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Local delivery of minocycline from metal ion-assisted self-assembled complexes promotes neuroprotection and functional recovery after spinal cord injury

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Abstract

Many mechanisms contribute to the secondary injury cascades following traumatic spinal cord injury (SCI). However, most current treatment strategies only target one or a few elements in the injury cascades, and have been largely unsuccessful in clinical trials. Minocycline hydrochloride (MH) is a clinically available antibiotic and anti-inflammatory drug that has been shown to target a broad range of secondary injury mechanisms via its anti-inflammatory, anti-oxidant, and anti-apoptotic properties. However, MH is only neuroprotective at high concentrations. The inability to translate the high doses of MH used in experimental animals to tolerable doses in human patients limits its clinical efficacy. In addition, the duration of MH treatment is limited because long-term systemic administration of high doses of MH has been shown to cause liver toxicity and even death. We have developed a drug delivery system in the form of hydrogel loaded with polysaccharide-MH complexes self-assembled by metal ions for controlled release of MH. This drug delivery system can be injected into the intrathecal space for local delivery of MH with sufficient dose and duration, without causing any additional tissue damage. We show that local delivery of MH at a dose that is lower than the standard human dose (3 mg/kg) was more effective in reducing secondary injury and promoting locomotor functional recovery than systemic injection of MH with the highest dose and duration reported in experimental animal SCI (90–135 mg/kg).

Graphical Abstract

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The authors declare no competing financial interest.

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1. Introduction

Traumatic spinal cord injury (SCI) causes partial or complete loss of motor, sensory, and autonomic functions below the injury site. The initial trauma is followed by a wave of secondary injury cascades that leads to progressive tissue damage and cavitation [1], resulting in deleterious functional loss. Thus, therapies that can limit secondary injury will reduce the extent of disability resulting from SCI and improve functional recovery. Many mechanisms contribute to the secondary injury, including inflammation, cellular damage from free radicals such as reactive oxygen species (ROS) and nitric oxide (NO), glutamate excitotoxicity, calcium influx, ischemia, hemorrhage, and edema [1–3]. However, current treatment strategies are highly specific, usually targeting only one or a few elements in the injury cascades, and have been largely unsuccessfully in clinical trials. Minocycline hydrochloride (MH) is a clinically available antibiotic and anti-inflammatory drug that also exhibits potent neuroprotective activities [4–6]. It has been shown to target all of the aforementioned secondary injury mechanisms via its anti-inflammatory, anti-oxidant, and anti-apoptotic properties [2, 7–14]. Consequently, MH was considered the highest scoring neuroprotective therapy for SCI in a recent systematic review of pre-clinical data [15].

A number of studies have shown that systemic administration of MH for 1–5 days significantly reduced secondary injury and improved functional recovery in experimental animal models of SCI [2, 3, 7, 16, 17]. However, the doses of MH used in these studies (90–135 mg/kg/day for 1–5 days) are much higher than that used in a recent Phase II clinical trial (12–22.5 mg/kg/day for 7 days [18]) and the standard human dose of 3 mg/kg/day [19]. The clinical trial shows that intravenous (IV) administration of MH for 7 days only resulted 2.3 µg/ml MH in the cerebrospinal fluid (CSF) at steady state. While this concentration is sufficient for the anti-inflammatory effect [17, 20, 21], it is still far below the fully neuroprotective level of 35–75 µg/ml (1.5–50 µg/ml for mitochondrial cell death pathways [22] and glutamate toxicity [23], 10–40 µg/ml for hemorrhage-induced toxicity [13], and 35–75 µg/ml for excitotoxicity and Ca^{2+} influx [11], in a dose-dependent manner). Thus, it is likely that the modest clinical benefit observed in the phase II trial is more related to MH's anti-inflammatory effect rather than its direct neuroprotective effect. Although the potentially sub-optimal treatment dosing regimen in the clinical trial was suggested to be safe, one patient displayed elevated liver enzymes, indicating hepatocellular toxicity [18]. Thus, further increasing the systemically delivered dose of MH to reach its full neuroprotective capacity may not be safe for human patients. In addition, clinically the duration of MH treatment must be limited because long term systemic administration of high doses of MH has been shown to cause morbidity, liver toxicity, and even death [7, 24]. Local

delivery of MH could potentially expose the injured spinal cord tissue to high local concentrations of MH that systemic administration cannot safely achieve and prolong the duration of MH treatment, while avoiding the deleterious side effects from systemic exposure. However, no study on local delivery of high concentrations of MH to promote neuroprotection after SCI has been reported; this may possibly be due to the limitations of current drug delivery systems.

