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Original Research

Cryogen spray cooling mitigates inflammation and injury-induced CISD2 decline in rat spinal cord hemisection model

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Therapeutic strategies for traumatic spinal cord injury generally involve rectifying concomitant destruction to the spinal cord from inflammation, mitochondrial dysfunction, and eventual neuronal apoptosis. Elevating the expression of spinal cord injury-attenuated CDGSH iron-sulfur domain-2 has been shown to mitigate the pathologies above. In the current work, hypothermia was induced via continuous cryogen spray cooling in a rat spinal cord hemisection model. Spinal cord injury was shown to elevate the mRNA expression of proinflammatory mediators, including NFκB, iNOS, TNF-α, and regulated upon activation, normal T-cell expressed and secreted as well as lower CDGSH iron-sulfur domain-2 expression. Cryogen spray cooling treatment was shown to attenuate inflammatory reactions and elevate CDGSH iron-sulfur domain-2 expression. Immunohistochemical analysis of the glial fibrillary acidic protein, caspase-3 and NeuN in spinal cord injured rats that underwent cryogen spray cooling treatment revealed notable reductions in injury-induced astrocytic activation, apoptosis, neuronal loss, and decline in CDGSH iron-sulfur domain-2 expression. These results demonstrate the CDGSH iron-sulfur domain-2 preserving effects of cryogen spray cooling, which could contribute to the prevention of astrocytic activation, astrocyte-mediated neuroinflammation, apoptosis, and neuron loss.

Keywords

Cryogen spray cooling; hypothermia; CISD2; inflammatory response; astrocyte activation; apoptosis; neuronal loss; spinal cord injury

1. Introduction

The incidence of acute spinal cord injury (SCI) in the general population ranges from 15-40 cases per million worldwide. The pathophysiology of acute SCI initially involves significant insult to the spinal cord, resulting in immediate structural damage, including cell membrane rupture, myelin and axon damage, and microvascular destruction. Secondary injuries include profound proinflammatory responses, excitotoxicity, hyperoxidation, mitochondrial dysfunction, and eventual apoptotic cell death (Bethea and Dietrich, 2002; Houle and Tessler, 2003). Several molecular mechanisms have been implicated in SCI's pathogenesis, including hypoxia, ischemia, lipid peroxidation, free radical production, neutral protease activation, prostaglandin production, and programmed cell death (Bethea and Dietrich, 2002; Hung et al., 2007; Tator and Fehlings, 1991). Therapeutic strategies targeting secondary damage focus on reducing neuronal loss.

Amino acid CDGSH iron sulfur domain 2 (CISD2) plays an important role in calcium metabolism (Shen et al., 2017) as well as anti-apoptotic (Chang et al., 2010) and anti-inflammatory activities (Kung et al., 2020; Lin, 2020). CISD2 helps preserve the mitochondrial membrane's integrity and thereby prevents mitochon-

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drial malfunction and ultimate cell death. CISD2 depletion has been linked to mitochondrial breakdown and dysfunction accompanied by autophagic cell death in CISD2 knockout mice (Chen et al., 2009). At the endoplasmic reticulum, CISD2 has been shown to attenuate calcium-mediated excitotoxicity through the binding of CISD2 to BCL2 and the inositol 1, 4, 5-triphosphate receptor (Chang et al., 2012). CISD2 has also been shown to enhance the combination of BCL2 and BECN1, which regulates cellular autophagy/apoptosis (Kang et al., 2011). CISD2 deficiency has been linked to an increase in iNOS and a reduction in BCL2 levels in SH-SY5Y cells challenged with lipopolysaccharide (LPS) (Lin et al., 2017). CISD2 deficiency has also been linked to augmented nuclear translocation of the NFκB (nuclear factor κB) p65 subunit and a shift of the M2 phenotype toward M1 microglia-associated inflammation in EOC microglia (Lin, 2020). Its reduction has even been linked to a decrease in PPAR-β expression in SH-SY5Y cells, indicating enhanced degradation of IκB (an NF-κB inhibitor) and consequent NFκB activation (Kung et al., 2020). In mice, CISD2 expression levels have been shown to decline as a function of aging (Lin et al., 2019) or spinal cord hemisection injury (Lin et al., 2017). In neural cell models involving CISD2 knockdown, CISD2 deficiency has been shown to augment inflammatory responses and mitochondrial dysfunction, as indicated by low DeltaPsi (m) levels, high reactive oxygen species (ROS) levels, and increased cellular apoptosis (Lin et al., 2019). Therapeutic strategies aimed at elevating CISD2 levels through curcumin administration (Lin et al., 2017, 2019) or wild bitter melon (Kung et al., 2020) have also been shown to mitigate the pathologies above.

