Ethamsylate attenuates mutilated secondary pathogenesis and exhibits a neuroprotective role in experimental model of spinal cord injury

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Abstract

Deficits in the neuronal connection that succumbs to the impairment of sensory and motor neurons are the hallmarks of spinal cord injury (SCI). Secondary pathogenesis, which initiates after the primary mechanical insult to the spinal cord, depicts a pivotal role in producing inflammation, lesion formation and ultimately causes fibrotic scar formation in the chronic period. This fibrotic scar formed acts as a major hindrance in facilitating axonal regeneration and is one of the root causes of motor impairment. Cascade of secondary events in SCI begins with injury-induced blood spinal cord barrier rupture that promotes increased migration of neutrophils, macrophages, and other inflammatory cells at the injury site to initiate the secondary damages. This phenomenon leads to the release of matrix metalloproteinase, cytokines and chemokines, reactive oxygen species, and other proteolytic enzymes at the lesion site. These factors assist in the activation of TGF- β 1 signalling pathway, which further leads to excessive proliferation of perivascular fibroblast, followed by deposition of collagen and fibronectin matrix, which are the main components of the fibrotic scar. Subsequently, this scar formed inhibits the propagation of action potential from one neuron to adjacent neurons. Ethamsylate, an anti-hemorrhagic drug, has the potential to maintain early hemostasis as well as restores capillary resistance. Therefore, we hypothesized that ethamsylate, by virtue of its anti-hemorrhagic activity, reduces hemorrhagic ischemia-induced neuronal apoptosis, maintains the blood spinal cord barrier integrity, and decreases secondary damage severity, thereby reduce the extent of fibrotic scar formation, and demonstrates a neuroprotective role in SCI.

Introduction

Spinal cord injury (SCI) is a traumatic life challenging neuro-deficit condition that negatively impacts the physical health, emotional health, financial condition, and social well-being of individuals and their beloved. Although, the present scenario is fairly better than the past decades as the survival rate and living expectancy have been drastically improved. Nevertheless, the current treatment is inadequate to deal with the complications associated with SCI. The ultimate cause of it is believed to be the pathophysiology of SCI, which is highly complex and consists of multiple events that depict a critical role in inducing scar formation. The whole pathology begins with the primary mechanical insult to the spinal cord due to any mechanical force, which induces structural damage to the spinal cord followed by the actual cellular and tissue destruction being marked from the initiation of the secondary pathogenesis [1]. This secondary injury poses the main threat to the irreversible damage imposed on the spinal cord *via* the formation of an inevitable scar in the long run. The prime suspect in initiating the cascade of secondary damages is believed to disrupt the blood spinal cord barrier (BSCB) [2]. It is a tight junction (TJ) of highly specialized capillaries consisting of non-fenestrated endothelial cells. This particular barrier protects the spinal cord from any pathogens, inflammatory cells, or toxins in normal conditions. However, in the case of SCI, mechanical insult causes barrier destruction and increases its permeability. This leads to infiltration of neutrophils, macrophages and other immune cells that ultimately mediates inflammation, edema and lesion formation [3]. MMP-9, a matrix metalloproteinase is a zinc containing endopeptidase

which is demonstrated to be tremendously upregulated in the acute phase of injury. Findings reveal that drastic upregulation of MMP-9, leads to the destruction of the basal lamina of BSCB, alters its permeability, and facilitates extravasation of harmful components [4]. On the other hand, TJ proteins, *viz* claudin and occludin are involved in maintaining the integrity of BSCB [5].

As mentioned above, mechanical destruction of BSCB is augmented by overproduction of MMP-9 and eradication of TJ proteins in the acute phase of injury. Thus, neutrophil infiltration is facilitated, followed by migration of other leukocytes and immune cells at the injury area. This leads to the accumulation of the immune or other inflammatory cells, which results in the excessive release of certain cytokines and chemokines at the injury site. This deposition of inflammatory mediators exacerbates the inflammatory condition, and subsequently, lesion formation occurs. In the chronic period, this lesion develops into a scar. The scar formed in SCI is composed of fibrotic scar, surrounded by glial scar. Fibrotic scar developed comprises fibroblast, neutrophils, macrophages, and excessive matrix protein accumulation such as fibronectin, collagen, and laminin [6]. The fibrotic scar formed has a pronounced effect on axonal degeneration and acts as a physical barrier in the axonal growth. This axonal degeneration interrupts the transfer of message from one neuron to another neuron, which causes loss of sensory and motor function, ultimately causing paralysis. Since decade ago, the theory behind the formation of fibrotic scar is linked with TGF- β 1 signaling [7]. It is supported by well-defined signaling pathways that depict that binding of active TGF- β 1 to the TGF- β 1 receptor on the membrane induces the activation of co-smad proteins, followed by its translocation into the nucleus. This accounts for promoting the activation of certain transcriptional factors that trigger the fibroblast proliferation and induces the generation of matrix proteins like fibronectin, collagen, and elastin [8]. Therefore, it can be said that disruption of BSCB together with TGF- β 1 mediated action plays a leading role in the formation of fibrotic scar. Besides, MMP-9 also promotes the activation of latent TGF- β 1 through the release of TGF- β 1 from its binding protein [9]. This clearly proves how MMP-9 aid in triggering TGF- β 1 signalling, indirectly contributing to the process of fibrotic scar formation.