MH is a highly water-soluble small-molecule drug (MW 494 Da). It is released very quickly (less than 24 h) from hydrophilic drug delivery systems such as hydrogels [25, 26]. In addition, MH degrades rapidly in aqueous solution, especially at body temperature [27]. Thus, pumps cannot be used for continuous intrathecal delivery of MH. Poly (lactic-co-glycolic acid) (PLGA) microspheres or nanoparticles have been used for sustained release of MH [28, 29]. However, the low drug loading efficiency for MH (up to 1.92%) makes it impossible to administer a sufficient amount of drug in the limited intrathecal space for local delivery. A recent study reported the development of PEGylated poly-ε-caprolactone-based nanoparticles (PCL) for intraparenchymal delivery of MH to modulate inflammation after SCI [30]. However, the drug loading efficiency of PCL is also low (0.1%) [31], which limits the amount of MH that can be applied locally for effective neuroprotection. MH can form positively charged chelates with divalent metal ions including Ca^{2+} and Mg^{2+} [27, 32]. Utilizing this property, one study reported encapsulating MH into polyion complex (PIC) micelles through electrostatic interaction between MH- Ca^{2+} chelates and negatively charged carboxymethyldextran-*block*-poly (ethylene glycol) (CMD-PEG) [27]. Although the loading efficiency of MH in the PIC micelles was high (50%), drug release only lasted for 24 h.

For optimum treatment effect, the dose and duration of local MH release should match the progression of secondary injury. Studies have shown that loss of grey matter was usually completed within 24 h, and loss of white matter extended up to 7 days after SCI [36, 37]. Approximately 7 days after injury, a delayed wave of oligodendrocyte apoptosis was observed to occur in the white matter. The number of apoptotic cells decreased by 3 weeks, and apoptosis was nearly complete at 6 weeks [36, 38–40]. This delayed oligodendrocyte apoptosis, triggered by inflammation and withdrawal of trophic signals after axonal loss [1, 36, 41, 42], causes demyelination of surviving axons and subsequent conduction deficits or failure [1, 43]. It has been shown that a low concentration of MH (0.5 ng/ml) was sufficient to inhibit the production of neurotoxic molecules by reactive glial cells that lead to apoptosis of oligodendrocytes [17]. Thus, a release profile with high doses of MH release for 7 days for neuroprotection, followed by low doses of MH release for 3–6 weeks targeting chronic inflammation and delayed oligodendrocyte apoptosis potentially matches the progression of secondary injury.

We have developed novel dextran sulfate (DS)-MH complexes self-assembled by metal ion binding-mediated interaction for sustained release of bioactive MH [33]. DS is a negatively charged biocompatible polysaccharide that has high binding affinity for metal ions [34]. We found that Ca^{2+} or Mg^{2+} ions could induce formation of insoluble complexes between DS and MH [33]. The strong metal ion-mediated interaction enabled high drug loading efficiency (45.2%) and stable long term MH release. In the present study, we encapsulated the complexes into injectable agarose hydrogel so that the drug delivery system can remain

localized in the intrathecal space at the injury site (Fig. 1A). This route of drug delivery is preferred over epidural or intraparenchymal delivery because it bypasses the dura mater as a diffusion barrier and is not anticipated to cause any additional tissue damage [35]. However, as we previously reported, MH release from DS-MH complexes was slow due to the strong metal ion-mediated interaction between MH and DS [33]. As a result, we found that encapsulating the complexes into agarose hydrogel resulted in sustained low-dose MH release for 37 days. To develop a formulation that can release high dose of MH for effective neuroprotection after SCI, in this study we investigated the factors that control MH release and developed a formulation capable of releasing high doses of MH at the acute stage for neuroprotection, followed by low dose of MH release at the chronic stage targeting chronic inflammation and delayed apoptosis of oligodendrocytes. We quantified MH release *in vivo* and found that local delivery of MH at a dose of 1.3 mg/kg generated significantly higher MH concentration in the local spinal cord tissue than intraperitoneal (IP) injection of 495 mg/kg MH over 5 days. This is the first time that high concentrations of MH could be delivered to the local neural tissue at a human safe dose. Using a clinically relevant rat unilateral cervical contusion injury model, we investigated the efficacy of local delivery of MH using this formulation in promoting neuroprotection, immunomodulation (by assessing inflammation and microglia/macrophage polarization), and locomotor functional recovery.

2. Materials and methods

2.1. Fabrication of DS-MH complexes within agarose hydrogel

For low-dose release formulation, 2.4 mg/ml DS (500 kDa, Sigma-Aldrich) solution was prepared in 2X Hank's Balanced Salt Solution (HBSS) supplemented with 28.8 mM MgCl_2 . Agarose (SeaPlaque, Lonza) was subsequently dissolved in the DS solution at 70°C at a concentration of 3% (w/v). 2 mg/ml minocycline solution was prepared in deionized (DI) water. After agarose in DS solution was cooled down to 37°C, an equal volume of MH solution was added to the agarose-DS solution and well mixed. DS-MH complexes were formed within the agarose solution. Then the mixture solution was allowed to gel by cooling at 4°C for 30 min. For the fast-release formulations, the DS/MH ratio was changed to 2:3 and no additional MgCl_2 was added into the system (the Mg^{2+} in the HBSS was used for complex formation). Chitosan (Sigma-Aldrich) was dissolved in 0.1 M acetic acid and added into MH solution. After mixing MH-chitosan solution with agarose-DS solution, an equivalent amount of NaOH was added into the mixture solution to neutralize the acid. Then the mixture solution was allowed to gel at 4°C.

