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GSNOR and ALDH2 alleviate traumatic spinal cord injury

Mushfiquddin Khan[*,1], **Fei Qiao**[2], **S.M. Touhidul Islam**[1], **Tajinder S. Dhammu**[1], **Pavan Kumar**[1], **Jeseong Won**[2], **Avtar K. Singh**[2,3], **Inderjit Singh**[1,3]

[1]Department of Pediatrics, Medical University of South Carolina, Charleston, SC

[2]Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, SC

[3]Ralph H Johnson VA Medical Center, Charleston, SC

Abstract

Traumatic spinal cord injury (SCI) enhances the activity of S-nitrosoglutathione reductase (GSNOR) and inhibits the mitochondrial aldehyde dehydrogenase 2 (ALDH2) activity, resulting in prolonged and sustained pain and functional deficits. This study's objective was to test the hypotheses that GSNOR's specific inhibitor N6022 mitigates pain and improves functional recovery in a mouse model of SCI. Furthermore, the degree of recovery is enhanced and the rate of recovery is accelerated by an ALDH2 activator Alda-1. Using both wild-type and GSNOR$^{-/-}$ mice, the SCI model deployed for groups was contusion at the T9-T10 vertebral level. The enzymatic activity of GSNOR and ALDH2 was measured, and the expression of GSNOR and ALDH2 was determined by western blot analysis. Functional improvements in experimental animals were assessed with locomotor, sensorimotor, and pain-like behavior tests. Wild-type SCI animals had enhanced GSNOR activity and decreased ALDH2 activity, leading to neurovascular dysfunction, edema, and worsened functional outcomes, including locomotor deficits and pain. Compared to wild-type SCI mice, GSNOR$^{-/-}$ mice had better functional outcomes. Monotherapy with either GSNOR inhibition by N6022 or enhanced ALDH2 activity by Alda-1 correlated well

[*]Corresponding author: Mushfiquddin Khan, Ph.D. Associate Professor, Department of Pediatrics, Medical University of South Carolina, 513 Darby Children's Research Institute (DCRI), 173 Ashley Ave, Charleston, SC 29425, khanm@musc.edu, Tel: (843) 792-7991, Fax: (843) 792-3653.

Authors' Contributions

This study is based on an original idea of MK, JW and IS. MK, TSD, FQ and TI wrote the manuscript and all authors reviewed the manuscript. PK, MK, TSD and FQ carried out animal and biochemical studies. MK, AKS, TSD, JW, TI, IS and PK critically examined the animal and biochemical studies. All authors have approved the manuscript.

Ethics approval and consent to participate

All animals received humane care in compliance with the Medical University of South Carolina's (MUSC) guidance and the National Research Council's criteria for humane care. Animal procedures were approved by the institutional animal care and use committee (IACUC) of MUSC. Human data or human tissue has not been used in this study.

Consent for publication

Not applicable

Conflict of interest

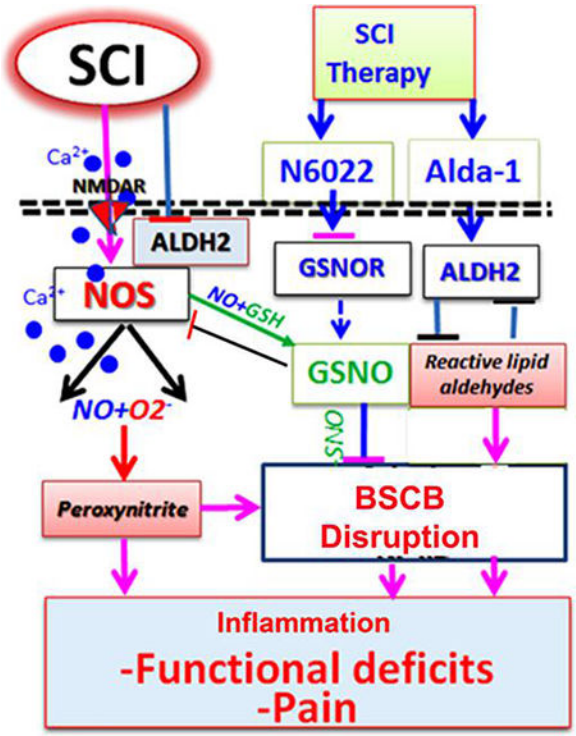
The author(s) declare that they have no conflict of interests.

Availability of supporting data

The data sets generated/analyzed during the current study are saved on the institutional server and are available.

with functional recovery and lessened pain. However, combination therapy provided synergistic pain-relieving effects and more significant functional recovery compared with monotherapy. Conclusively, dysregulations in GSNOR and ALDH2 are among the causative mechanisms of SCI injury. Either inhibiting GSNOR or activating ALDH2 ameliorates SCI. Combining the specific inhibitor of GSNOR (N6022) with the selective activator of ALDH2 (Alda-1) provides greater protection to the neurovascular unit and confers greater functional recovery. The study is novel, and the combination therapy (N6022+Alda-1) possesses translational potential.

Graphical Abstract



Combining N6022 with Alda-1 provides greater functional recovery than monotherapy with N6022 or Alda-1 in SCI.

Keywords

Alda-1; ALDH2; BMS score; GSNOR; N6022; pain; reactive aldehydes; reactive oxygen species; SCI; S-nitrosylation

1. Introduction

Traumatic spinal cord injury (SCI) is a life-changing incident because it leaves most patients impaired and in pain. The restricted number of inadequate therapeutic options, such as methylprednisolone or pain medicine (Wang et al., 2019), suggests the need to investigate pharmacological interventions that are directly relevant to SCI mechanisms and thus effective in mitigating pain as well as aiding and improving functional recovery.

In SCI pathology, several deleterious mediators, especially peroxynitrite (Khan et al., 2018; Xiong and Hall, 2009) and reactive aldehydes including 4-HNE and acrolein (Carrico et al., 2009; Lin et al., 2018; Liu-Snyder et al., 2006; Singh et al., 2007), play critical roles. Formation of peroxynitrite results in decreased nitric oxide (NO) and S-nitrosoglutathione (GSNO) levels, which contribute to disease pathology. Like deleterious peroxynitrite, reactive aldehydes are also harmful to cellular functions.

NO invokes its major effect on cellular functions via S-nitrosylation of proteins/peptides (Zhang et al., 2017). S-nitrosylation is a physiological signaling process regulating multiple cellular activities, including the stress response, by changing protein activity, localization, stability, and protein-protein interactions (Hess and Stamler, 2012; Khan and Singh, 2019). Decreased NO bioavailability stems from an instantaneous reaction between NO and superoxide in the same compartment, thus forming deleterious peroxynitrite and reducing the formation of beneficial GSNO. Endogenous GSNO, via S-nitrosylation, can inhibit the aberrant activity of nitric oxide synthases (NOS), thus decreasing peroxynitrite formation and improving neurovascular function. However, SCI conditions also increase the activity of the GSNO catabolizing enzyme GSNO reductase (GSNOR), which reduces cellular levels of GSNO (Chen et al., 2013; Zhang et al., 2017). Although exogenously administered GSNO has been used in both human and animal studies (Broniowska et al., 2013), its use as a therapeutic drug suffers from limitations. GSNO treatment of thromboembolic patients causes hypotension (Molloy et al., 1998), and its pharmacokinetics, short half-life, and lesser stability do not favor exogenous GSNO as a suitable drug candidate (Broniowska et al., 2013). In contrast, endogenous GSNO produced by the GSNOR inhibition invokes the beneficial effects of GSNO without causing the adverse effects associated with exogenous administration. However, the role of endogenously available GSNO, by the inhibition of GSNOR, has not been studied in the context of SCI pathology. Our initial studies showing the efficacy of GSNOR inhibition by N6022 and its safety profile support investigating it as a candidate for SCI therapy. Therefore, we investigated the efficacy of N6022 (Sun et al., 2011) for neurovascular protection and functional recovery in SCI.