Ethamsylate, an anti-hemorrhagic agent, is a well-established drug in the market. It is well-tolerated, with no significant adverse effects being reported. In clinical settings, it is used for menorrhagia, post-partum bleeding, and periventricular hemorrhage. Its mode of action is to increase the platelet adhesiveness and capillary resistance [10]. Moreover, a previous study carried out by Fan and his colleague reveals that Ethamsylate, an anti-hemorrhagic drug, has the potential to maintain early hemostasis and thereby improves functional recovery in contusion and transection induced model of SCI [11]. However, this study is carried out till the intermediate phase of SCI (DPI-10), and other long-term beneficial effects of ethamsylate are still unknown. Hence, the current study investigated the role of ethamsylate on secondary pathogenesis, vascular stabilization, and fibrotic scar formation in the chronic compression model of SCI. Therefore, we hypothesized that ethamsylate protects the BSCB *via* its action on capillaries, reduces the accumulation of cytokines and chemokines at the injury site, and decreases matrix destruction. Moreover, excessive bleeding results in loss of oxygen and nutrient supply to the neuronal cell, causing hemorrhagic ischemia resulting in apoptosis and necrosis at the injury site. This sets the particular area suitable for fibrotic scar formation. Therefore, ethamsylate being an anti-hemorrhagic

drug, can reduce bleeding, restores capillary resistance, and protect the spinal cord from secondary damages. Hereby, it contributes in reducing the extent of fibrotic scar formation and confers a neuroprotective role in SCI.

Materials And Methods

Model Induction: compression model

A total of 64 C57BL/6J female mice were used to carry out this study. All the animals were procured with approval from Institutional Animal Ethics Committee (IAEC/20/017). Surgical procedures and postoperative care were carried out under the guidelines and instructions provided by IAEC. Animals were anesthetized by injecting 1:4 of ketamine and xylazine intraperitoneally. After that, an incision was done, followed by exposure of the spinal cord through a laminectomy. For inducing compression model, the spinal cord was compressed at T9 ~ T10, using a clamp for 30 secs. Following that, an incision was sutured, and 10 % w/v povidone was applied at the incision site. For sham, as a surgical control, the only laminectomy was performed with no injury being done to the spinal cord via clamp compression.

Administration of Drug

Ethamsylate (TCI, Details required) was dissolved in 10% glucose solution. Treatment group animals were administered with total seven doses; ethamsylate (100mg/kg) was administered 1 hour after SCI, through intravenous injection. After that, from DPI-1 (Day post-injury) to DPI-3, animals were given ethamsylate (100mg/kg) orally twice a day. Vehicle groups were treated with 10% glucose solution only.

Western blot analysis

After 24hrs of compression-induced SCI, animals were sacrificed. Total length of ~ 2 cm long spinal cord was isolated with the injury site at its center. The spinal samples obtained were homogenized with RIPA lysis buffer and PMSF. After that, centrifugation of homogenized tissues was carried out at 12,000 rpm for 7 mins at 4 °C. Next, the supernatant was taken out, and protein concentration was estimated using a BCA assay kit (Bio-Rad). Protein samples of 30µg were loaded and separated on the SDS page, and then the gel is transferred to PVDF membrane. The membranes were blocked with 5% BSA (bovine serum albumin) for 1hr at room temperature. After that, membranes were incubated in primary antibody against MMP-9 (1:1000, Abcam), Claudin-1 (1:1000, Thermo scientific), Occludin (1:1000, Invitrogen) and GAPDH (1:10000, Abcam) for overnight at 4°C. Next day, three washing were given with TBS-T (tris-buffered saline solution with tween-20). This was followed by incubation of membranes in horseradish peroxidase-conjugated secondary antibodies (Abcam) for 1hr at room temperature. Then, bands were visualized using gel documentation system (Bio-Rad), and densitometric analysis of bands was done using ImageJ software.