2.2. In vitro MH release

Agarose hydrogels loaded with DS-MH complexes were injected at room temperature and were incubated at 37°C in HBSS (pH 7.4) for quantification of MH release. The release media were changed every 24 h. The amount of MH released every 24 h was measured by UV absorbance at 245 nm (the other components in the drug delivery system have negligible absorbance at this wavelength).

2.3. Bioactivity of released MH

Rat cortical neurons were used to assess the neuroprotective activity of released MH as previously reported [33]. Briefly, neurons were isolated from E17 rat embryos in accordance with protocols approved by Drexel University's IACUC committee. The cells were maintained in Neurobasal medium supplemented with 2% B27 supplement and 2 mM L-glutamine. On day 7, the culture medium was replaced with Neurobasal medium supplemented with B27 minus antioxidants (AOs) to remove the neuroprotective AOs from the culture medium. Then the neurons were treated with 100 μ M hydrogen peroxide (H_2O_2) to induce oxidative stress. 40 μ g/mL fresh MH or MH released on day 1 (diluted to 40 μ g/mL) was added to the H_2O_2 -treated cultures to assess the neuroprotective activity of MH. After 6 h, the cells were stained with 2 μ M calcein AM (live staining) and imaged with an inverted fluorescence microscope (Leica). The number of live cells were quantified using Image J software.

RAW264.7 murine macrophages were used to assess the anti-inflammatory activity of released MH. The macrophages (kindly provided by Dr. Narayan Avadhani, University of Pennsylvania) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were stimulated with 300 pg/ml lipopolysaccharide (LPS) for upregulation of NO, a neurotoxic molecule produced by reactive microglia and macrophages after SCI. Fresh MH (0.5 μ g/ml) or MH released from the hydrogel on day 44 (diluted to 0.5 μ g/ml) was added to the LPS-stimulated cultures to assess the anti-inflammatory activity of released MH. After 48 h, the levels of accumulated nitrite in the culture medium, as an indicator of NO, was determined using Griess reagent (Promega).

2.4. Animal surgery and administration of hydrogel/complexes

All experimental procedures were performed in compliance with protocols approved by Drexel University's IACUC committee and followed National Institutes of Health guidelines for the care and use of laboratory animals. Rats were randomly assigned to experimental groups, and different treatments were randomly distributed across these rats. We utilized a well-characterized and clinically relevant unilateral cervical contusion injury model in this study [44, 45]. Briefly, female Sprague-Dawley rats (250–300 g) were anesthetized with a cocktail of ketamine (60 mg/kg), xylazine (6 mg/kg) and acepromazine (0.5 mg/kg). A unilateral laminectomy was performed at the C5 level to expose the underlying spinal cord segment. The vertebral column was stabilized and the exposed spinal cord was impacted at a force of 200 kDyne, using an Infinite Horizons (IH) device (Precision Systems and Instrumentation, Lexington, KY). The diameter of the impactor tip was 1.6 mm. For histological and behavioral evaluations, four experimental groups were used in this study: (1) untreated injury control (n = 9); (2) 30 μ L agarose hydrogel loaded with DS-MH complexes at room temperature was injected into the intrathecal space at the injury site until the injured spinal cord was full covered with gel, then the remaining gel (~ 5 μ L) and another 100 μ L gel of the same formulation was applied on top of the dura to further increase MH concentration in the local spinal cord tissue (n=11); (3) blank agarose hydrogel was applied following the same procedure as group 2 (n=9); and (4) IP injection of MH at a dose of 90 mg/kg immediately after SCI, and then at a dose of 45 mg/kg every 12 h for 5

days (highest doses and longest duration reported in experimental animal SCI [2]) as positive control (n=9). In addition, SCI rats receiving MH treatments as described in experimental group (2) and (4) were used for quantification of MH concentration in the local spinal cord tissue (n=4).

2.5. Quantification of MH concentration in spinal cord tissue

1, 3, 7 and 21 days after MH administration, the rats were euthanized with Euthazol. MH concentrations in C5 segments were measured by HPLC as reported by Milane et al. [46]. Each segment was homogenized in 100 μ L ice-cold phosphate buffer (0.01 M, pH 7.4). 2.5 μ L of phosphoric acid (H_3PO_4) was added to the homogenate, and the mixture was vortexed and centrifuged at 2,000 rpm for 30 min at 4°C. MH was extracted to the supernatant. The precipitate was re-dissolved in 100 μ L phosphate buffer and centrifuged. The second supernatant was combined with the first supernatant for HPLC analysis to measure MH concentration. HPLC analysis was performed on a Waters 1525 Binary HPLC system (Milford, MA) equipped with a UV/visible detector and a reversed phase C18 column (Waters, 75 $\times$ 4.6 mm, 3.5 μ m). The mobile phase consists of phosphate buffer (25 mM, pH 3.0)/methanol/acetonitrile at a volume ratio of 91:6:3 and ran at a flow rate of 1.53 mL/min. The sample injection volume was 203 μ L and the UV detection wavelength was 2453 nm.