N6022 specifically and reversibly inhibits GSNOR (Green et al., 2012; Sun et al., 2011), and it has been classified as a “first-in-class” drug (Colagiovanni et al., 2012). In rat studies, lower doses ranging from 1-10 mg/kg/day are well tolerated (Colagiovanni et al., 2012). Two clinical trials with N6022 have been completed (Nivalis Therapeutics, Inc.). The trials showed that N6022 was a safe drug, well-tolerated after intravenous (iv) administration in healthy volunteers (NCT01147406, NCT01339897) as well as in adult patients with mild asthma (NCT01316315) or cystic fibrosis (NCT01746784). The 5 mg/kg body weight dose in rodents has been recommended in a pharmacokinetic evaluation study of N6022 (Colagiovanni et al., 2012).

Together, these studies support that the dose of N6022 used in our studies (5 mg/kg, iv) is justified. There are many other GSNOR inhibitors available in the market. We opted for N6022 because neither its iv administration to humans (Que et al., 2018) nor its oral administration to mice with experimental autoimmune encephalomyelitis (EAE) (Saxena et al., 2018) resulted in any adverse effect. However, our SCI studies with GSNOR-knockout

mice indicated that the absence of GSNOR activity alone is not sufficient to provide optimal protection.

In SCI, reactive aldehyde-induced loss of mitochondrial function is a critical mechanism that is regulated, at least in part, by the mitochondrial detoxifying enzyme ALDH2. Reactive aldehydes are metabolized and detoxified by ALDH2. The activity of ALDH2 can be enhanced by its agonist Alda-1 (Luo et al., 2014; Perez-Miller et al., 2010). Alda-1 [N-(1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide] is a selective ALDH2 agonist (Perez-Miller et al., 2010). It is stable, solid, and cell-permeable. The treatment of normal rats with Alda-1 enhances the activity of ALDH2 in the brain, likely via crossing the blood-brain barrier (Rivera-Meza et al., 2019). Alda-1 reduces the Km value (Michaelis constant) and increases the V-max (maximum velocity) by binding to ALDH2 near the catalytic site (Chen et al., 2014; Perez-Miller et al., 2010). Therefore, Alda-1 is an agonist as an allosteric activator of ALDH2 by increasing productive enzyme-substrate interactions. It is also reported to function as a chemical chaperone, exerting its allosteric effect to restore a catalytically inhibited enzyme (Chen et al., 2014; Perez-Miller et al., 2010). So far, Alda-1 has not been investigated in human studies; however, its administration to animals is not associated with any adverse effects. The dose of 10 mg/kg body weight has been reported to be effective with significant efficacy (He et al., 2018; Lu et al., 2017). In animal studies, Alda-1 provides robust protection against post-cardiac arrest myocardial dysfunction (Zhang et al., 2020), myocardial infarctions (Hua et al., 2018), hepatic ischemia (Zhang et al., 2018), and CNS ischemia-reperfusion (Liu et al., 2017).

Therefore, we investigated the efficacy of combination therapy of N6022 and Alda-1 and compared those effects with monotherapy following SCI. The data provide evidence that the combination therapy (N6022+Alda-1) was more effective in reducing blood-spinal cord barrier (BSCB) disruption, mitigating pain, and improving neurobehavioral functions in a young adult mouse model of contusion SCI.

2. Results

2.1. GSNO or N6022 treatment for four weeks improves locomotor function (BMS score), pain-like behavior (thermal algesia) and depressive-type behavior (FST) following SCI

Previous studies from our laboratory have shown the efficacy of exogenously-administered GSNO in a rat model of contusive SCI (Chou et al., 2011; Khan et al., 2018). This study's objective was to investigate the comparative efficacy of exogenous GSNO vs. endogenous GSNO in terms of functional recovery following SCI (Fig 1). To increase endogenous levels of GSNO, we used N6022, a selective inhibitor of GSNOR. BMS score almost reached a plateau in the SCI group, whereas the score significantly improved in both the GSNO ($p<0.001$) and the N6022-treated ($p<0.001$) groups (Fig 1). Similar efficacy of GSNO and N6022 was observed for pain ($p<0.001$) (Fig 1B) and depression-like behaviors ($p<0.05$) (Fig 1C). However, no significant difference was observed in any of the functional outcomes between the two treated groups (GSNO vs. N6022).

2.2. Impact on locomotor function (BMS score) and pain-like behavior (thermal analgesia) of Alda-1 treatment for 30 days in wild-type and GSNOR-deficient mice following SCI

Based on significant functional recovery in N6022-treated SCI animals (Fig 1), we used GSNOR-knockout (KO) mice in anticipation of greater functional recovery following SCI (Fig 2). Surprisingly, the results of the BMS score (Fig 2A) and thermal algesia (Fig 2B) were similar in wild-type (WT) SCI vs. GSNOR KO SCI groups, indicating that complete deletion of GSNOR is not helpful for functional recovery in SCI. Because GSNOR activity is also associated with formaldehyde detoxification, and the mitochondrial ALDH2 enzyme also detoxifies formaldehyde, we combined the ALDH2 potent agonist Alda-1 to determine whether ALDH2 activation provides synergistic benefits with GSNOR inhibition. Indeed, Alda-1 treated GSNOR KO SCI mice group showed significantly greater functional recovery ($p<0.05$) compared with the Alda-1-treated WT SCI mice group (Fig 2).

2.3. N6022 and/or Alda-1 treatment for four weeks improves locomotor function (BMS score) and pain-like behavior (thermal algesia) following SCI

The observation that Alda-1 provides significantly greater functional improvements to GSNOR KO mice than WT mice following SCI (Fig 2) indicates that combining Alda-1 with the GSNOR inhibitor N6022 may provide additional functional recovery benefits. To test this hypothesis, WT SCI animals were treated with Alda-1+N6022 to compare WT SCI animals' treatment with each drug independently. The combination therapy conferred greater improvements in BMS scores (Fig. 3A) and pain behaviors (Fig. 3B) ($p<0.05$) compared with monotherapy.

2.4. N6022 and/or Alda-1 treatment for 72 h protects against blood-spinal cord barrier (BSCB) leakage and edema formation and down-regulates neurovascular pro-inflammatory mediator ICAM-1 following SCI

Remarkable functional recovery in the drug-treated animals indicates therapeutic activity associated with N6022 and/or Alda-1 during the disease's late phases. However, the effects of these drugs during the early phase of contusive SCI are not known. Because the acute phase of injury is associated mainly with blood-spinal cord barrier (BSCB) disruption, edema formation, and neurovascular inflammation (Lee et al., 2018), we investigated the effects of the treatments on BSCB disruption, edema, and the expression of ICAM-1. Although treatment with Alda-1 or N6022 protected against BSCB damage edema, the combination therapy showed greater protection ($p<0.05$), as shown by Evan’s blue extravasation (Fig 4A, B). Similar effects of the treatments were also observed on edema (Fig 4 C). The expression of ICAM-1 was also reduced in all the treated groups compared with the SCI group; however, a significant difference in the expression levels was not observed among the treated groups (Fig 4D, E).