Evans blue permeability assay

This assay was performed to measure the integrity of BSCB [12]. After 24hrs of SCI, animals were administered with 0.5ml of 2% Evans blue (Sigma Aldrich) intravenously, and after 30 mins, animals were sacrificed with transcardial perfusion. Spinal cord were removed and ~ 2 cm long spinal cord was isolated with injury area at the middle. After that, spinal samples were homogenized with 50% trichloroacetic acid and later centrifuged (Thermo scientific) at 10000g for 10 min. The supernatant was collected and at 620nm, spectrometric absorbance was measured using a multimode reader (Thermo Scientific). Standard curve with known concentration of Evans blue was plotted. For quantification, microgram (μg) of Evans blue per milligram (mg) of the tissue was used for the determination of the concentration of Evans blue in the sample with reference to the standard curve obtained.

Quantitative real-time PCR

Approximately 2 cm long spinal cord samples were collected, with the lesion area being at the middle. From the spinal samples, total RNA was extracted using TRIzol reagent (Fisher Scientific), followed by synthesis of cDNA with the help of reverse transcriptase kit (Bio-Rad) in thermocycler (Bio-Rad). Accordingly, 10 μl PCR reaction was prepared using Sybr-green reaction kit (Bio-Rad) and specific primers (Sigma Aldrich). Quantitative RT-PCR were performed using PCR machine (Bio-Rad). Data were collected in delta Ct values, and the increase in fold change was calculated and compared between different groups. Mentioned below is the list of primer sequence used in this study: IL-1 Mouse: forward primer, 5'-TTGTGGCTGTGGAGAAGCTGT-3' and reverse primer, 5'-AACGTCACACACCAGCAGGTT-3'; IL-6 Mouse: forward primer, 5'-GCTACCAAAGTGGATATAATCAGGA-3' and reverse primer, 5'-CCAGGTAGCTATGGTACTCCAGAA-3'; TNF- Mouse: forward primer, 5'-AGCAAACCACCAAGTGGAGGA-3' and reverse primer, 5'-GCTGGCACCACTAGTTGGTTGT-3'.

Tissue fixation and paraffin block preparation

After 28 days of SCI, animals were anesthetized and transcardial perfusion were performed by delivering 0.9% saline and 4% paraformaldehyde (PFA) transcardially. After that, the spinal cord was removed and the spinal cord was immersed in 4% PFA (Sigma Aldrich) overnight at 4°C. Tissue processing was carried out by immersing the spinal cord for 30 mins each in increasing ethanol concentrations (70, 80, 90, and 100%) and xylene (for overnight). After this, paraffin infiltration was done for 2 hrs and paraffin blocks were prepared using a paraffin embedder (Thermo Scientific). The blocks were cut longitudinally, and thin sections of 5 μm were obtained using a microtome (Leica). Sections were taken on slides and deparaffinized by treating 3 times with xylene, for 5 mins each. Followed by rehydration steps carried out with the help of different concentrations of ethanol in the sequence of 100, 90, 80, and 70% and rinsed in distilled water for 5 mins. After the slide is ready and rehydrated, the following techniques are performed.

Immunohistochemistry

After rehydration, sections were treated with pepsin (Sigma Aldrich) for 10 mins for antigen retrieval and then sections were further treated with 3% H_2O_2 (Fisher scientific) in methanol for 5 mins to inhibit endogenous peroxidase. Sections were then blocked with a protein blocker (Abcam) for 5 mins and primary antibody incubation was done overnight at 4°C. The primary antibodies used are BDNF (1:200,

Abcam), Collagen-IV (1:500, Abcam), Fibronectin (1:1000, Abcam), GFAP (1:500, Invitrogen), Laminin (1:50, Invitrogen), NF-H (1:1000, Abcam), and TGF- β 1 (1:200, Abcam). Three washing was given with PBS-T, and sections were then incubated in Alexa Fluor conjugated secondary antibodies (Abcam) for 1hr at room temperature, away from the light. All sections were stained with DAPI (4 '6-diamidino-2-phenylindole) for 10 mins to detect the nuclei. Then sections were washed again with PBS-T (two times) and mounted with DPX. Slides were visualized under confocal microscopy (Leica) at the required laser wavelength to obtain a fluorescence image. The intensity of the immunofluorescent images are quantified using ImageJ software.

Luxol fast blue (LFB) staining

This staining method was used to assess the degree of myelin degeneration in the white matter of the spinal cord [13]. For this, sections were dipped into 0.1% w/v Luxol fast blue solution (Sigma Aldrich) and incubated in oven at 56 °C for 2hrs. Excess stains were washed with 95 % alcohol and followed by rinsing off with distilled water. Sections were then immersed in 0.05% lithium carbonate solution for 10 secs for differentiation. It was then again dipped into 95% alcohol (1min) to continue differentiation and washed with distilled water. Differentiation was analyzed microscopically, and after complete differentiation was achieved between grey and white matter, sections were rinsed in distilled water and 95% ethanol. Followed by two washing in 100% ethanol for 5 mins and dipped in xylene for 5 mins. Sections were mounted using DPX and visualized under a microscope (Leica).