2.6. Histological assessment of lesion volume, tissue sparing and inflammation

Six weeks post-injury, animals were euthanized and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Cervical spinal cords were harvested, post-fixed in 4% paraformaldehyde at 4°C overnight, and transferred to 30% sucrose in PBS for cryoprotection. Cryostat sections (20 μ m thick) were cut in the transverse plane. A series of sections 160 μ m apart were stained for cresyl violet for Nissl bodies and euryochrome cyanine (Sigma) for myelin. The lesion area on each section was analyzed and quantified using ImageJ software to calculate the total lesion volume.

For immunohistochemistry, the tissue sections were permeabilized and blocked with 4% normal goat serum/0.3% Triton X-100 (Sigma-Aldrich) in 0.01 M PBS for 1 h, and then incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: anti-myelin basic protein (MBP, 1:200, Covance) to identify myelin, anti-neurofilament-H (NF-H, 1:500, Millipore) to identify axons, anti-CD68 (1:1000, AbD Serotec) to identify reactive microglia/macrophages, anti-iNOS (1:5000, Millipore) to identify M1 phenotype and anti-arginase 1 (1:500, Santa Cruz Biotechnology) to identify M2 phenotype. After washing, the sections were incubated with secondary antibodies conjugated to Alexa fluorophores (1:200, Invitrogen) at room temperature for 2 h. All the sections were counterstained with nuclear dye 4'-6-Diamidino-2-phenylindole (DAPI, Molecular Probes). For visualization of CD68, iNOS and arginase 1 stainings, tissue sections were imaged with a Zeiss fluorescence inverted light microscope. For visualization of MBP and NF-H staining, confocal fluorescence images were captured using an Olympus FV1000 laser scanning confocal microscope. For quantification of the number of MBP⁺/NF-H⁺ myelinated axons, CD68⁺ reactive microglia/macrophages, iNOS⁺/CD68⁺ M cells and arginase 1⁺/CD68⁺ M2 cells, tissue sections were imaged with a Zeiss AxioObserver Wide Field inverted microscope equipped with Slidebook 6 software with stereology module. All

the quantifications were conducted by an experimenter who was blinded to the experimental groups. The percentages of M1 and M2 microglia/macrophages were determined by dividing the number of iNOS⁺/CD68⁺ (M1) and arginase 1⁺/CD68⁺ (M2) cells by the number of CD68⁺ (pan-microglia/macrophage) cells, respectively. A ratio of the percentage of M1 cells to M2 cells was also calculated as follows:

$$\text{M1:M2} = \% \text{M1 cells} / \% \text{M2 cells}$$

2.7. Behavioral analysis

Prior to and weekly after injury, behavioral tests were conducted by 2–3 observers in a blinded manner for up to 6 weeks before sacrifice. Forelimb Locomotor Scale (FLS) test was used to assess the animals' abilities to utilize their affected forepaw during locomotion in the open-field. FLS is a 17-point scale that evaluates deficits based on range of joint movements, degree of weight support, and forepaw placement [44, 45]. Additionally, skilled locomotion on a grid platform was performed to further evaluate the sensory motor coordination after SCI. The performance of grid walk test was evaluated by the percentage of correct steps over total steps.

2.8. Statistical analysis

Repeated-measures ANOVA followed by post-hoc analysis (Bonferroni's test for FLS and Tukey's test for grid walking) for multiple comparisons was used for behavioral analysis. For all the other studies, we used one-way ANOVA followed by Tukey's post-hoc tests for multiple comparisons between groups.

3. Results and discussion

3.1. *In vitro* MH release from hydrogel loaded with DS-MH complexes

We have previously reported metal ion-assisted self-assembly of DS-MH complexes (with size ranging from 50 to 100 nm) for sustained release of MH [33]. Given that calcium is potentially neurotoxic and magnesium is a neuroprotective agent that has been evaluated in large clinical trials of neural injuries [15, 47], we used Mg²⁺-based DS-MH complexes in the present study. We encapsulated the DS-MH complexes in injectable agarose hydrogel so that the complexes can remain localized in the intrathecal space at the injury site (Fig. 1A). As shown in Fig. 1B, the hydrogel can be easily injected from a 30G needle that is attached to a Hamilton syringe. It remained as hydrogel at 37°C both *in vitro* and *in vivo*.

At the loading of 1 mg MH/mL hydrogel, sustained low-dose MH release for 37 days was achieved (Fig. 2A, low-dose). The slow release from DS-MH complexes was mediated by the strong metal ion-mediated interactions between DS and MH [33]. However, high doses of MH release is required for effective neuroprotection after SCI. We found that increasing drug loading increased the release duration, but had little effect on the dose of drug release. As shown in Fig. 2B, for the low-dose formulation increasing drug loading from 0.3 to 1 mg/mL greatly reduced the release rate, resulting in prolonged duration from 11 to 37 days but only mild increase in dose released daily. To accelerate MH release to achieve high dose release at the acute stage, we investigated the factors that can accelerate MH release. We

