SCI treatments. Because of its unique properties, such as biocompatibility, mechanical strength, suitable degradation rate, appropriate pore size, and high surface area, chitosan hydrogel offers new opportunities for the treatment of SCI (16,18).

Although there are various pathways implicated in the degenerative processes of SCI, it is widely thought that oxidative stress and adenosine monophosphate-activated protein kinase (AMPK) play a crucial role in its development. Jiang et al. showed that AMPK activation by irisin can alleviate the inflammatory responses and oxidative stress in the SCI model in rats and improve behavior tests, including the BBB and inclined plane (19). Met is a well-known antidiabetic drug that can activate AMPK and reduce ROS generation. In this study, we fabricated a Met-loaded CH hydrogel to evaluate its neuroprotective effects it on the SCI model in rats.

The FTIR analysis confirms that CH and Met have an effective interaction. The SEM scans also revealed the porosity of the CH/Met hydrogel. These characteristics have the potential to improve Met's capacity to mitigate secondary harm from SCI. Our data demonstrated that only CH hydrogel containing Met at a dose of 12.5 mg/mL after 48 hours of exposure increased the cell viability of U87 cells. However, the same results were not observed

24 or 72 hours after exposure. It is possible that metformin is released gradually over time, achieving a threshold concentration that exerts significant effects at 48 hours. In addition, metformin may modulate various signaling pathways that have different timelines for activation. The utilization of CH hydrogels, which are loaded with varying doses of Met (10, 50, and 100 mg/kg), can effectively modulate the expression of apoptosis-related genes, Bax and Bcl-2. However, we did not see significant changes between the NC group and CH/Met 10 mg/kg and Met has similar efficacy at 50 and 100 mg/kg.

According to Afshari et al, when compared to dosages of 10 and 100 mg/kg, Met at 50 mg/kg had the greatest impact on facilitating locomotor recovery and reducing problems related to SCI (20). Another study found that a 50 mg/kg dose of Met was more effective than high doses of 100 and 200 mg/kg in reducing the inflammatory response (21). These findings are in line with our data, despite the fact that, in our investigation, there was no significant difference in the downregulation and upregulation of the Bax and Bcl2 gene expression between the 50 and 100 groups compared to the NC group. The potential cause of this phenomenon may be attributed to the specific carrier employed in this investigation, as Afshari and colleagues' study utilized an intraperitoneal injection route. In a separate study, Zhang et al proposed that Met, administered at a dosage of 50 mg/kg, may potentially decrease inflammation and apoptosis, as well as facilitate functional recovery in rats with SCI via stimulating the Wnt/β-catenin signaling pathway (22). The histological and IHC findings demonstrated a significant enhancement in the overall histopathological characteristics of the spinal cord within the CH/Met hydrogel group. Our study showed that the use of CH hydrogel containing 50 and 100 mg/kg of Met significantly decreased the expression of S100β protein compared to the NC group. In this work, we show that CH/Met hydrogel reduces apoptotic cell death induced by SCI and has neuroprotective properties via regulating S100β and apoptosis. The absence of locomotor scoring limits our ability to assess functional recovery post-injury. Additionally, the lack of longitudinal studies to capture dynamic histopathological changes over time represents a significant limitation of our study, as such data could provide deeper insights into the progression of injury and recovery.

Conclusions

Our results suggest that the CH/Met hydrogel has neuroprotection effects in an SCI-induced model in rats. CH hydrogel containing Met, especially at 50 and 100 mg/kg significantly modulated histopathological changes and apoptosis-related gene expression in SCI. As well, S100β protein expression reduced in the CH/Met hydrogel received group compared to the NC group. This data suggests that CH/Met hydrogel has the potential to be a therapeutic agent for the treatment of SCI.

Authors' Contribution

Conceptualization: Erfan Sheikh-Akbarizadeh, Ahmad Asghari.
Data curation: Erfan Sheikh-Akbarizadeh, Ahmad Asghari, Pejman Mortazavi.
Formal analysis: Erfan Sheikh-Akbarizadeh, Ahmad Asghari, Pejman Mortazavi.
Funding acquisition: Erfan Sheikh-Akbarizadeh.
Investigation: Erfan Sheikh-Akbarizadeh, Ahmad Asghari.
Methodology: Erfan Sheikh-Akbarizadeh, Ahmad Asghari, Pejman Mortazavi.
Project administration: Ahmad Asghari.
Resources: Ahmad Asghari, Pejman Mortazavi.
Software: Erfan Sheikh-Akbarizadeh.
Supervision: Ahmad Asghari, Pejman Mortazavi.
Validation: Ahmad Asghari, Pejman Mortazavi.
Visualization: Erfan Sheikh-Akbarizadeh, Ahmad Asghari, Pejman Mortazavi.
Writing–original draft: Erfan Sheikh-Akbarizadeh, Ahmad Asghari.
Writing–review & editing: Erfan Sheikh-Akbarizadeh, Ahmad Asghari.

Conflict of Interests

The authors declared no conflict of interest in this study.

Ethical Issues

The Ethics Committee of Islamic Azad University, Sciences and Research Branch, Tehran, Iran confirmed the study protocol (Code: IR.IAU.SRB.REC.1402.014).

Financial Support

Self-funded.