2.5. N6022 and/or Alda-1 treatment for 72 h decreased GSNOR activity without changing its expression following SCI

In this study, we tested the hypothesis that N6022 alone or in combination with Alda-1 invokes a protective neurovascular protective effect by inhibiting GSNOR activity. The activity of GSNOR was significantly increased in the SCI group compared with the sham

group (Fig 5A). N6022 treatment of the SCI group significantly inhibited GSNOR activity ($p<0.01$) (Fig 5A). While Alda-1 had no effects on GSNOR activity, the combination (N6022+Alda-1) treatment group had decreased activity of GSNOR (Fig 5A). Interestingly, the expression of GSNOR remained unchanged among the experimental groups (Fig 5B, C).

2.6. Alda-1 treatment for 72 h increased ALDH2 activity following SCI

Alda-1 is a selective agonist of mitochondrial ALDH2, and animals treated with Alda-1 show significantly increased activity of ALDH2 in rat brain and mouse heart (Casin et al., 2018; Rivera-Meza et al., 2019). ALDH2 overexpression is also reported to reduce neuronal death, leading to improved locomotor function in a mouse model of spinal cord ischemia and reperfusion (Liu et al., 2017). In this study, the activity of ALDH2 was significantly ($p<0.001$) decreased in the SCI group compared with the sham group (Fig 6A). Treatment with Alda-1 of SCI significantly increased ALDH2 activity ($p<0.001$) (Fig 6A), which correlated with neurovascular protection in the acute phase of injury (Fig 4). As indicated by the western blot analysis, the expression of ALDH2 remained unchanged among the groups (Fig 6B, C).

3. Discussion

For the first time, this study examined the role of two critical metabolic enzymes, GSNOR and ALDH2, in SCI. Both enzymes' activity was dysregulated, and either the inhibition of GSNOR or the activation of ALDH2 improved functional outcomes in a mouse model of contusion SCI. Based on significant improvements with monotherapy, we investigated and observed that the combination therapy provided even greater and accelerated functional recovery beyond either treatment by itself following SCI.

The efficacy of exogenous GSNO via S-nitrosylation has been documented in several CNS diseases, including SCI from our laboratory (Chou et al., 2011; Khan et al., 2018). Because GSNO’s pharmacokinetics are less favorable for developing it as a therapeutic drug, we tested the efficacy of endogenous GSNO using a potent and specific inhibitor of GSNOR, N6022, and compared the results with exogenously administered GSNO (Fig 1). Interestingly, N6022 was as effective as GSNO in improving neurobehavioral functions, including locomotor, pain, and depression-like behaviors following SCI (Fig 1). Similar efficacy of exogenous GSNO versus N6022 was also observed in a mouse model of experimental stroke (Khan et al., 2019). Our laboratory has also reported the efficacy of N6022 in two different animal models of CNS injury (cerebral ischemia/reperfusion and experimental autoimmune encephalomyelitis) (Khan et al., 2019; Khan et al., 2020; Saxena et al., 2018). As a first-in-class drug, N6022 showed a strong safety profile in animal and human studies (Colagiovanni et al., 2012; Que et al., 2018; Sun et al., 2011). Taken together, these observations supported using N6022 as a therapeutic drug for neuroprotective activity in SCI.

In addition to metabolizing/degrading GSNO, GSNOR also acts as a formaldehyde dehydrogenase, and thus it detoxifies formaldehyde (Staab et al., 2009). Therefore, GSNOR inhibition may result in the accumulation of cellular toxic formaldehyde. Formaldehyde is also detoxified by mitochondrial ALDH2, and its activation is shown to reduce the levels of

formaldehyde in a GSNOR knockout mouse model of heart ischemia-reperfusion injury (Casin et al., 2018). Therefore, our approach to combine the GSNOR inhibitor N6022 with the ALDH2 activator Alda-1 for optimal functional recovery was logical. The combination therapy approach is further supported by our finding of limited functional recovery in GSNOR KO mice following SCI (Fig 2). As observed in GSNOR KO mice, excessive formaldehyde is reported to affect cognitive functions and other toxic effects (Ai et al., 2019). These observations indicate that the complete absence of GSNOR activity, as in GSNOR KO mice, or prolonged and sustained inhibition by a GSNOR inhibitor, may not be helpful for overall functional recovery. However, partial inhibition of GSNOR by N6022 [a specific inhibitor with shorter half-life ranging from 1.7-4.7 h in rat plasma (Colagiovanni et al., 2012)] improved locomotor (Fig 3A) as well as sensory functions (Fig 3B) compared with non-treated animals. Based on our data (Fig 2) and the protective effect of Alda-1 in GSNOR KO mice (Casin et al., 2018), combining N6022 with Alda-1 for optimal functional recovery following SCI was justified.

Edema and vessel injuries contribute to ischemic injury in the perilesional region in the early phases of the injury. Down regulating such neurovascular injuries in the early phase is associated with subsequent neuroprotection and functional recovery. A clinically transformational SCI therapy would address this early injury and alleviate the secondary neurovascular injury and stimulate functional recovery later in the recovery phase. Previously, we have documented that exogenously administered GSNO protects the neurovascular unit (Chou et al., 2011; Khan et al., 2009; Khan et al., 2012); however, the neurovascular effects of N6022 and Alda-1 on BSCB protection and edema were previously unknown in SCI. Like in experimental stroke studies (Khan et al., 2019; Li et al., 2018), both drugs significantly reduced edema and Evans blue extravasation (Fig 4) and decreased the expression of ICAM-1, indicating their neurovascular protective effects. The combination therapy had greater improvements in both edema and BSCB leakage, supporting the combination of N6022 and Alda-1 to invoke synergistic neurovascular protective and anti-inflammatory effects.

Because the study design is based on the mechanisms that N6022 invokes its beneficial effects via the inhibition of GSNOR and Alda-1 provides protective effects via the activation of ALDH2, we investigated the effects of N6022 and Alda-1 on the activity and the expression of GSNOR. SCI-induced increased activity of GSNOR was inhibited by N6022 and not by Alda-1 (Fig 5A). Because Alda-1 had no effects on GSNOR activity, any additional effect of Alda-1 on the activity was not observed in the combination group. Furthermore, neither SCI, N6022, nor Alda-1 altered the expression of GSNOR in the early phase (72 h after SCI) of the injury (Fig 5B, C). Unlike the increased activity of GSNOR, the activity of ALDH2 was suppressed following SCI (Fig 6A). The treatment with Alda-1 significantly enhanced ALDH2 activity, which correlated with neurovascular protection, supporting our hypothesis that ALDH2 plays a beneficial role. It’s also clear from the finding of no-change in ALDH2 expression (Fig 6B, C) that Alda-1 invokes its effect by enhancing the ALDH2 activity rather than regulating its expression.