found that this could be achieved by reducing DS/MH ratio and Mg^{2+} concentration (Fig. S1A and B), through weakening the interactions between DS and MH. Furthermore, adding chitosan, a biocompatible cationic polysaccharide, to compete with MH for the binding to DS and Mg^{2+} ions also accelerated MH release (Fig. S1C). We also increased MH loading to 3 mg/mL to allow for sufficient dose and duration of MH release. By modulating these factors that control MH release, we have developed a formulation capable of releasing high-dose of MH at the acute stage to target neuroprotection, followed by sustained low-dose of MH to target chronic inflammation and delayed apoptosis of oligodendrocytes (Fig. 2A, high-dose). MH release from the high-dose formulation was significantly higher than that from the low-dose formulation throughout the duration of release except on day 32. The loading efficiency of MH in the high-dose formulation complexes was 40.6%. Fig. 2B shows that the release rate of MH from the high-dose formulation (3 mg/mL) was close to that from the low-dose formulation (1 mg/mL) despite three times increase in drug loading. This result confirmed that reducing DS/MH ratio and Mg^{2+} concentration, and adding chitosan accelerated MH release.

We have previously shown that released MH retained the same bioactivity as fresh MH throughout the duration of release from Mg^{2+} -based DS-MH complexes [33]. To obtain high dose of MH release at the acute stage and localize the complexes at the injury site, in the present study we added chitosan in the complex and encapsulated the complexes into agarose hydrogel. To investigate whether these modifications affected the bioactivity of MH, we performed neuroprotective and anti-inflammatory assays using MH released from the high-dose formulation at acute and chronic stages, respectively. H_2O_2 is one of the neurotoxic reactive oxygen species that is elevated acutely after SCI [48]. Fig. 2C shows that H_2O_2 treatment induced massive neuronal loss (82%). Both fresh and released MH significantly increased neuron survival by 3-fold compared to H_2O_2 -treated group, and the cell viability of both groups were not significantly different from each other. This result suggests that fabrication of the drug delivery system did not affect the bioactivity of MH. Fig. 2D shows that LPS stimulation significantly upregulated NO production by macrophages. NO is a neurotoxic free radical produced by reactive microglia/macrophages after SCI. Both fresh MH and MH released on the last day of release (day 44) significantly inhibited NO production by LPS-stimulated macrophages. There was no significant difference between the two groups, suggesting that MH released from this drug delivery system retained the same bioactivity as fresh MH throughout the duration of release.

3.2. *In vivo* MH concentration in the local spinal cord tissue

Most SCI patients have injuries at the cervical level and the injuries are typically contusive [43, 50, 51]. We used a clinically relevant unilateral cervical contusion injury model in which spinal cords of adult rats were impacted at C5 level. In this injury model, only the grey and white matter ipsilateral to the injury was damaged. Agarose hydrogel loaded with MH was injected into the intrathecal and epidural space. The procedure to inject a medication in these places are within the normal practices of clinical medicine currently and are performed thousands of times a day in the United States. The injections most often performed currently are epidural injections. The practice of performing intrathecal injections is also utilized for anesthesia purposes (i.e. spinal anesthesia) or placement of intrathecal

catheters. Thus, our route of drug delivery can be used for human patients. MH concentrations in the C5 segments were measured by HPLC. Fig. 3 shows that hydrogel loaded with DS-MH complexes (MH gel) was capable of locally delivering high doses of MH for at least 3 days *in vivo* (35.74 to 24.73 $\mu\text{g/mL}$ from day 1 to 3 in the local spinal cord tissue), and MH concentration in the C5 segment on day 1 was significantly higher than that of the rats receiving IP treatment. MH concentrations in both groups were not significantly different from each other on days 3 and 7. At day 21, we could still detect a low MH concentration of 1.00 $\mu\text{g/mL}$ in the C5 segments of rats receiving MH gel treatment. This concentration is still sufficient to target chronic inflammation and delayed apoptosis of oligodendrocytes [17, 20, 21]. MH was undetectable at 28 days. It is noteworthy that although MH concentrations in MH gel and IP groups at day 3 to 7 were similar, the dose used in the IP group (495 mg/kg over 5 days) was substantially higher than that used in the clinical trial (99 mg/kg over 7 days) and cannot be used on human patients. In contrast, the total amount of MH administered by MH gel is estimated to be 1.3 mg/kg over at least 21 days. This dose was much lower than the standard human dose of 3 mg/kg/day, which is safe to take for 10.5 months [49]. Thus, the dose delivered by MH gel is safe for human use. This study confirmed our hypothesis that local delivery of MH at a dose that is lower than the standard human dose can expose the injured spinal cord tissue to higher concentrations of MH than systemic administration can safely achieve.

3.3. Local MH treatment was more effective than high doses of IP treatment in reducing lesion volume

Six weeks after SCI, Nissl myelin-stained sections spanning the entire lesion were used to determine the total lesion volume. Fig. 4 shows that local MH gel treatment significantly decreased the total lesion volume by 58.5% compared to untreated control. In contrast, IP injection of MH with the highest dose and duration reported in experimental animal SCI only decreased lesion volume by 29.8%, resulting in significantly higher lesion volume compared to the MH gel group. Moreover, the lesion volume of animals receiving blank gel treatment without MH was not significantly different from that of untreated control, indicating that the neuroprotective effect was from MH and this delivery method (drug delivery system and route of delivery) did not cause any additional tissue damage. Thus, this drug delivery method represents a safe and non-invasive approach for local delivery of MH.