Haematoxylin and Eosin (H and E) staining

To detect the pathological changes after SCI, sections were stained with H&E [14]. Sections were dipped in hematoxylin solution (Abcam Kit) for 1min and washed three times in tap water for 1 min each. After that, sections were dipped in bluing solution (Abcam Kit) for 10–15 secs. This was followed by washing with distilled water and then in 100% alcohol for 1 min. Next, sections were immersed into Eosin solution (Abcam kit) for 10 mins, and thereafter, sections were rinsed in 100% alcohol with three changes each for 1 min. later, sections were mounted with DPX and viewed under microscope (Leica).

Evaluation of Functional recovery

Two different types of behavioral assessment: BMS and grip strength, were performed to analyse the functional recovery over 28 days after SCI was induced. Behavioral studies were performed at DPI-1,7,14, and 28.

Basso mouse scale (BMS) and Grip Strength

Mice were allowed to move freely in an open field. Hind limb locomotion, ability to lift the tail and trunk was observed and scored accordingly [15]. BMS score ranges from 0 to 9, with zero being an indication of complete paralysis and 9 for normal movement of the hind limb. Movement of mice were video recorded, and two independent observers evaluated the video and subjected score accordingly.

Grip strength is done to assess the motor strength of the mice. The mice were kept in the room 30 mins prior to the experiment for acclimatization. After that, mice were allowed to grasp the bar of the instrument as it was slowly and steadily pulled away from its tail. The maximal force applied for grasping the bar is recorded and noted down. Three independent readings were taken with 30 mins gap between each reading [16]. Average of these 3 readings were taken and graph was plotted.

Statistical analysis

All the data are analyzed using one-way analysis of variance and represented as mean $\pm$ SEM. For comparison between different groups, Tukey's post hoc test was used. * $P < 0.05$ was accepted as statistically significant.

Results

Ethamsylate preserves the integrity of BSCB and attenuates the production of inflammatory mediators

MMP-9 has a significant impact on the degradation of BSCB, and the drastic increase in expression of MMP-9 in the acute phase of SCI is well-reported [17]. Therefore, we analyzed the effect of ethamsylate on MMP-9 expression *via* western blot analysis of DPI-1 samples. This study includes, three groups, sham group, vehicle group [10% glucose], and treatment group [Ethamsylate (100mg/kg)]. Treatment with ethamsylate significantly (* $P < 0.05$) attenuated the MMP-9 expression compared to the vehicle group [Figure 1A-B]. Next, we examined the expression of TJ proteins such as claudin-1 and occludin, as it is well-understood that TJ proteins act as a backbone of BSCB by maintaining its structural integrity [18]. Various studies reported alteration in the expression of claudin-1 and occludin in the acute phase of injury [19]. From our study, we found a profound difference in TJ protein expression in vehicle and ethamsylate group as demonstrated in Fig. 1 (C) and (D). The expression of tight junction proteins was preserved in ethamsylate treated group, which indicates that ethamsylate inhibits the degradation of TJ proteins [Figure 1C-D]. These data suggest that ethamsylate has a protective effect on BSCB as it decreases the MMP-9 production and attenuates the degradation of TJ proteins in the acute phase of SCI.

Furthermore, we performed evans blue permeability assay to evaluate the alteration in the permeability of the BSCB [20]. As it was confirmed that ethamsylate reduces the expression of MMP-9 and preserves the TJ proteins present in endothelial cells. It was noted that penetration of Evans blue dye in the spinal cord of ethamsylate treated group was drastically lower than the vehicle group. Interestingly, there was no penetration of evans blue dye into the spinal cord of sham animals (Figure-1H). Quantitative analysis suggests the significant difference in the permeability of evans blue dye in the spinal cord of treatment and vehicle group with $###P < 0.01$ as shown in Figure-1 F. The notable difference in the extravasation of evans blue dye into the spinal cord of vehicle and treatment group reveals that ethamsylate has a protective effect on BSCB.

BSCB disruption after SCI facilitates the infiltration of neutrophils and other inflammatory cells at the injury site [21]. Neutrophil infiltration occurs within the 24hrs of injury leading to increased production and release of the inflammatory mediators *viz.* IL-1 β , IL-6, and TNF- α [22]. This process eventually leads to severe inflammation and aggravates tissue destruction as well as spreads the damage to the surrounding tissue. Thus, we analyzed the spinal samples through qRT-PCR to determine the mRNA expression of these inflammatory mediators at DPI-1. We found that ethamsylate treated group tremendously reduced the upregulation of IL-1 β , IL-6, and TNF- α as depicted in Fig. 1 (A), (B) and (C). Our data reveals that ethamsylate reduces the production of these inflammatory mediators and inhibit the exacerbation of secondary damages.