The present study design had several limitations. First, the study was based on animal experiments only, and thus the cause-and-effect relationship was not investigated *in vitro.*

Second, the study is based on male animals only; therefore, the data do not address any gender-based differential efficacy of N6022 or Alda-1. Third, the study endpoints did not exceed a month. To achieve greater improvements in pain may take a longer time. Fourth, we did not determine the effects of N6022 on the expression of ALDH2. Finally, the treatment with drugs was initiated first at two h after SCI, which may be early following an injury to maximize any translational potential.

4. Conclusions

GSNO is a natural component of the human body, and increasing its endogenous levels using the GSNOR-specific inhibitor N6022 aids in functional recovery following SCI. Similarly, the ALDH2 agonist Alda-1 improves functional outcomes following SCI, and its use in preclinical studies has shown a strong safety profile. Based on highly remarkable improvements in the combination group, we anticipate that combination therapy (N6022+Alda-1) will offer a novel correction of SCI's disturbed NO metabolome and dysregulated mitochondrial functions, leading to a potential human SCI therapy.

5. Experimental Procedure

5.1. Reagents

N6022 (3-(5-(4-(1H-imidazol-1-yl) phenyl)-1-(4-carbamoyl-2-methylphenyl)-1H-pyrrol-2-yl) propanoic acid) was from Axon Medchem (Reston, VA, USA). Alda-1 [N-(1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide] (Item#: 21555) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

5.2. Animals

Animals were young adult male C57BL/6J mice, obtained from Jackson Laboratory (Bar Harbor, ME, USA) weighing 25-30 g at the time of spinal cord injury procedure. GSNOR KO (B6 background) are bred in our animal facility. Breeding pairs of GSNOR KO, generated with the B6 mice background (Casin et al., 2018), were received from Drs. Mark Kohr and Shyam Biswal (Johns Hopkins University, School of Medicine, Baltimore, MD, USA). All animals received humane care in compliance with the Medical University of South Carolina's (MUSC) guidance and the National Research Council's humane care criteria. Animal procedures were approved by the institutional animal care and use committee (IACUC) of MUSC. Eight animals/group in behavior studies and four animals/group in biochemical (enzyme activity and expression) studies were used. Eight animals/group in BSCB studies were also used. The animal number in each group is also included in figure legends.

5.3. Drugs (N6022 and Alda-1) treatment

N6022 (5.0 mg/kg body weight in 20% 100 μl DMSO/saline, iv) was slowly administered by tail vein at 2h after spinal cord contusion. The same dose of N6022 was administered daily by the iv route until the endpoints. Sham and SCI animals were administered 20% DMSO in saline (vehicle) as previously described by our laboratory (Saxena et al., 2018) and others

(Blonder et al., 2014; Bodas et al., 2017). In our studies, a 5 mg/kg body weight dose of N6022 had no effects on physiological parameters (heart rate, mean arterial blood pressure and body/rectal temperature) when measured at 30 min after the administration of the N6022 preparation (5.0 mg/kg body weight in 40% DMSO/saline, 100 μl, ip) in mice (Table 1).

Alda-1 was also administered at 2 h after spinal cord contusion. Sham and SCI animals were administered vehicle (20% DMSO/saline, 100 μl ip). The same dose of Alda-1 was administered daily by the ip route until the endpoints. In our studies, 10 mg/kg body weight dose of Alda-1 had no effects on physiological parameters (heart rate and body/rectal temperature) when measured at one h after the administration of the Alda-1 preparation (10.0 mg/kg body weight in 20% DMSO/saline, 100 μl ip) in mice (Table 1). Alda-1 was administered first in the combination group, followed by N6022 using the same dose and route described for Alda-1 and N6022.

5.4. Physiologic parameters

Using wild type normal animals, the physiologic parameters were measured at 30 min or one hour after the treatment with vehicle (20% DMSO/saline, 100 μl ip), N6022 (5.0 mg/kg body weight, iv) or Alda-1 (10.0 mg/kg body weight, ip) as described in Table 1. Mean blood pressure (MBP) and heart rate (HR) were measured without anesthesia. Body/rectal temperature was monitored MBP and HR were measured using an NIBP system (Kent Scientific, Torrington, CT). It is a non-invasive computerized tail-cuff system using an automated inflation/deflation pump.

5.5 Mouse contusion SCI model

Including the sham group, all mice were anesthetized with Ketamine (90 mg/kg, ip) and Xylazine (10 mg/kg, ip). Analgesic buprenorphine (0.05-0.1 mg/kg, sq, 12 hourly) was administered pre-emptively and after the surgery to alleviate pain. The toe was firmly pinched to elicit the withdrawal response. The animal was then placed in a stereotaxic frame. SCI at the T9-T10 level was produced on the exposed spinal cord following laminectomy between T-9-T10. SCI was induced using a computer-controlled impactor device described by Dr. Bilgen (Bilgen, 2005) and used in our studies (Chou et al., 2011; Khan et al., 2018). SCI was performed with 1-2 mm tissue deformation and an impact velocity of 1.5 m/s and contusion time of 85 ms. These parameters and conditions produce reproducible moderate spinal cord injury. Sham animals followed the same procedures, with the exception of the impact. During impact, body temperature was maintained at 37 °C by a heating pad. Immediately after injury, the muscle and skin were closed using nylon suture, and 2% lidocaine jelly was applied to the lesion site to minimize any possible discomfort. The bladders of all animals were expressed two to three times per day initially and later as needed. The body weight and humane endpoints were regularly monitored. The animals were euthanized at the endpoint by decapitation under deep anesthesia to harvest the spinal cord for biochemical estimations. The spinal cords were snap-frozen and stored at −70 °C for assays later on as needed.

5.6. Basso mice scale (BMS) score

The BMS was developed to assess open field locomotion deficits in spinal cord injured mice (Basso et al., 2006). This scoring system is widely used as an indicator of locomotor function recovery in mouse models of SCI. Later, modifications were added to improve the scale as described (Pajoohesh-Ganji et al., 2010). BMS scoring was performed at the indicated time points, as shown in Fig 1. BMS scores were evaluated by two observers blinded to the identity of animal groups.

5.7. Sensory functions (pain sensitivity)

Pain-like behaviors were determined by mechanical allodynia (Khan et al., 2015b) and hyperalgesia (Oghbaei et al., 2020) methods. Improvement in mechanical threshold and thermal withdrawal latency by drug treatments were used to indicate improved sensory function leading to reduced pain-response behavior.

i) Mechanical allodynia: Animals were acclimatized to the animal chamber of dynamic plantar aesthesiometer (DPA) for about 15 minutes. DPA is an automated version of the von Frey hair analysis (Ugo Basile, Italy). It is used to assess changes in sensation or the development of mechanical hyperalgesia resulting from neuropathic pain (Obata et al., 2004). Animals were placed individually in a small enclosed testing area with a wire mesh floor. The DPA device was positioned beneath the animal so that the filament was directly beneath the foot's plantar surface to be tested. The instrument raised the filament to touch the foot and progressively increased the force applied until it reached a maximum of 20 g. The force at which the foot was withdrawn was recorded with the software supplied by the manufacturer.

ii) Thermal algesia: Mice were gently restrained by placing them in a restrainer, and then the distal 1.0 cm of their tail was dipped into the hot water bath maintained at 49 ± 1.0°C. The latency (time in seconds) between exposure to the hot water and the sudden tail withdrawal was recorded. A cut-off time of 30 s was established to minimize the possibility of tissue damage from heat exposure.