Local MH treatment was more effective in tissue protection than IP injection possibly because it generated higher MH concentration in the spinal cord tissue and provided longer duration of MH treatment (at least 21 days) than IP treatment. Studies have shown that 35 $\mu\text{g/mL}$ was the lowest concentration of MH that can protect neurons from excitotoxicity and Ca^{2+} influx, which are important contributors to the secondary injury after SCI [1–3]. Our HPLC study shows that 24 h after administration, MH concentration in the local spinal cord tissue of rats receiving MH gel treatment was 35.74 $\mu\text{g/mL}$, whereas that in the IP group was 24.97 $\mu\text{g/mL}$ which is insufficient to target the two important secondary injury mechanisms. In addition, the prolonged MH treatment delivered by MH gel could modulate the persistent chronic inflammation contributing to delayed apoptosis of oligodendrocytes, which provided further tissue protection.

3.4. Local MH treatment was more effective than IP treatment in protecting axons and myelin

Axons traversing the injury site are the only remaining connections between the brain and caudal spinal neurons, therefore the functional consequences of SCI are mostly determined by the level and extent of damage to the white matter [1, 50, 52]. In this study, preservation of axons and myelin was assessed by quantification of the number of myelinated axons as a function of distance from the lesion epicenter. Confocal images (Fig. 5A) show that at the lesion epicenter as well as at distances 0.96 mm rostral and caudal to the epicenter, only animals receiving local MH gel treatment preserved many myelinated axons in the spared tissue surrounding the injury cavity. Fig. 5B confirmed that local MH gel treatment preserved significantly more myelinated axons compared to the other three groups at the lesion epicenter as well as at distances 0.96 and 1.92 mm rostral and caudal to the epicenter, whereas IP injection of high doses of MH had no significant effect on white matter protection at these locations. At distances 2.88 mm rostral and caudal to the lesion epicenter, both local MH gel and IP treatments significantly increased the number of myelinated axons compared to untreated control, and there was no significant difference between the two treatments. The result that local MH gel treatment was effective in protecting white matter at all locations studied, whereas IP treatment was only protective at distances further away from the lesion epicenter suggests that in the region where tissue was directly injured from the impact (close to lesion epicenter), the neuroprotective effect of local MH gel treatment was more pronounced than systemic IP treatment. It is possible that more severe injury requires higher concentration of MH for effective protection, whereas modest injury is less sensitive to MH concentration. Loss of white matter has been reported to extend to 7 days after SCI, followed by delayed apoptosis of oligodendrocytes which was nearly complete at 6 weeks. In the present study, we show that local delivery of high concentrations of MH for 3 days followed by low doses of MH for at least 21 days demonstrated superior efficacy in protecting white matter. However, MH concentration in the local spinal cord tissue was lower than the fully neuroprotective level of 35 µg/mL at day 3 and 7 after MH gel administration. It is possible that further increasing the dose of local MH treatment within the first 7 days after SCI would be more effective in promoting neuroprotection, which warrants further investigation. The interplay among the four factors (drug loading, DS/MH ratio, Mg^{2+} concentration, and adding chitosan) that modulate MH release is complex. Changing one parameter will change the optimum value of another parameter. We are still working on optimizing this drug delivery system to achieve higher drug release in the first 7 days. At all the locations quantified, blank gel treatment did not significantly change the number of myelinated axons compared to untreated control, further confirming the safety of this drug delivery method.

3.5. Both local MH treatment and IP injection were effective in modulating inflammation

Inflammation is an important mediator in the secondary injury cascades after SCI [1, 3]. M1 (classical activation) and M2 (alternative activation) microglia/macrophages are the two primary subsets of microglia/macrophages at the injury site [53]. The M1 phenotype produces proinflammatory cytokines that exacerbate the inflammatory response and releases neurotoxic molecules contributing to tissue damage, whereas the M2 phenotype is immunomodulatory and is involved in repair and regeneration [53]. MH is a potent anti-

inflammatory agent that has been shown to reduce microglial reactivity after SCI [2, 7]. Moreover, it has been shown to selectively inhibit M1 polarization of microglia *in vitro* and *in vivo* [30, 54].

To assess inflammation and microglia/macrophage polarization, 6 weeks after SCI, the total numbers of CD68⁺ reactive microglia/macrophages, iNOS⁺/CD68⁺ cells (M1 phenotype) and arginase 1⁺/CD68⁺ cells (M2 phenotype) within a 3.84 mm segment spanning 1.92 mm rostral and caudal to the lesion epicenter were quantified stereologically using images taken at a high magnification (20x). Low magnification images were used to show the overall distribution of CD68⁺ cells (Fig. 6A). Fig. 6B shows that both local MH gel and IP treatments significantly reduced the total number of CD68⁺ cells compared to untreated control. In addition, the number of CD68⁺ cells in the MH gel group was significantly less than that in the IP group. This result suggests that local MH treatment was more effective than IP injection in reducing inflammation. It is possible that the higher MH concentration from local delivery exerted a stronger anti-inflammatory effect in a dose-dependent manner. In addition, since inflammation following SCI is caused by tissue injury, reduced secondary injury in the MH gel group may also led to reduced inflammation.