Therapeutic hypothermia aimed at halting tumor progression was first reported in 1945 (Kwon et al., 2008). Over the last half-century, therapeutic hypothermia has been evaluated for its neuroprotective effects on the brain and spinal cord in situations such as ischemic or traumatic brain injury (TBI) (Bernard and Buist, 2003; Hansebout et al., 1984; Lo Jr et al., 2009; Yu et al., 2000). Therapeutic hypothermia can be managed systemically or locally. Systemic treatment involves the cooling of external surfaces or internally via the endovascular system. Local hypothermia avoids the side effects of systemic hypothermia while allowing a more pronounced reduction in temperature at the injury site (Jordan and Carhuapoma, 2007). Researchers have demonstrated therapeutic hypothermia's efficacy in attenuating secondary injury by reducing neuroinflammation and cellular apoptosis in ischemic brain injury (Ohmura et al., 2005). Therapeutic hypothermia has been shown to preserve damaged tissue and promote impaired locomotor function in experiments on SCI rats and SCI patients undergoing systemic hypothermia (Lo Jr et al., 2009; Yu et al., 2000) or local spinal cord cooling (Hansebout et al., 1984).

In 1994, Nelson et al. (1995) introduced cryogen spray cooling (CSC) to induce local hypothermia during the spatially selective photocoagulation of biological tissue. This method involves spraying cryogen on the skin's surface for a short duration, such that the effects of cooling remain localized in the epidermis and the temperature of deeper blood vessels remains unaffected (Nelson et al., 1996; Waldorf et al., 1997). As mentioned above, neural injuries can lead to CISD2 downregulation, resulting in exaggerated inflammatory reactions. In the current work, we sought to verify the neuroprotective (anti-inflammatory and CISD2-preserving) effects of therapeutic hypothermia induced through the continuous delivery of CSC in a rat spinal cord hemisection model.

2. Materials and methods

2.1 Hemisection of the spinal cord in rats

Male Sprague-Dawley (SD) rats (Academia Sinica, Taipei, Taiwan) weighing 280-330 g were maintained at a density of two animals per cage for at least 5 days after they arrived at our laboratory. The rats were given *ad libitum* access to food and water while housed within a room with a 12 : 12 h light-dark cycle. All rats were treated as per the guidelines stipulated by the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of Chang Gung University (IACUC Approval No.: CGU14-052).

The surgical procedure used in the lateral hemisection of the spinal cord in rats can be found in our previous studies (Lin et al., 2011, 2012). Briefly, isoflurane was delivered to the rats as general anesthesia, before the animals were fastened within a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). An adjustable wire knife was used to create a spinal cord lesion on the left side. Control animals were subjected to a sham operation (laminectomy without hemisection). Posterior decompression was achieved via laminectomy at the eleventh thoracic vertebrae using a fine diamond drill. The wire knife guide was positioned vertically adjacent to the lateral surface at the spinal cord's lower thoracic level. This permitted analysis of the healthy cephalad and caudal segments of the spinal cord. The knife was turned medially and then extended 1.5 mm, at which point the guide was lifted by 4.0 mm to hemi-transect the spinal cord. Iridectomy scissors were then used to cut to ascertain the thoroughness of the hemisection. The wound was closed in layers.

2.2 Cryogen spray cooling for SCI animals

The rats were divided into three groups: (1) Vehicle-treated sham-operated control (sham; laminectomy only) (n = 6); (2) Vehicle-treated SCI group (SCI) (n = 6); (3) SCI rats post-treated with CSC treatment (SCI + CSC) (n = 6).

The cryogen is 1, 1, 1, 2-tetrafluoroethane, an FDA-approved replacement for Freon, biodegradable, non-toxic, and incombustible. Following spinal cord hemisection, cryogen spurts were immediately sprayed onto the injured spinal cord through an electrically powered nozzle from a distance of 3 cm above the dural surface. Coolant was delivered continuously in spurts of 20 milliseconds at intervals of 5 spurts per minute for 10 minutes. CSC treatment lowered the surface temperature in the treatment region to -10 °C. The surface temperature returned to normal within roughly 500 milliseconds after CSC treatment (Anvari et al., 1996; Smithies et al., 1998). Note that spraying was precisely regulated using a programmable digital delay generator. A schematic diagram showing the SCI model and CSC treatment are presented in Fig. 1A.