5.8. Forced swim test (FST)

The forced swim test (FST) was developed to measure depressive-like behavior in mice following CNS trauma. The test was performed as previously described (Can et al., 2012). Briefly, a cylindrical tank (30 cm height × 20 cm diameter) made of Plexiglass was used in the study. The water level was maintained 15 cm from the bottom; thus, the mice could not touch the bottom of the tank either with their hind limbs or tail. The entire experiment was recorded with a video camera. Tests were performed individually in sequence. The mice were forced to swim for 8 min 24 h before the actual test. On the day of the test, each mouse was given a forced swim period of 6 min. The last 4 min of the total 6 min was used for the final calculation. Periods of mobility and immobility were calculated as described by Can et al. (Can et al., 2012).

5.9. Evans blue (EB) extravasation analysis

Blood-spinal cord barrier (BSCB) leakage was determined as described (Cabrera-Aldana et al., 2017) with slight modification. The mice were administered (iv) 100 μl of a 5% solution of EB in saline at 4 h following SCI. At 72 h, cardiac perfusion was performed under deep anesthesia with 200 ml of saline to remove the circulating EB. The spinal cord was isolated and photographed. The spinal cord was homogenized in 750 μl of N, N-dimethyl formamide (DMF). The homogenized spinal cord was kept at room temperature in the dark for 72 h and then centrifuged at 10,000 × g for 25 min. The supernatant was spectrofluorometrically analyzed (lex 620 nm, lem 680 nm) to determine the content of EB.

5.10. Measurement of Edema (spinal cord water content)

At 72 h following SCI, mice were euthanized to assess spinal cord water content (edema) as described earlier (Cabrera-Aldana et al., 2017). Fresh spinal cord from non-perfused mice was collected, and a segment of 0.5 cm of the spinal cord containing the lesion was weighed. Each sample was dried at 60°C for 72 h, and the dry weight was recorded. Tissue water content was calculated as water content (%) = (wet weight – dry weight)/wet weight × 100.

5.11. GSNOR Activity

GSNOR activity was measured using GSNO-dependent consumption of NADH as described (Hayashida et al., 2019). Briefly, brain samples were homogenized in a 50 mmol Tris-HCl (pH 8.0), 150 mmol NaCl, 1 mmol EDTA, 0.1% Triton X-100, and 1:100 protease inhibitor cocktail, and centrifuged at 10,000g for 10 min. Samples were diluted to 0.1mg/ml protein in reaction buffer (20 mmol Tris-HCl (pH 8.0) and 0.5 mmol EDTA). 100 μl of each sample was incubated with 100 μmol NADH in the presence or absence of 150 μmol GSNO. GSNO-dependent consumption of NADH was monitored via absorbance at 340nm for 15 min at 25°C. Heart homogenate was used as a positive control (Casin et al., 2018).

5.12. ALDH2 activity

ALDH2 activity in tissue lysates was measured using an ALDH2 enzymatic activity assay kit from Abcam (Cat# ab115348) according to the manufacturer's protocol and as described by Hua et al. (Hua et al., 2018). As described in the western blot analysis section, the frozen spinal cord (5mm segment) was homogenized and centrifuged at 10,000 × g for 30 min at 4°C. The activity was measured at 25°C. The NADH production level was determined spectrophotometrically by monitoring the alterations in absorbance intensity at 340 nm every 30 sec for 5 min. The reaction rate of ALDH2 was expressed as μmol NADH/min/mg protein.

5.13. Western blot analysis

The animals were euthanized at the endpoints by decapitation under deep anesthesia, and the spinal cord was collected for biochemical experiments. The spinal cords were snap-frozen and stored at −80 °C for subsequent assays.

Using 5 mm of spinal tissue (~1mm epicenter; 2mm caudal and 2 mm rostral from the epicenter) from the injured cord, the western analysis was performed as described earlier

(Khan et al., 2018). The following antibodies were used for western blot. ICAM-1 (Thermo Fisher Scientific Cat# MA5407, RRID:AB_223596, 1:1000 dilution), GAPDH (Cell Signaling Cat # 5174S, RRID:AB_610622025, 1:5000 dilution), GSNOR (Abcam Cat# ab91385, RRID:AB_2049142), β-actin (Abcam Cat# Ab8226, RRID:AB_306371, 1:10,000 dilution), and horseradish peroxidase-conjugated, goat anti-rabbit secondary antibody (Jackson ImmunoResearch Lab Cat# 111-035-045, RRID:AB_2337938, 1:10,000 dilution). All antibodies were diluted with 1XTBST with 2% non-fat dry milk. Protein concentrations were determined using a protein assay kit from Bio-Rad Laboratories (Hercules, CA). Twenty microgram protein was used for western analysis. Densitometry of protein expression was performed using ImageJ software (NIH, Bethesda, MD).

5.14. Statistical evaluation.

Statistical analysis was performed as described previously from our laboratory (Khan et al., 2015a; Khan et al., 2015b). Statistical significance was determined by one-way ANOVA followed by the post-hoc Tukey test using Graph pad Prism software 5.01. Data are expressed as mean ±standard deviation (SD) of n determinations. Statistical significance was set as a p value<0.05.

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Highlights

- SCI disturbs nitric oxide metabolome and compromises mitochondrial function.
- Key metabolic enzymes, GSNOR and ALDH2, turn aberrant after SCI.
- Inhibition of GSNOR by N6022 or activation of ALDH2 by Alda-1 protects against SCI.
- Combination therapy with N6022 and Alda-1 had greater efficacy than monotherapy with either.
- Targeting GSNOR and ALDH2 is a novel approach for SCI therapy.