Ethamsylate reduces axonal demyelination, decreases cystic cavity formation, and thereby facilitates functional recovery after SCI

Axonal demyelination is a prominent characteristic observed in the case of SCI [23]. Due to this, sensory and motor signals are delayed or disrupted. This leads to impairment in sensory and motor neurons, which might cause paralysis. Hence, LFB staining was performed to evaluate the sparing of myelinated white matter on treatment with ethamsylate. For this, longitudinal sections of the spinal cord were stained with LFB, to examine the extent of myelin degeneration. This dye stains the myelin sheath covering the axonal fibers in the white matter with blue color. Myelinated and intact axons were present throughout the section in sham animals after DPI-28, as evidenced by a bluish color. Whereas, in the case of the vehicle group, white matter appears to be very pale at the lesion site, denoting the occurrence of myelin degeneration. Interestingly, major tissue sparing was observed in ethamsylate treated group at the lesion area, compared with the vehicle group (Fig. 2A). The degree of demyelination was lower in the ethamsylate group as compared to the vehicle group. Hence, ethamsylate maintains the axonal integrity after SCI.

Histopathological changes are reported in SCI with necrosis and cystic cavities formation occurring at the lesion area [24]. This lesion formation in the chronic period leads to scarring. The morphological and structural changes in the chronic phase (DPI-28) was evaluated through H&E staining. In sham section, healthy neuronal projections with no cystic cavities was observed (Fig. 2B). While in the case of the vehicle group, pathological abnormalities with the formation of cystic cavities, necrosis, and accumulation of immune and inflammatory cells were observed near the lesion area as depicted in Fig. 2B. Ethamsylate treated group was comparatively found to have lesser cavity formation with decreased accumulation of inflammatory cells at the lesion area. These observations in the pathophysiological changes summarise that ethamsylate reduces lesion and cystic cavity formation.

The improvement in functional recovery was evaluated through behavioral studies such as BMS and Grip strength performed at different day points such as DPI-1,7,14,21 and 28. BMS was performed to analyze the functional recovery of the hind limb. BMS score consists of scoring numbers 0 to 9, 0 signifies complete paralysis, that is zero movement of hindlimbs, whereas 9 score is subjected when the mice

move normally [26]. Animals in the sham group have regular locomotor activity, so they were assigned with 9 scores. All the animals were paralyzed entirely in the vehicle and ethamsylate group after SCI (Fig. 2C-i). Treatment with ethamsylate showed faster recovery compared with vehicle group (Fig. 2C-ii). Considerable difference in the score was seen between vehicle and treatment, supporting our evidence that ethamsylate promotes functional recovery after SCI.

Next, grip strength was used to evaluate the strength of both the forelimb and hind limb. This test is particularly useful to analyze any motor deficit in the limbs after SCI as it measures the flexor muscle strength. Moreover, as hind limb becomes completely paralyzed after inducing a compression injury at the thoracic region. At DPI-1 a paramount decrease observed in the grip strength of both vehicle and ethamsylate treated groups (Fig. 2D-i). However, prominent increased in the grip strength can be seen in case of ethamsylate treated group as compared with the vehicle group. Our behavioral data reveal that the grip strength of the sham group remained constant throughout the study. At DPI-28, a vast difference was observed between the grip strength of vehicle and ethamsylate group as depicted in Fig. 2D-iv. A notable improvement in the locomotor function was observed in ethamsylate treated group, supporting its role in improving functional recovery.

Ethamsylate reduces the extent of Fibrotic scar formation

In the chronic period of SCI, scar formation is inevitable pathogenesis. It is reported in numerous studies that scar formation is the ultimate cause of the neuronal deficit and locomotor impairment [27]. Fibrotic scar formation is well-reported in SCI, it is suggested that fibrotic scar formation contributes to a huge extent in functional damage by acting as a physical barrier, which disrupts the axonal connections between the two adjacent neurons [28]. Therefore, it is believed that reducing the level of fibrosis could lessen the extent of fibrotic scar formation and improve the neuronal network for proper motor and sensory function. TGF- β 1 is highly upregulated in fibrosis and undergoes a downstream signalling pathway by binding with the TGF- β type 2 receptor. The TGF- β 1 signalling pathway leads to the proliferation of fibroblast, which causes excess deposition of fibronectin, laminin, and collagen-IV [29]. Fibrotic scar formed near the injured area can be distinguished by excessive accumulation of perivascular fibroblast and deposition of matrix protein fibronectin [30]. It was observed that on treatment with ethamsylate, the fluorescence intensity of TGF- β 1 and fibronectin was significantly decreased at the injury area as represent in Fig. 3C and D. Whereas, in the case of vehicle group, the intensity of both TGF- β 1 and fibronectin was quite high. This indicates that matrix formation of fibronectin, a major component of fibrotic scar, is significantly reduced on treatment with ethamsylate. In the sham group, fibronectin was uniformly distributed in the ECM (Fig. 3B). Whereas in the case of vehicle group, it is accumulated and results in matrix formation at the injury site. Our data indicate that TGF- β 1 mediated fibrotic scar formation is significantly reduced on treatment with ethamsylate.