At 24 h post-treatment, all of the animals in all groups underwent deep anesthesia via isoflurane. The animals were then sacrificed, and the spinal cord tissue was removed to investigate mRNA expression (n = 3 in each group) and perform immunohistochemical analysis (n = 3 in each group).

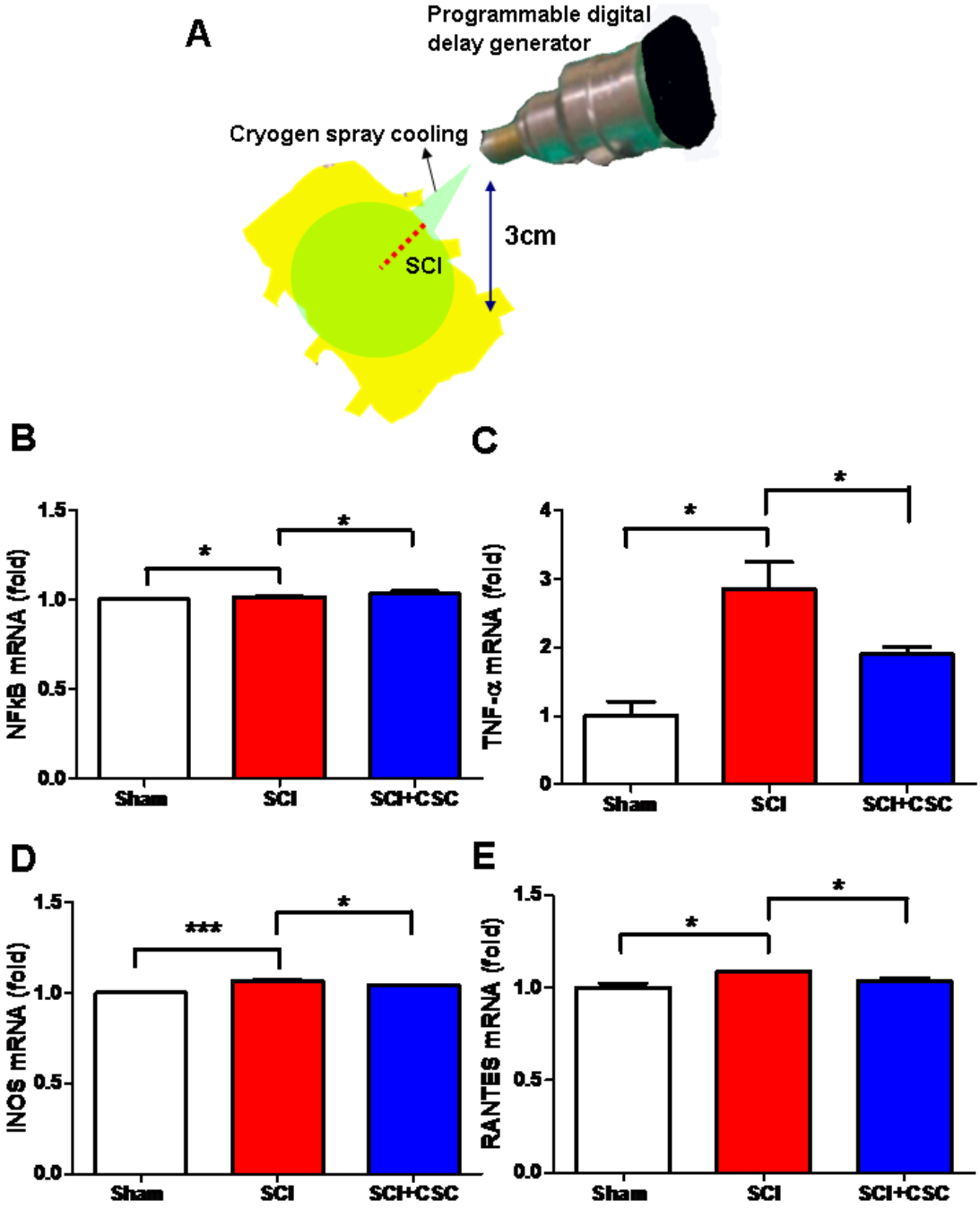

Fig. 1. CSC reduced injury-induced inflammation following spinal cord hemisection in rats. (A) Schematic illustration of the CSC technique applied to tissue adjacent to the spinal cord hemisection under the following conditions: (i) Sham controls; (ii) SCI rats undergoing SCI; (iii) SCI rats undergoing SCI followed by CSC (SCI + CSC); mRNA expression levels of (B) NFκB P105, (C) TNF-α, (D) iNOS, and (E) RANTES. Vertical bars indicate the mean ($\pm$ SEM) mRNA expression (n = 3 in each group). *, $P < 0.05$ and ***, $P < 0.001$ indicate the level of statistical significance.