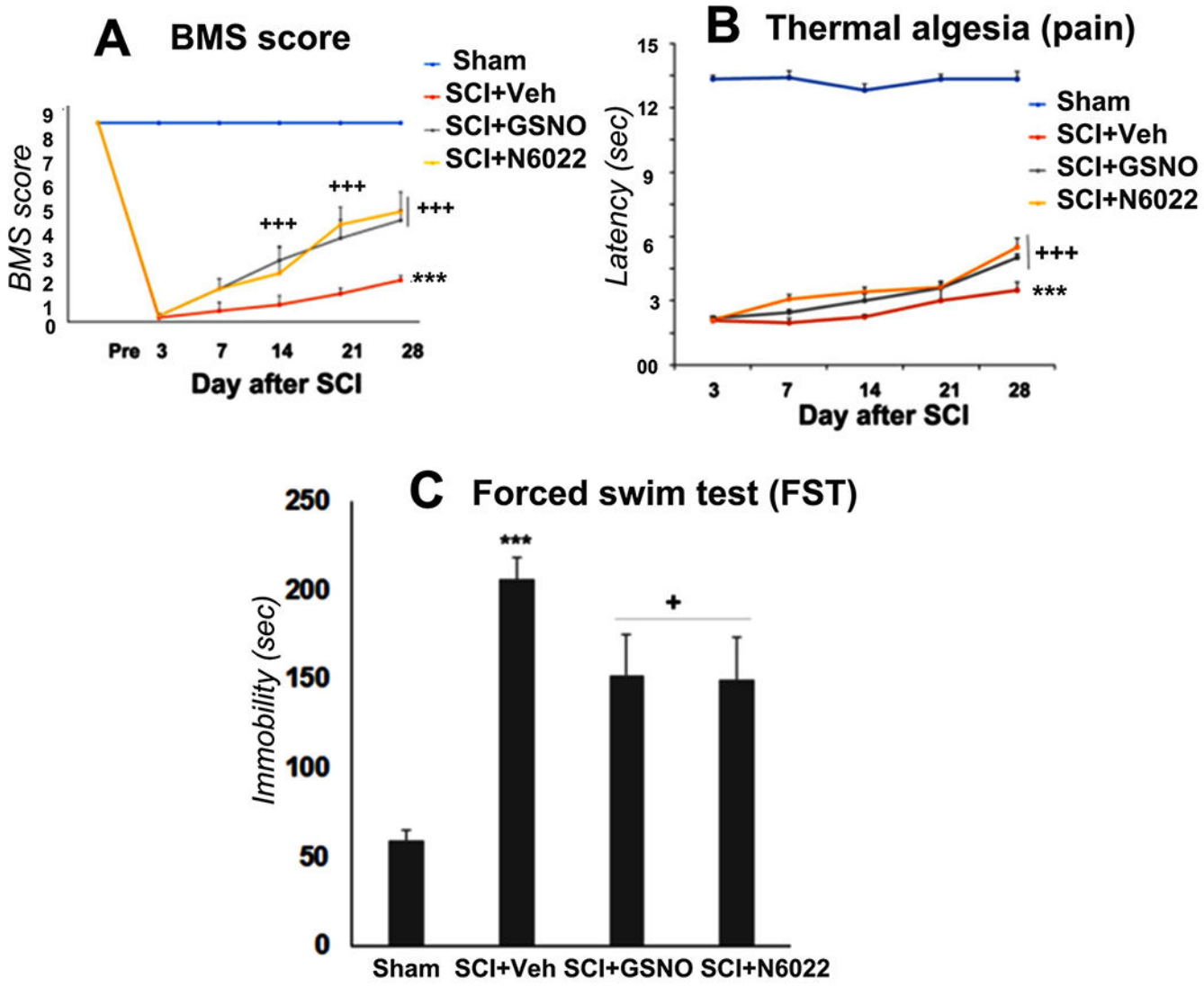

Fig 1.

Effect of N6022 and GSNO on locomotor function and pain- and anxiety-like behaviors in a 28 day wild type (WT) mouse model of SCI. Studies on locomotor function were performed at indicated days using BMS locomotor rating scale (A) and pain-like behavior using the thermal algesia method (B). Anxiety-like behavior was evaluated on the 28th day after SCI using FST (C). Data are presented as mean ± SD (n = 8). ***p < 0.001 vs. Sham, +++p<0.001, +p<0.05 vs. SCI+Veh.

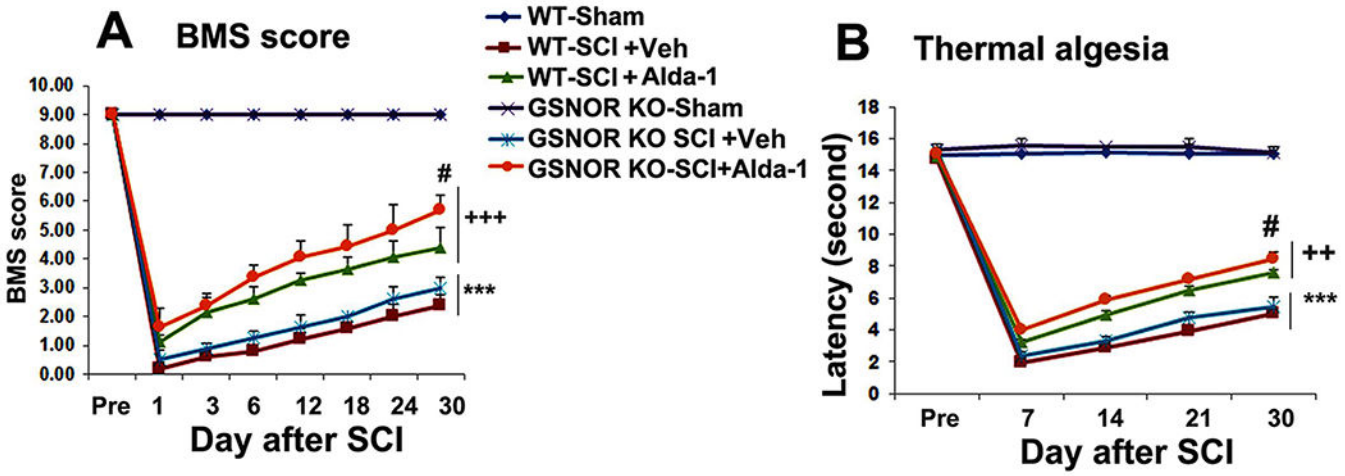

Fig 2.

Effect of N6022 and Alda-1 on locomotor function and pain-like behavior in a 30 day wild type (WT) and GSNOR$^{-/-}$ (GSNOR KO) mouse model of SCI. Studies on locomotor function were performed at indicated days using BMS locomotor rating scale (A) and pain-like behavior using the thermal algesia method (B). Data are presented as mean ± SD (n = 8). ***p < 0.001 vs. Sham, ++p<0.01 vs. SCI+Veh, #p<0.05 vs. WT-SCI+Alda-1.

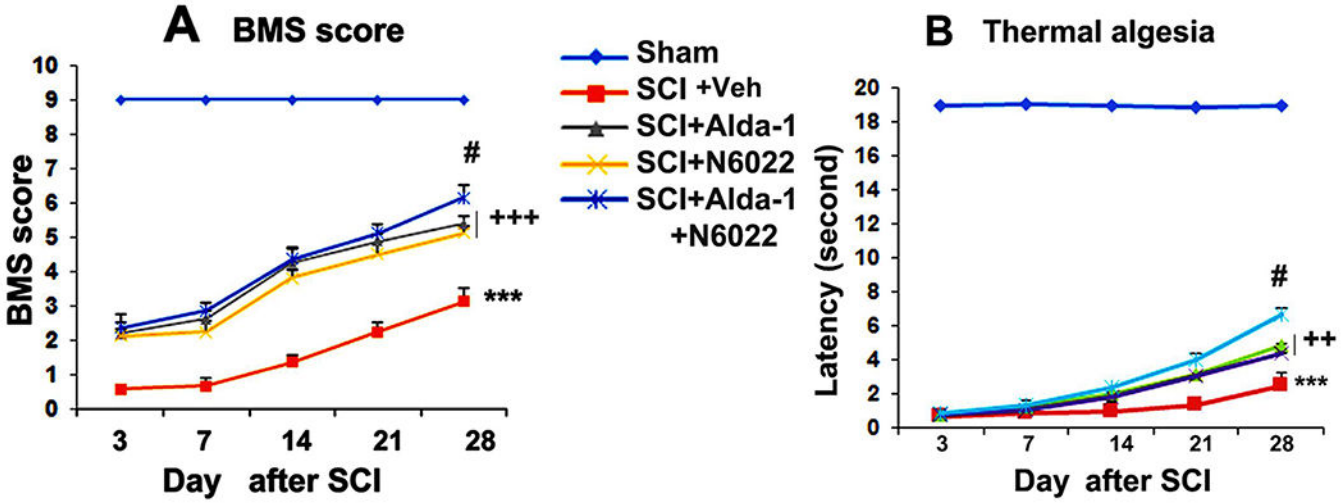

Fig 3.