Further examination of microglia/macrophage phenotype revealed significant reduction of the percentage of M1 cells (%M1), but no significant change of %M2 in the MH gel group compared to untreated injury control and blank gel groups (Fig. 7A and B). This is consistent with a previous report showing that selective targeting resident microglia but not recruited macrophages with MH reduced M1 proinflammatory phenotype but had no effect on M2 phenotype in a mouse model of SCI [30]. It is interesting that systemic IP treatment did not significantly reduce %M1, but significantly increased %M2 phenotype compared to untreated control. It is unclear why local and systemic MH treatments had opposite effect on microglia/macrophage polarization. Both resident microglia and recruited peripheral macrophages constitute the reactive microglia/macrophage population after SCI [54]. A previous study shows that in a SOD1^{G93A} mouse amyotrophic lateral sclerosis (ALS) model, IP injection of MH selectively inhibited M1 polarization of microglia/macrophages, whereas M2 polarization was not affected [54]. However, in this transgenic disease model only very few recruited macrophages could be found in the spinal cord [56]. Therefore, this study also only studied the effect of MH on local resident microglia, but not recruited macrophages. Our MH gel treatment could target both resident microglia and recruited macrophages in the injured spinal cord, which led to selective inhibition of M1 polarization. This result suggests that once the peripheral macrophages entered the injured spinal cord, the effect of MH on these cells was the same: selective inhibition of M1 polarization. Conversely, under IP treatment the peripheral macrophages were exposed to MH before they entered the injury site. It is possible that IP injection may cause some systemic effects on peripheral macrophages, and the complex interplay between the recruited macrophages that were pre-modulated by MH and resident microglia contributed to enhanced M2 polarization in the injured spinal cord tissue.

We further analyzed M1/M2 ratio, which is suggested to have significant implications for CNS repair [53]. Fig. 7C shows that both local MH gel and systemic IP treatments significantly reduced the M1/M2 ratio compared to untreated control and blank gel groups,

and there was no significant difference between the two treatments. During successful normal wound healing the M1/M2 ratio has been shown to increase in the first few days and go back to baseline level 10 days after injury, whereas for SCI and non-healing diabetic wounds, the M1/M2 ratio remains to be elevated [53, 57]. Our study shows that both local and systemic MH treatments could effectively reduce the M1/M2 ratio, although the mechanisms are different: one through inhibition of M1 polarization and the other through enhancing M2 polarization. It is possible that combining both treatments can modulate both phenotypes simultaneously and further decrease the M1/M2 ratio. It is noteworthy that although IP treatment led to a similar M1/M2 ratio as MH gel treatment, the latter demonstrated a much stronger neuroprotective effect and tissue sparing as shown in Fig. 4 and 5. This result suggests that immunomodulation was not the only mechanism that contributed to tissue protection from MH treatment, as MH has direct neuroprotective effect at high concentrations. There was no significant difference between control and blank gel groups in the number of CD68⁺ reactive microglia/macrophages, %M1, %M2, or M1/M2 ratio, suggesting that this drug delivery method did not have any effect on inflammation after SCI.

3.6. Local MH treatment was more effective than high doses of IP injection in improving locomotor functional recovery

Prior to and weekly after injury, the rats underwent behavioral tests including FLS and grid walking. Both tests have previously been used in the unilateral cervical injury model, and were able to detect significant, permanent deficits in untreated, injured animals after an initial period of spontaneous recovery [44]. Fig. 8 shows that local MH gel treatment significantly improved FLS and grid walking scores compared to untreated control and blank gel groups over the time course of 1–6 weeks after injury, whereas IP injection of high doses of MH (6–7.5 times higher than that used in clinical trials) only significantly improved FLS and grid walking scores at earlier time points (week 1–3 for FLS and 1–4 for grid walking), but not at later time points (week 4–6 for FLS and 5–6 for grid walking) compared to untreated control. It is noteworthy that the insignificant improvement by IP treatment at later time points was due to the increased variance from multiple comparisons of all four groups. If we only do multiple comparisons between IP, MH gel, and untreated control groups to evaluate the efficacy of the two MH treatments, then IP treatment significantly improved both FLS and grid walking scores 1–6 weeks after injury. From this perspective, our result is consistent with previous studies showing that IP injection of high doses of MH significantly improved functional outcomes [2, 3, 7, 16, 17]. Nonetheless, the behavior results from the present study suggest that local MH gel treatment was more effective than IP injection of high doses of MH in promoting locomotor functional recovery. There was no significant difference between blank gel and untreated control groups for both behavioral tests at any time point studied (Fig. 8), confirming the safety of our drug delivery system and route of delivery.