2.3 Reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was prepared through the direct lysis of animal tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, reverse transcribing the mRNA into cDNA using oligo-dT, and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR was performed using the ABI StepOne sequence detector system (Applied Biosystems, Foster City, CA, USA) in accordance with the SYBR Green methodology, normalized to the expression of a housekeeping gene (GAPDH), as previously described (Lee et al., 2015). The primer sets and product size of each cDNA of interest were as follows: rat CISD2 (Gene ID: 295457, accession number: NM_001191608, 5'- CCCTTCCTTGGTGTACTTGCA -3' and 5'- TTTCGTTCACCACTTTGGGATT -3', 129 bp); NFκB

P105 (Gene ID: 81736, accession number: NM_001276711, 5'- GGGCCTGCAAAGGTTATCGT -3' and 5'- TGCCGTCCTCACAGTGCTT -3', 128 bp); TNF-α (Gene ID: 24835, accession number: NM_012675, 5'- GACGTGGAACTGGCAGAAGAG -3' and 5'- AGGAATGAGAAGAGGCTGAGACA -3', 147 bp); iNOS (Gene ID: 24599, accession number: NM_012611, 5'- GTCTTTGACGCTCGGAACTGT -3' and 5'- CGACCTGATGTTGCCATTGT -3', 101 bp); RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) (Gene ID: 81780, accession number: NM_031116, 5'- AACCCCCTACTCCCACTCATTC -3' and 5'- GGCCATAGGAGAGGACACAGTT -3', 106 bp); and GAPDH (Gene ID: 24383, accession number: NM_017008, 5'- CGTGTTCCTACCCCCAATGT -3' and 5'- CCTGCTTCACCACCTTCTTGAT -3', 103 bp).

2.4 Immunohistochemical staining

On the second day after SCI, the animals underwent deep anesthesia via isoflurane before being sacrificed. They were then thoroughly perfused with phosphate-buffered saline (PBS), followed by cold 4% paraformaldehyde in 0.15 M sodium phosphate buffer. The spinal cords were removed immediately, fixed at 4 °C for 8 h, and cryoprotected in 15% and 30% sucrose for 3 days. Tissue from the spinal cords was frozen (-80 °C) before analysis. Sections (5 μm in thickness) were cut from the center of the spinal cord hemisection on a frozen stage using a sliding microtome. The sections were then washed in PBS and incubated in 3% normal goat serum with 0.3% Triton X-100 in PBS for 1 h. The free-floating sections were incubated at 4 °C with the following antibodies: Anti-GFAP (Catalog: 16825-1-AP, Proteintech, Chicago, USA); Anti-caspase-3 (Catalog: 19677-1-AP, Proteintech, Chicago, IL, USA); Anti-NeuN (clone A60, Catalog: MAB377, Merk, Taipei City, Taiwan); Anti-CISD2 (Catalog: 13318-1-AP, Proteintech, Chicago, IL, USA). Immunoreactivity was visualized using the Vectastain Elite ABC Peroxidase method (Vector Laboratories, Burlingame, CA, USA) with diaminobenzidine (DAB) as the chromagen. The morphological and quantitative analysis focused on injured grey and white matter adjacent to the spinal cord hemisection (epicenter), which is indicated as the region of interest (ROI) within the dashed box in Fig. 3A. Cell counting was performed at a magnification of 400× on every sixth section stained using the antibodies mentioned above. Only cells presenting visible indications of staining were counted. All data are presented as mean ± SEM of three consecutive measurements.

2.5 Statistical analyses

Independent two-sample *t*-tests were used to compare data between the experimental groups. One-way analysis of variance (ANOVA) was used to determine whether there were significant differences between the means of three or more independent (unrelated) groups. In cases where the differences were apparent, multiple comparisons were performed using the Newman-Keuls method. Data are presented as mean ± SEM. All statistical assessments were two-sided and evaluated at a 0.05 level of significance. Statistical analysis was performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc; La Jolla, CA).