Effect of N6022 and Alda-1 and comparison of monotherapy with combination therapy (N6022+Alda-1) on locomotor function and pain-like behavior in a 28 day wild type mouse model of SCI. Studies on locomotor function were performed at indicated days using BMS locomotor rating scale (A) and pain-like behavior using the thermal algesia method (B). Data are presented as mean ± SD (n = 8). ***$p < 0.001$ vs. Sham, +++$p<0.001$, ++$p<0.01$ vs. SCI+Veh, #$p<0.05$ vs. SCI+Alda-1 and SCI+N6022.

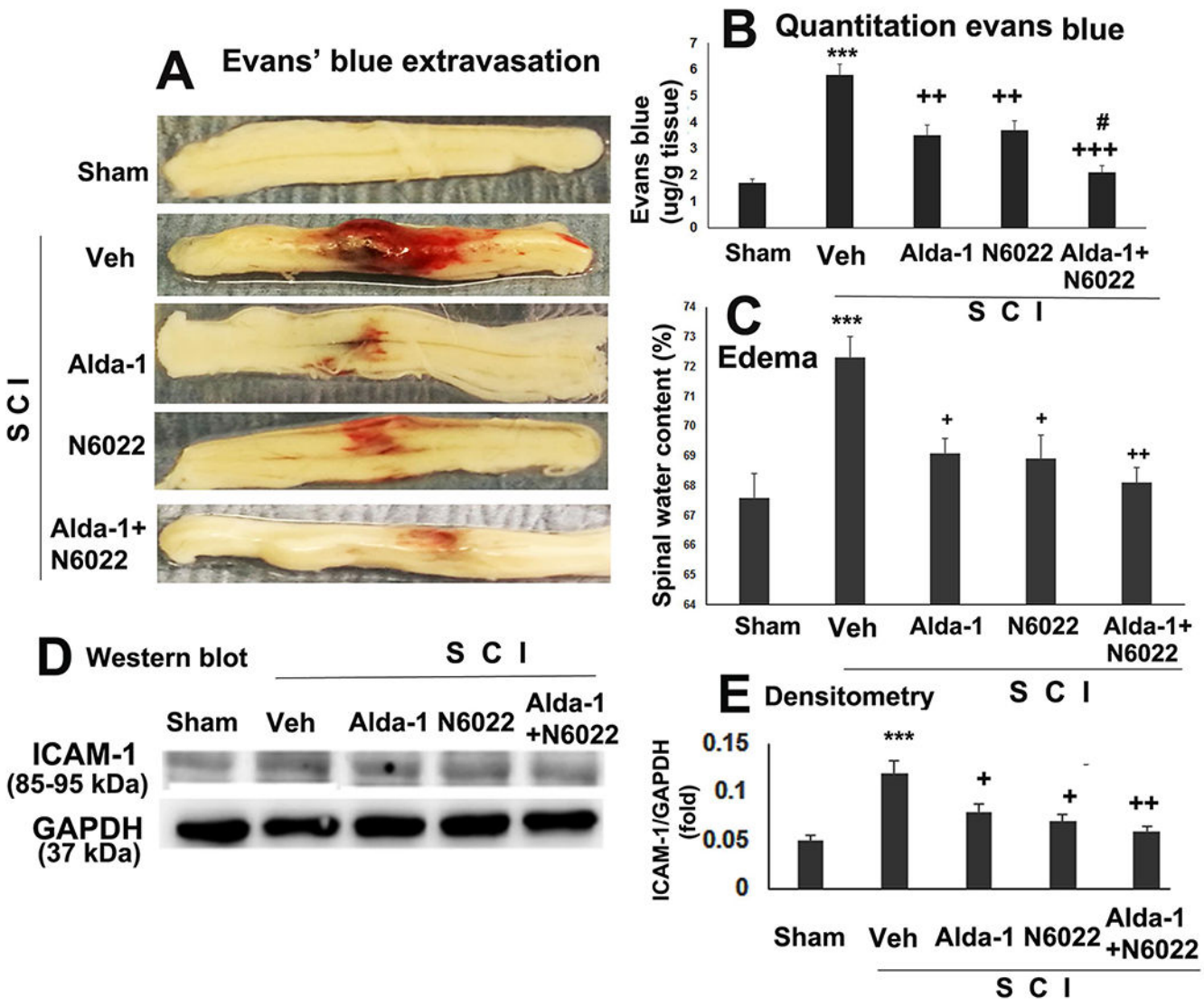

Fig 4.

Effect of N6022 and Alda-1 and comparison of monotherapy with combination therapy (N6022+Alda-1) on blood-spinal cord barrier (Evans blue extravasation), edema, and the expression of ICAM-1 in the acute phase (72h) of SCI using a wild type mouse model of SCI. Representative whole spinal cords showing Evans blue dye extravasation into the spinal cord (A) and quantification of the Evans blue extravasation (B), levels of edema (spinal water content) (C), Western blot of the expression of ICAM-1 (D) and densitometry of ICAM-1 (E). Data are presented as mean ± SD (n = 8). ***p < 0.001 vs. Sham, ++ +p<0.001, ++p<0.01, +p<0.05 vs. SCI+Veh, #p<0.05 vs. SCI+Alda-1 and SCI+N6022.