Ethamsylate plays a role in the regulation of ECM remodeling

ECM remodeling has been extensively reported in the chronic phase of SCI. ECM remodeling observed in the SCI is due to the excessive deposition of collagen, particularly collagen-IV and laminin at the injury site[31]. ECM remodeling in the chronic state leads to fibrosis due to over deposition of these ECM proteins at the injury site. ECM proteins *viz* collagen-IV, laminin and fibronectin are uniformly distributed in the extracellular matrix in normal physiological conditions. But during the chronic phase of injury, the fibrotic matrix formation occurs at the injury site due to the accumulation of fibrous protein such as collagen-IV. So far, we have seen that ethamsylate acts via TGF- β 1 pathway and regulates the expression of these ECM components. However, MMPs are also widely known for their ability to regulate the ECM remodeling, particularly MMP-9[32]. And since our western blot data shows decrease in MMP-9, on treatment with ethamsylate. It can be proposed that ethamsylate might be having a role in ECM remodeling. Moreover, our immunohistochemical study revealed that, matrix deposition of collagen-IV and laminin is quite high at the injury area in the vehicle group as compare to the ethamsylate treated group as depicted in Fig. 4A and B. As our result shows that ECM protein deposition is quite reduced on treatment with ethamsylate, it indicates that ethamsylate has a significant role in regulating ECM remodeling after SCI.

Ethamsylate improves neuronal function and exhibits neuroprotective effect.

To determine whether ethamsylate has any role in neuroprotection, we checked the expression of neurofilament-heavy (NF-H) and brain-derived neurotrophic factor (BDNF) after SCI. NF-H is an intermediate filament present abundantly in the cytoplasm of neurons and modulates axonal transport and signaling. It is a critical determinant for evaluating the neuroprotective effect in SCI [33]. While BDNF is a neurotrophic factor widely known for its essential role in neuronal development, maintaining synaptic transmission and plasticity. Besides, it is also reported that BDNF promotes pronociceptive effects and plays a critical role in facilitating spinal cord nociceptive plasticity[34]. In our study, it was observed that, on treatment with ethamsylate, the fluorescence intensity of NF-H and BDNF is significantly low as compare to intensity in vehicle group as shown in Fig. 5C and D. It signifies that ethamsylate limits neuronal damage and improves neuronal function. This particularly indicates that ethamsylate has a neuroprotective role in SCI.

Discussion

Therapy for SCI remains an ongoing battle in the present era. Limited therapy in SCI is what drives to focus on the therapeutic benefits in the field of SCI. The main obstacle which makes the treatment inadequate is the formation of scar. So far, no medication is available to eradicate the scar formation or restore the neuronal circuit [35]. Even though, Methylprednisolone is approved by FDA for SCI treatment, it is still not considered an ideal therapy due to its severe adverse effect and uncertainty in the risk-benefit ratio [36]. Moreover, the therapeutic drugs used in clinical settings to deal with SCI, remain largely symptomatic therapy. Therefore, these limitations create the need for a therapeutic treatment that can reduce fibrotic scar formation and delineate the neuroprotective effect with minimal side-effects. Herein we studied the effect of ethamsylate, a homeostatic or anti-hemorrhagic drug, widely known for its safe

action. It has a multi-faceted mechanism of action, which can be beneficial for its use in SCI. Previous findings suggest that ethamsylate can maintain early hemostasis and thereby improve functional recovery in SCI-induced rodent model [11]. Therefore, we decided to explore the role of the ethamsylate on fibrotic scar formation as well as its effect on mediating secondary damages and exhibiting neuroprotection. So that, it can be repurposed for use in SCI.