3. Results

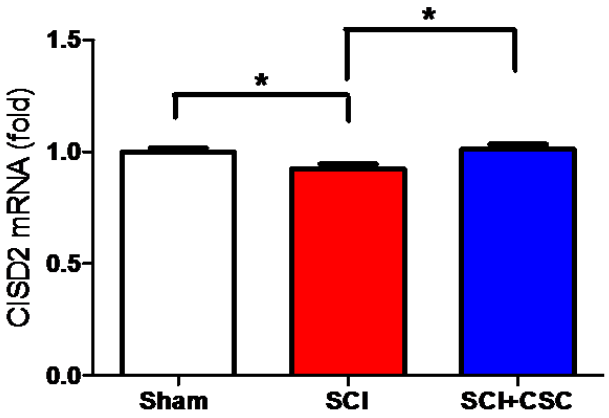

Fig. 2. Spinal cord hemisection produced a corresponding decrease in CISD2 expression. CSC mediated the injury-induced decrease in CISD2 expression. Vertical bars indicate the mean (± SEM) mRNA expression (n = 3 in each group). *, $P < 0.05$ and ***, $P < 0.001$ indicate the level of statistical significance.

3.1 CSC treatment attenuated the expression of proinflammatory mediators in mRNA levels at 24 h after SCI

In the current spinal cord hemisection model, rats were subjected to CSC treatment involving short spurts (20 milliseconds) at intervals of 5 spurts per min for 10 min, or untreated with CSC (Fig. 1A). qRT-PCR was used to examine the mRNA expression of proinflammatory mediators at the site of SCI. Compared to the sham control group, animals in the SCI only group presented higher expression levels of NFκB (P105), TNF-α, iNOS, and RANTES (*$P < 0.05$, Fig. 1B; *$P < 0.05$, Fig. 1C; ***$P < 0.001$, Fig. 1D; and *$P < 0.05$, Fig. 1E, respectively). Compared to the SCI only group, animals in the SCI + CSC group presented significantly lower mRNA expression levels of NFκB (P105), TNF-α, iNOS, and RANTES (*$P < 0.05$, Fig. 1B; *$P < 0.05$, Fig. 1C; *$P < 0.05$, Fig. 1D; and *$P < 0.05$, Fig. 1E, respectively). Overall, these findings indicate that CSC treatment had an anti-inflammatory effect on rats previously subjected to SCI.

3.2 CSC treatment upregulated the expression of CISD2 mRNA at 24 h after SCI

The qRT-PCR analysis revealed that CISD2 expression levels were significantly lower in the SCI only group than in the sham control group ($P < 0.05$, Fig. 2). CISD2 expression levels were considerably higher in the SCI only group than in the SCI + CSC group ($P < 0.05$). In a previous paper, we reported on the anti-inflammatory effects of CISD2 (Lin et al., 2017). In the present work, CSC treatment was shown to attenuate the injury-induced pro-inflammatory response (i.e., SCI-induced reduction in CISD2 expression).

3.3 CSC treatment attenuated GFAP expression following SCI

Astrocytes can be stimulated to release various pro-inflammatory mediators in response to SCI, as indicated by GFAP over-expression. Immunostaining for GFAP (arrow in Fig. 3C) was more robust in the SCI only group than in the sham control group (Fig. 3B) (***$P < 0.001$, Fig. 3E). Immunostaining for GFAP was lower in the SCI + CSC treatment (indicated by