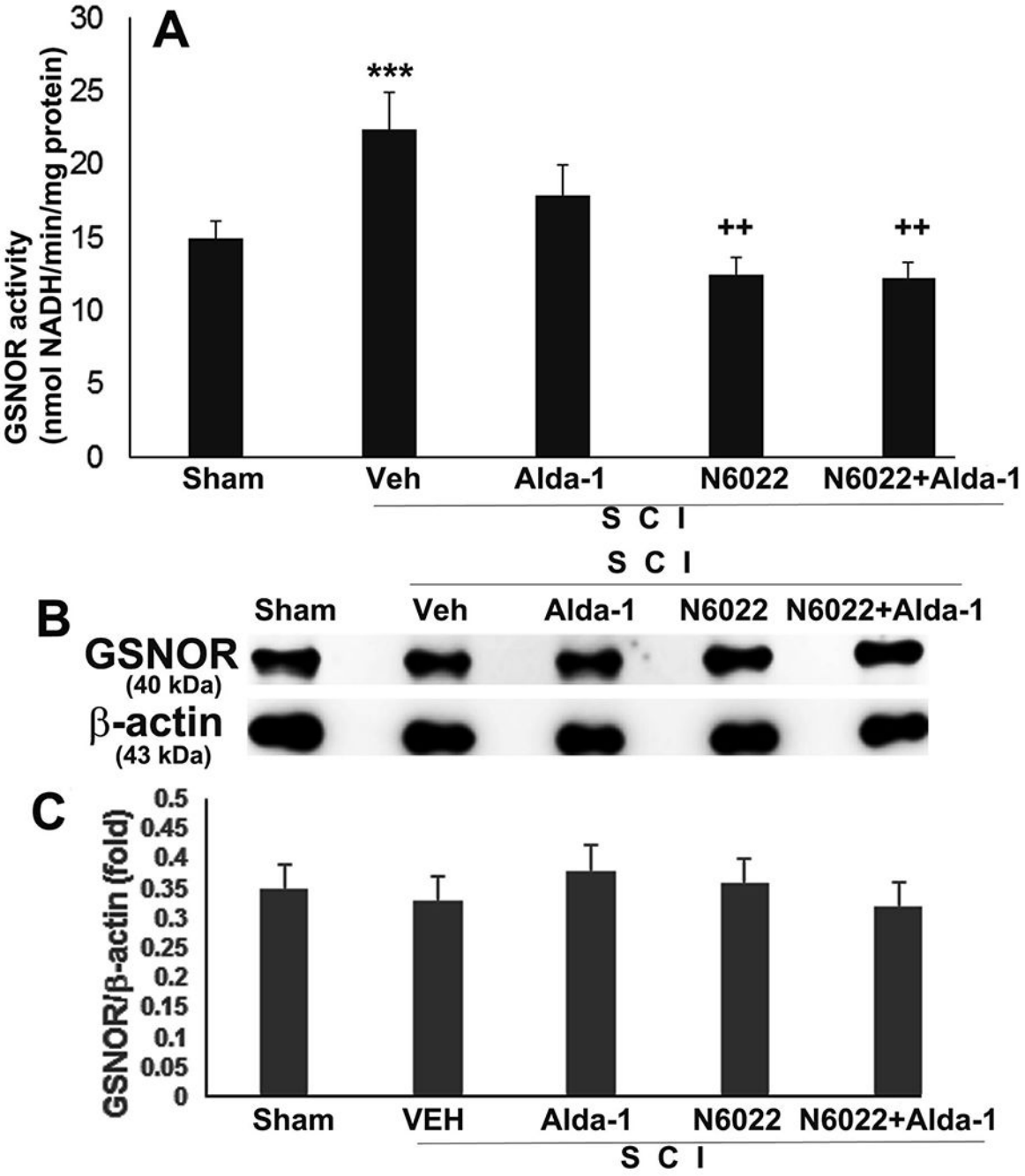

Fig 5.

Effect of N6022 and Alda-1 and comparison of monotherapy with combination therapy (N6022+Alda-1) on the activity and the expression of GSNOR in the acute phase (72h) of SCI using a wild type mouse model of SCI. GSNOR activity was measured as the consumption of NADH (A). The expression of GSNOR was determined by western blot (B) and its densitometry (C). Data are presented as mean ± SD (n = 4). ***p<0.001 vs. Sham, ++p<0.01 vs SCI+Veh.

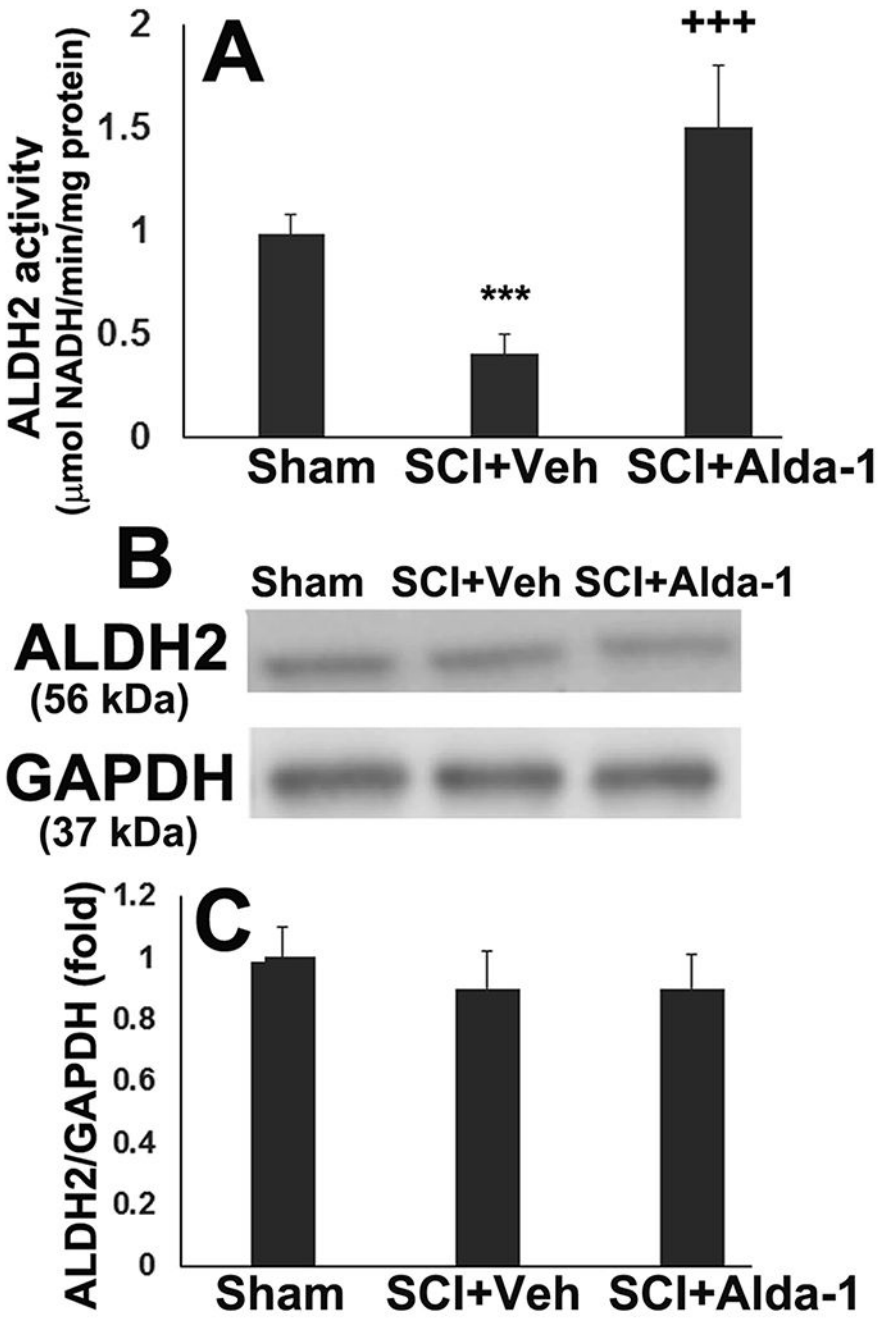

Fig 6.

Effect of Alda-1 and N6022 on the activity of ALDH2 in the acute phase (72h) of SCI using a wild type mouse model of SCI. ALDH2 activity was measured as the generation of NADH. Data are presented as mean ± SD (n = 4). *p<0.05 vs. Sham, +++p<0.001, +p<0.05 vs SCI+Veh, #p<0.05 vs. SCI+Alda-1.

Table 1.

Physiological parameters

Parameters	Vehicle-treated (measured after 1 h)	Alda-1-treated (measured after 1 h)	N6022-treated (measured after 30 min)
Rectal Temp (°C)	37.4±0.3	36.7±0.3	37.4±0.4
MABP(mm Hg)	168.11±9.0	155.6±13.2	170.5±17.4
HR (beat/min)	750.0±180.0	739.4±255.1	753.0±170.9

Measurements were performed after the treatment with vehicle (20% 100 μl DMSO/saline ip), Alda-1 (10 mg/kg body weight, in 20% 100 μl DMSO/saline, ip) or N6022 (5.0 mg/kg body weight, in 20% 100 μl DMSO/saline iv). Data are presented as means ± SD for n = 8. Alda-1, ALDH2 activator; N6022, GSNO reductase inhibitor; MABP, mean arterial blood pressure; HR, heart rate. No significant differences were observed among the groups.