In our study, we have administered the ethamsylate intravenously within the 8 hrs of injury, followed by oral treatment for three days. The reason behind opting this treatment strategy is the fact that 8 hours after the traumatic SCI is the most crucial period which determines the long-term effect of the trauma. Therefore, treatment administered within these 8 hrs shows a highly beneficial effect [37]. This is related to the pathophysiology of SCI, in which the primary mechanical damage to the spinal cord causes severe structural damages. In contrast, secondary pathogenesis, which shows cellular damage and tissue destruction, begins within few hours of injury [38]. So, early treatment can reduce the onset of secondary damages. In the long run, these secondary damages lead to the fibrotic scar formation, which acts as a barrier and disrupts the neuronal signal between the two adjacent neurons. This causes the loss of sensory and motor function, as observed in SCI patients. Ethamsylate by virtue of its anti-hemorrhagic and hemostatic effect, decreases the hemorrhagic ischemia in the acute phase of injury, thereby protects the neurons from necrosis and apoptosis. It is well reported that, in SCI, due to mechanical impact, BSCB gets disrupted, which results in the migration of neutrophils and other leukocytes to the injury site [39]. This particular event initiates the cascade of secondary damages. Therefore, maintaining the integrity of BSCB is of foremost importance in SCI. MMP-9, a matrix proteolytic enzyme, is highly upregulated in the acute phase and is involved majorly in the disruption of BSCB *via* degradation of basal lamina [40]. Thus, it is derived from our findings that ethamsylate preserves the integrity of BSCB as it drastically reduces the expression of MMP-9 as well as increases the expression of TJ proteins such as occludin and claudin-1. Hence, ethamsylate *via* its ability to decrease the expression of MMP-9 and restoration of TJ protein level protects the BSCB and decreases its permeability. This reduces infiltration of leukocytes and other inflammatory cells to the injury site, thus decreasing the release of cytokines and chemokines at the injury site. Our findings also demonstrated that there was minimal penetration of Evans blue dye into the spinal cord of ethamsylate treated group, which further supports our finding that ethamsylate preserves the integrity of BSCB. After that, we checked the effect of ethamsylate on inflammatory mediators, which initiates the cascade of secondary damages and causes tissue destruction. We noted a significant change in the mRNA expression of IL-1 β , IL-6, and TNF- α between the vehicle and ethamsylate-treated group. So, from this, we can conclude that ethamsylate decreases the release of inflammatory components and reduces the severity of secondary pathogenesis.

Next, we moved on to the histopathological study of the DPI-28 spinal section using H&E staining. We found a reduced cystic cavity formation at the injured area on treatment with ethamsylate compared to the vehicle group. Moreover, LFB staining of myelin sheath demonstrated increased white matter sparing in the treatment group with respect to the vehicle group. This particularly denotes that ethamsylate reduces myelin degeneration at the lesion area and therefore preserves axonal integrity. These histopathological changes can be correlated with functional recovery observed in the ethamsylate treated

group. Besides, behavioral studies data displays a noticeable difference in functional recovery, with the BMS score of the ethamsylate treated group reaching upto 8 at DPI-28 and the vehicle group remained at an average of 3. Then, we further proceeded to look for TGF- β 1 level and ECM remodeling at the injury site to determine the extent of fibrotic scar formation. Our immunofluorescence data reveals that TGF- β 1, known as the prime suspect behind the fibrosis, is tremendously decreased in the ethamsylate treated group. Moreover, excessive accumulation of fibronectin, collagen-IV, and laminin at the lesion area can be seen in the case of the vehicle group, whereas in the treatment group, the deposition of these extracellular components at the injury site is relatively low. It is reported that MMP-9 predominantly activates TGF- β 1 [41], which in physiological condition remains bound with a binding protein, that is in the form of latent TGF- β binding protein (inactive form). MMP-9 cleaves the TGF- β 1 from its binding protein, and only activated TGF- β 1 binds with type 2 TGF- β receptor and undergoes a downstream signaling cascade which results in excessive proliferation of fibroblast and release of fibronectin, collagen-IV, and laminin, which are the main components of fibrotic scar [8].

As our study depicts that ethamsylate decreases the expression of MMP-9, it directly reduces the activation of TGF- β 1, which inhibits the initiation of the fibrotic process and attenuates the fibrotic scar formation. Moreover, TGF- β 1 are predominantly secreted by neutrophils and monocytes [42], and migrates to the lesion site due to increased permeability of BSCB after SCI. Ethamsylate stabilizes the BSCB; therefore reduces the production of TGF- β 1 at the injured area. Since our findings suggest that ethamsylate can reduce hemorrhagic ischemia, secondary damages, and fibrotic scar formation, it can also be rationalized to exhibit a neuroprotective role. Therefore, we evaluated the expression of NF-H and BDNF in the chronic period. We found that the fluorescence intensity of NF-H and BDNF in the treatment group was relatively high compared to the vehicle-treated group. This uncovers the fact that ethamsylate might also be having a neuroprotective role in SCI. In conclusion, the outcomes of the present study demonstrate that ethamsylate decreases the severity of secondary damages, reduces fibrotic scar formation, and promotes neuroprotection after SCI. Besides, ethamsylate is already a well-established drug in the market with a good safety profile [43]. Therefore, clinical trials of ethamsylate can be performed in SCI patients in the near future as it has the potential to be used in clinical settings for SCI treatment.