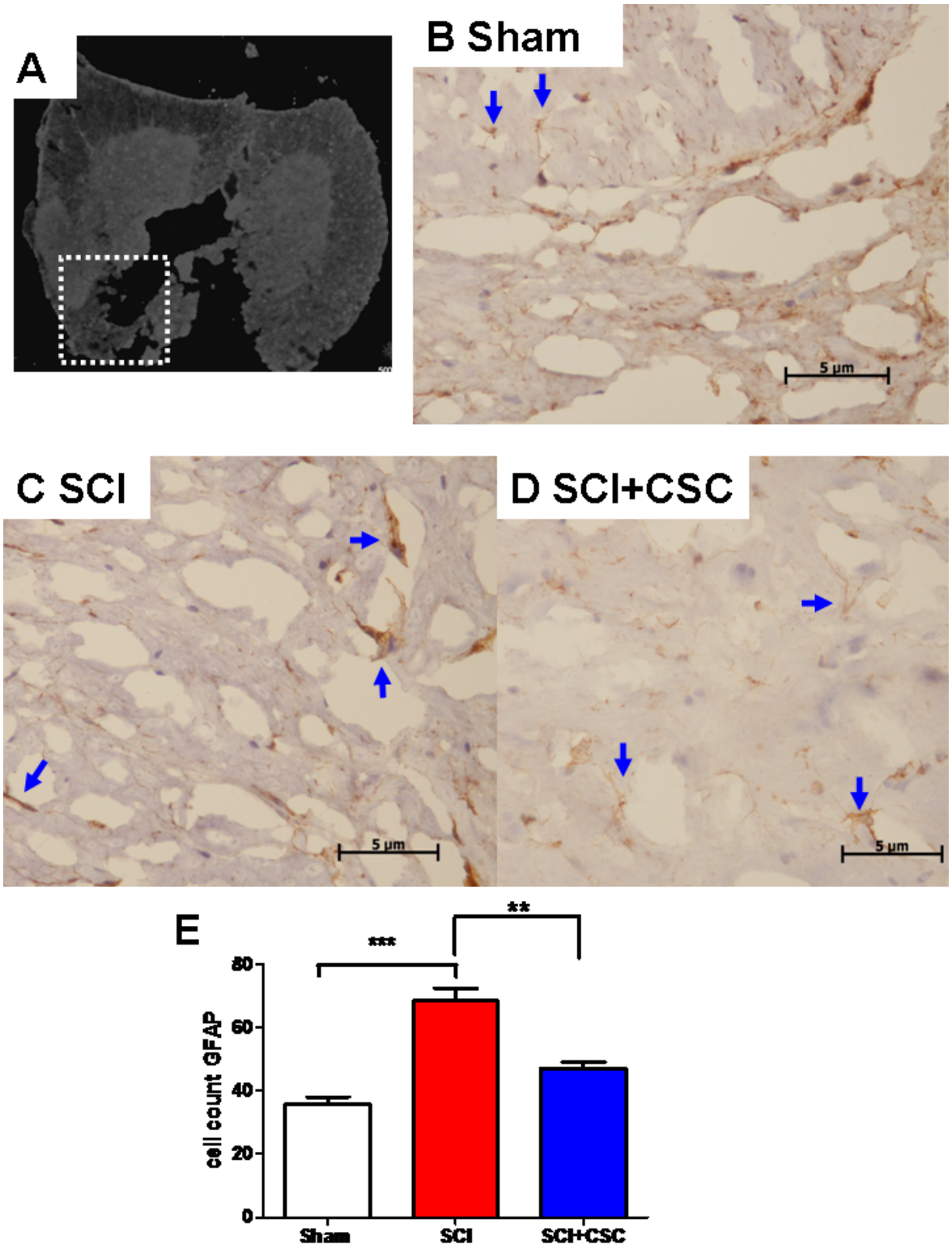

Fig. 3. Representative photographs of GFAP-stained sections of tissue samples were obtained from the spinal cords of rats in the sham control group (B), SCI (C), and SCI + CSC (D) after SCI. (A) Schematic depiction of ROI (labeled as the dashed box) in injured spinal cords for all groups. Staining of GFAP-positive cells was weaker in the sham group (B) than in the SCI group (C). CSC treatment decreased GFAP immunofluorescence. (A-C: magnification 400×). (E) Vertical bars indicate the mean (± SEM) number of GFAP-stained cells per tissue section in each group (n = 3). *, $P < 0.05$ and ***, $P < 0.001$ indicate the level of statistical significance.