References

1. Wang H, Zheng Z, Han W, et al. Metformin Promotes Axon Regeneration after Spinal Cord Injury through Inhibiting Oxidative Stress and Stabilizing Microtubule. Oxid Med Cell Longev. 2020;2020:9741369.
2. Gilbert EAB, Livingston J, Garcia-Flores E, Kehtari T, Morshead CM. Metformin Improves Functional Outcomes, Activates Neural Precursor Cells, and Modulates Microglia in a Sex-Dependent Manner After Spinal Cord Injury. Stem Cells Translational Medicine. 2023;12(6):415-28.
3. Guo Y, Wang F, Li H, et al. Metformin Protects Against Spinal Cord Injury by Regulating Autophagy via the mTOR Signaling Pathway. Neurochem Res. 2018;43(5):1111-7.
4. Jia Z, Zeng H, Ye X, Dai M, Tang C, Liu L. Hydrogel-based treatments for spinal cord injuries. Heliyon. 2023;9(9):e19933.
5. Li Y, Zou Z, An J, Wu Q, Tong L, Mei X, et al. Chitosan-modified hollow manganese dioxide nanoparticles loaded with resveratrol for the treatment of spinal cord injury. Drug Deliv. 2022;29(1):2498-512.
6. Michetti F, Corvino V, Geloso MC, Lattanzi W, Bernardini C, Serpero L, et al. The S100B protein in biological fluids: more than a lifelong biomarker of brain distress. J Neurochem. 2012;120(5):644-59.
7. Zigouris A, Kafritsas G, Alexiou GA, Voulgaris S. Chapter 15 - S100B in spinal cord injury. In: Rajendram R, Preedy VR, Martin CR, editors. Diagnosis and treatment of spinal cord injury. Academic Press; 2022:191-198.
8. Apostolova N, Iannantuoni F, Gruevska A, Muntane J, Rocha M, Victor VM. Mechanisms of action of metformin in type 2 diabetes: Effects on mitochondria and leukocyte-endothelium interactions. Redox Biol. 2020;34:101517.
9. Zhou LY, Chen XQ, Yu BB, et al. The effect of metformin on ameliorating neurological function deficits and tissue damage in rats following spinal cord injury: A systematic review and

network meta-analysis. Front Neurosci. 2022;16:946879.
10. Lipska KJ, Yao X, Herrin J, et al. Trends in Drug Utilization, Glycemic Control, and Rates of Severe Hypoglycemia, 2006-2013. Diabetes Care. 2017;40(4):468-75.
11. Khedkar A, Lebovitz H, Fleming A, et al. Impact of Insulin Tregopil and Its Permeation Enhancer on Pharmacokinetics of Metformin in Healthy Volunteers: Randomized, Open-Label, Placebo-Controlled, Crossover Study. Clin Transl Sci. 2019;12(3):276-82.
12. Li T, Jing P, Yang L, et al. CAQK modification enhances the targeted accumulation of metformin-loaded nanoparticles in rats with spinal cord injury. Nanomedicine. 2022;41:102526.
13. Kim Y, Zharkinbekov Z, Raziyeva K, et al. Chitosan-Based Biomaterials for Tissue Regeneration. Pharmaceutics. 2023;15(3):807. doi: 10.3390/pharmaceutics15030807.
14. Zhou Z, Liu Y, Jiang X, et al. Metformin modified chitosan as a multi-functional adjuvant to enhance cisplatin-based tumor chemotherapy efficacy. Int J Biol Macromol. 2023;224:797-809.
15. Kaczmarek-Szczepańska B, Ostrowska J, Kozłowska J, et al. Evaluation of Polymeric Matrix Loaded with Melatonin for Wound Dressing. Int J Mol Sci. 2021;22(11):5658. doi: 10.3390/ijms22115658..
16. Gholami M, Gilanpour H, Sadeghinezhad J, Asghari A. Facile fabrication of an erythropoietin-alginate/chitosan hydrogel and evaluation of its local therapeutic effects on spinal cord injury in rats. Daru. 2021;29(2):255-65.
17. Wang W, Huang X, Li J, et al. Methane Suppresses Microglial Activation Related to Oxidative, Inflammatory, and Apoptotic Injury during Spinal Cord Injury in Rats. Oxid Med Cell Longev. 2017;2017:2190897.
18. Ashammakhi N, Kim HJ, Ehsanipour A, et al. Regenerative Therapies for Spinal Cord Injury. Tissue Eng Part B Rev. 2019;25(6):471-91.
19. Jiang X, Shen Z, Chen J, et al. Irisin Protects Against Motor Dysfunction of Rats with Spinal Cord Injury via Adenosine 5′-Monophosphate (AMP)-Activated Protein Kinase-Nuclear Factor Kappa-B Pathway. Front Pharmacol. 2020;11:582484.
20. Afshari K, Dehdashtian A, Haddadi NS, et al. Anti-inflammatory effects of Metformin improve the neuropathic pain and locomotor activity in spinal cord injured rats: introduction of an alternative therapy. Spinal Cord. 2018;56(11):1032-41.
21. Araújo AA, Pereira A, Medeiros C, et al. Effects of metformin on inflammation, oxidative stress, and bone loss in a rat model of periodontitis. PLoS One. 2017;12(8):e0183506.
22. Zhang T, Wang F, Li K, Lv C, Gao K, Lv C. Therapeutic effect of metformin on inflammation and apoptosis after spinal cord injury in rats through the Wnt/β-catenin signaling pathway. Neurosci Lett. 2020;739:135440.