Declarations

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Authors contribution

1. SD: concepts and design of study, investigation, methodology, data interpretation, data interpretation, and writing the original draft.
2. KA and TM: analysis of behavioral data, performing surgery, and draft review.

3. AR and ZP : methodology and editing of the draft.

4. HK: Conceptualization, supervision, review, and editing of the final draft.

Availability of data and material

All data is provided with the manuscript and it can be requested from corresponding author upon reasonable request.

Compliance with ethical standards

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2. Reasearch involving human participants or animals: NOT applicable since it is a not a clinical manuscript.

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Figures

Figure 1

Ethamsylate preserves the integrity of the blood spinal cord barrier and attenuates the expression of inflammatory mediators after 24 h of spinal cord injury (A) represents the bands of MMP-9, claudin-1 and occludin with GAPDH as an internal control. (B) Depicts the densitometric analysis MMP-9 band with #P<0.05 vs vehicle and ***P<0.001 vs sham. (C) shows quantification of bands of claudin 1 with significant difference #P<0.01 vs vehicle and (D) occludin with ##P<0.01 vs vehicle. (E) It demonstrates the image of sham, vehicle and ethamsylate treated spinal cord, isolated after Evans blue administration. (F) Quantification of Evans blue in the spinal tissue sample, performed through spectrophotometric analysis at absorbance wavelength 620nm with ***P<0.001 vs sham and ##P<0.01 vs ethamsylate. Effect of ethamsylate on mRNA expression of inflammatory mediators such as (G) IL-1β; **P<0.01 vs sham and ##P<0.01 vs vehicle (H) IL-6; ***P<0.001 vs sham and ###P<0.001 vs vehicle and (I) TNF-α; #P<0.05 vs vehicle. The data are represented as mean ± SEM using one-way ANOVA followed by Tukey's post hoc comparison test (n=3).

Figure 2

Ethamsylate facilitates functional recovery through restoration of myelin sheath and reduction of cystic cavity formation at the injury site after DPI-28 (A) Represents the longitudinal section of spinal cord stained with luxol fast blue, visualized at 4x and 10x. Scarring in white matter with pale color is observed at injury site that denotes demyelination, which is drastically reduced in case of ethamsylate group. Complete bluish color is observed in the sham section, signifying intact myelin sheath. (B) depicts the longitudinal section of spinal cord stained with H&E and visualized at 4x and 10x. Formation of cystic cavities can be observed at the site of injury in vehicle group, which is reduced in case of ethamsylate treated group. Healthy neuronal projection can be seen in sham section. (C) Effect of ethamsylate on functional recovery evaluated via BMS score with significant differences visible from DPI-7 between vehicle and ethamsylate treated group with $###P<0.001$. D) represents the effect of ethamsylate on grip strength of animals with drastic differences being observed from DPI-7 with $***P<0.001$ vs sham and $###P<0.001$ vs ethamsylate. The data are analyzed using one-way ANOVA, followed by Tukey's post hoc comparison test (n=5).

Figure 3

Ethamsylate reduces the extent of fibrotic scar formation via decreasing the activation of TGF- β 1 and formation of fibronectin matrix (A) depicts the fluorescence image of TGF- β 1, scale bar =25 μ m and (B) fibronectin, scale bar=100 μ m. (C) represents the quantification of (A) with ** P <0.01 vs sham and ## P <0.01 vs ethamsylate. (D) denotes the fluorescence intensity of (B) with ** P <0.01 vs sham and ## P <0.01 vs ethamsylate at DPI-28. The above data are analyzed using one-way ANOVA, followed by Tukey's comparison test (n=3).

Figure 4

Ethamsylate plays a role in regulation of ECM remodelling Representative fluorescence image of (A) laminin and (B) collagen-IV, under 10x and scale bar=100µm. (C) depicts the florescence intensity quantification of (A) with ***P<0.001 vs sham and ###P<0.001 vs ethamsylate. (D) represents the fluorescence intensity of (B) with ***P<0.001 vs sham and ###P<0.001 vs ethamsylate (n=3).

Figure 5

Ethamsylate improves neuronal function and exhibits neuroprotective effect. Representative fluorescence image of (A) BDNF, visualized under 40x, scale bar=10 µm and (B) NF-H, scale bar=100µm. (C) denotes the intensity of (A) with #P<0.05 vs vehicle and (D) depicts the fluorescence quantification of (B) with ***P<0.001 vs sham and #P<0.05 vs vehicle. The data are analyzed using one-way ANOVA followed by Tukey’s post hoc test (n=3).

Supplementary Files

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