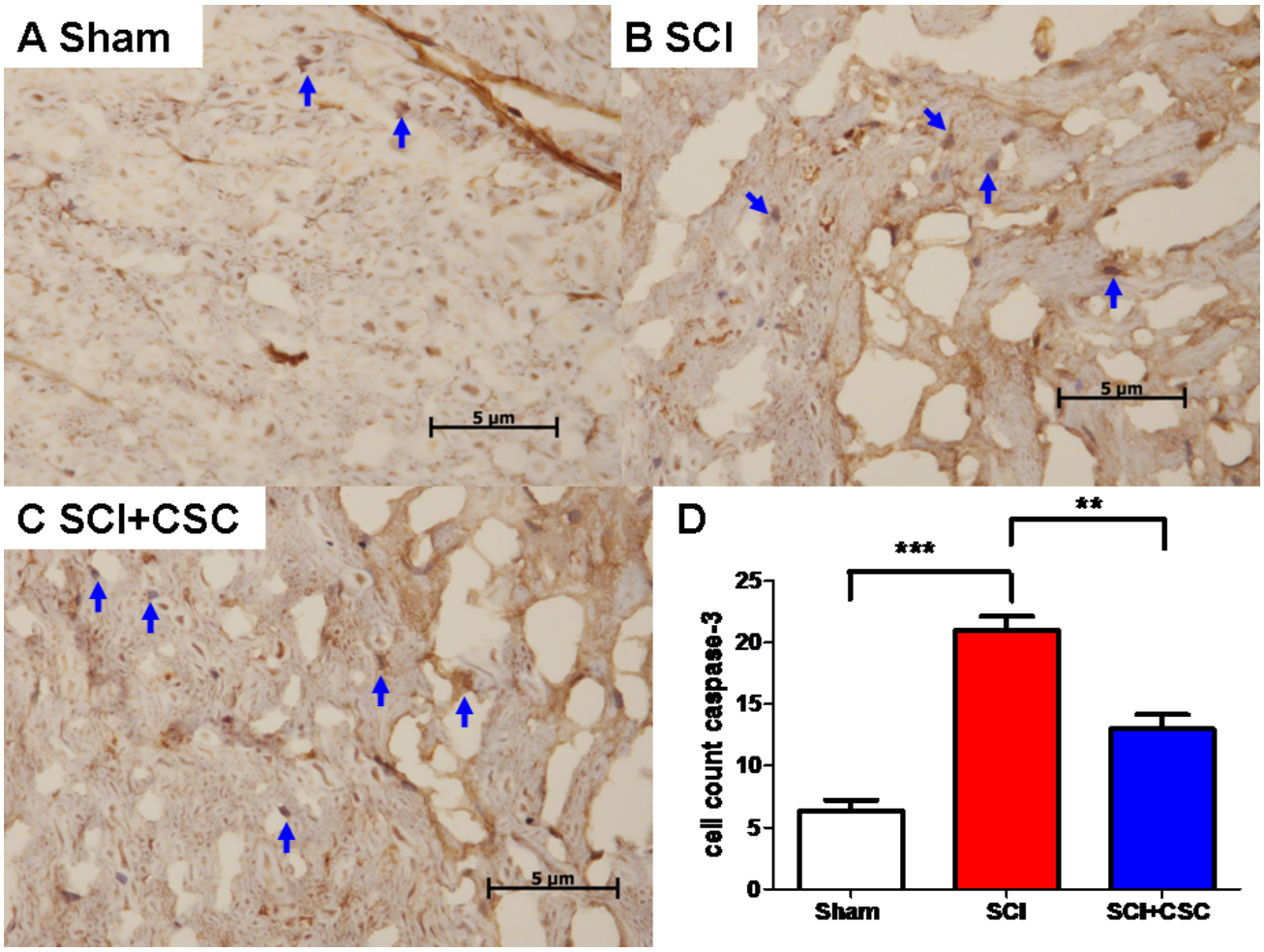

Fig. 4. Representative photographs of caspase-3-stained sections of tissue samples obtained from the spinal cords of rats in the (A) sham control group, (B) SCI only group, and (C) SCI + CSC group (A-C: 400×). The blue arrows in A-C indicate caspase-3-positive cells. (D) Vertical bars indicate the mean (± SEM) number of caspase-3-stained cells per tissue section in each group (n = 3). **, $P < 0.01$ and ***, $P < 0.001$ indicate the level of statistical significance.

an arrow in Fig. 3D) than in the SCI only group (**$P < 0.01$, Fig. 3E). These results indicate that CSC therapy mediated the activation of astrocytes and astrocyte-mediated proinflammatory responses following SCI.

3.4 CSC treatment could limit apoptosis in SCI animals

The blue arrows in Fig. 4A-C indicates the immunoreactivity of caspase-3 as a proxy of cellular apoptosis. Following spinal cord hemisection, immunostaining for caspase-3 was significantly higher in the SCI only group than in the sham control group (***$P < 0.001$, Fig. 4D). Immunostaining for caspase-3 was significantly lower in the SCI + CSC group than in the SCI only group (**$P < 0.01$, Fig. 4D), indicating that CSC attenuated cellular apoptosis.

3.5 CSC treatment could reduce neuronal damage following spinal cord hemisection

The blue arrows in Fig. 5A present the immunohistochemistry indicative of the neuron-specific marker NeuN in the sham group. As indicated by the blue arrows in Fig. 5B, the morphological integrity was far worse in the SCI only group than in the sham control group, and neuronal loss was also evident (***$P < 0.001$, Fig. 5D). The blue arrows in Fig. 5C indicate that NeuN staining was more preserved in the SCI + CSC group than in the SCI only group, and neuronal loss was less evident (*$P < 0.05$, Fig. 5D). Overall, the anti-inflammatory and anti-apoptotic effects of CSC could potentially help to prevent neuronal deficits.