4. Conclusions

The potential of MH to target many secondary injury mechanisms and protect neural tissue from multiple neurotoxic insults after SCI has been well documented. However, the inability

to translate the high doses used in experimental animals to tolerable doses in human patients limits its clinical efficacy. In this study, we developed a novel polysaccharide-based drug delivery system for local delivery of MH with appropriate dose and duration. We demonstrated that local delivery of MH at a safe human dose could expose the injured spinal cord tissue to high concentrations of MH that systemic administration cannot safely achieve, and was more effective in promoting neuroprotection and functional recovery than systemic injection of MH at doses that are 6–7.5 times higher than the highest tolerable human dose. This study for the first time proves that high concentrations of MH are required for effective treatment of SCI. All the materials used in the drug delivery system are biocompatible natural polysaccharides which did not elicit any adverse effect. Thus, this drug delivery system has great therapeutic potential for effective treatment of SCI. In addition, numerous studies have demonstrated the remarkable capacity of MH to provide neuroprotection in a variety of CNS injuries and neurodegenerative diseases, such as traumatic brain injury, stroke, intracerebral hemorrhage, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis. However, the effectiveness of MH treatment for these disorders has been compromised by the inability to locally deliver high concentrations of this medication. Thus, the technology developed from this study not only can be used for spinal cord repair, but may also have great therapeutic potential for the treatment of a variety of other debilitating neurological disorders and injuries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Agarose hydrogel loaded with DS-MH complexes can be injected into the intrathecal space for controlled local delivery of MH. (A) Schematic illustrating intrathecal injection of complex-loaded hydrogel to bypass the diffusion barrier of dura mater. (B) The complex-loaded hydrogel can be easily injected from a Hamilton syringe.

Fig. 2.

In vitro MH release and bioactivity of released MH. (A) MH release from agarose hydrogel loaded with different formulations of complexes (n=3). (B) Effect of drug loading on MH release (n=3). (C) Neuroprotective activity (n=3) and (D) Anti-inflammatory activity of released MH (n=4). *** $P < 0.001$ compared to H₂O₂ or LPS-treated group. Data shown are average $\pm$ SEM.

Fig. 3.

In vivo MH concentration in the C5 segments. * $P < 0.05$ compared to IP injection. Data shown are average $\pm$ SEM ($n = 4$).

Fig. 4. Local delivery was more effective than systemic injection of MH in reducing lesion volume. (A) Nissl Myelin staining 6 weeks after SCI. Scale bar = 1 mm. (B) Quantification of total lesion volume. * $P < 0.05$ and **** $P < 0.0001$ compared to untreated control; +++++ $P < 0.0001$ compared to blank gel; # and $P < 0.05$ compared to IP injection. Data shown are average $\pm$ SEM.

Fig. 5.

Local delivery was more effective than systemic injection of MH in white matter protection. (A) Confocal images show the integrity of white matter 6 weeks after SCI. Tissue sections were double stained for MBP (myelin, green) and NF (axon, red). Scale bar = 20 μ m. (B) Quantification of the number of myelinated axons. *** P < 0.001 and **** P < 0.0001 compared to untreated control; + P < 0.05, ++ P < 0.01, +++ P < 0.001 and ++++ P < 0.0001 compared to blank gel; ## P < 0.01, ### P < 0.001 and #### P < 0.0001 compared to IP injection. Data shown are average $\pm$ SEM.

Fig. 6.

Local delivery was more effective than systemic injection of MH in reducing inflammation. (A) CD68 staining for reactive microglia/macrophages 6 weeks after SCI. Scale bar = 1 mm. (B) Quantification of total number of CD68⁺ cells within a 3.84 segment centered at the lesion epicenter. * $P < 0.05$ and **** $P < 0.0001$ compared to untreated control; ++++ $P < 0.0001$ compared to blank gel; # $P < 0.05$ and ## $P < 0.01$ compared to IP injection. Data shown are average $\pm$ SEM.

Fig. 7.

Both Local and systemic MH treatments modulated microglia/macrophage polarization. (A) 6 weeks after SCI, tissue sections at the lesion epicenter were double stained for CD68 (red) and iNOS (green) to identify M1 polarization or CD68 (red) and arginase 1 (green) to identify M2 polarization. Scale bar = 200 μ m. (B) Quantification of percentage of iNOS⁺/CD68⁺ M1 cells, percentage of arginase 1⁺/CD68⁺ M2 cells, and M1/M2 ratio within a 3.84 segment centered at the lesion epicenter. * P < 0.05, ** P < 0.01 and *** P < 0.001 compared to untreated control; + P < 0.05 and +++ P < 0.001 compared to blank gel; # P < 0.05, ## P < 0.01 and ### P < 0.001 compared to IP injection. Data shown are average $\pm$ SEM.

Fig. 8. Local delivery was more effective than systemic injection of MH in improving behavioral outcomes after SCI. (A) FLS and (B) Grid walking scores. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$ compared to untreated control; + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$ and ++++ $P < 0.0001$ compared to blank gel. Data shown are average $\pm$ SEM.