3.6 CSC treatment upregulated injury-attenuated CISD2 expression following spinal cord hemisection

As in our previous research, we determined in the current study that spinal injury led to a decline in CISD2 expression, as evidenced by a reduction in CISD2 immunoreactivity in the SCI only group (***$P < 0.001$, Fig. 6D). Far more CISD2-positive cells were observed in the sham group (blue arrows in Fig. 6A) than in injured tissue (showing signs of vacuolation) at the site of the injury in the SCI only group (dashed circle in Fig. 6B). The number of CISD2-positive cells in the same injured tissue in the SCI + CSC group (dashed circles in Fig. 6B and 6C) was far higher than in the SCI only group (*$P < 0.05$, Fig. 6D). Our results demonstrate the CISD2-preserving beneficial effects of CSC. The eventual anti-inflammatory property could contribute to the prevention of astrocytic activation, astrocyte-mediated neuroinflammation, apoptosis, and neuron loss.

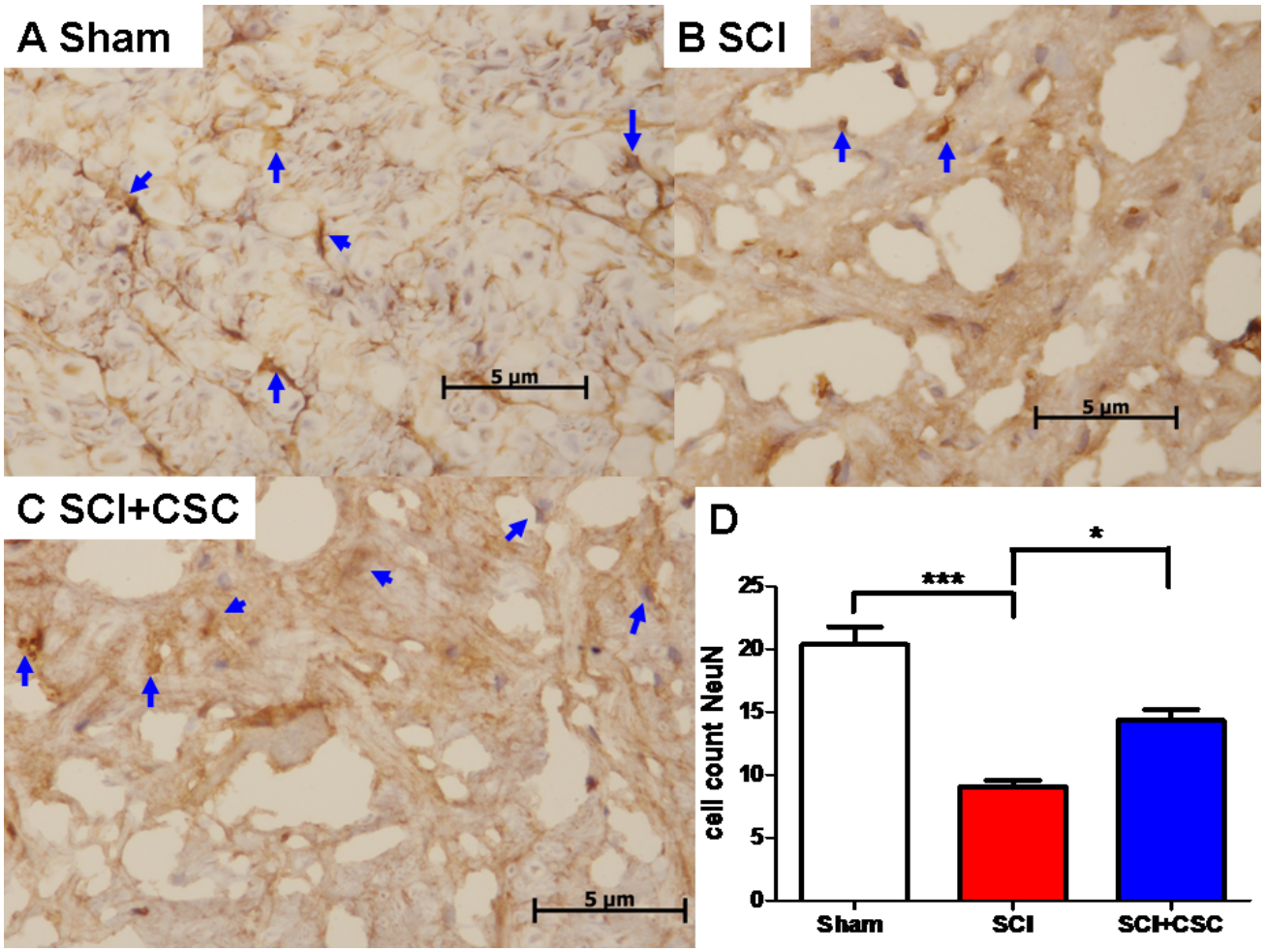

Fig. 5. Representative photographs of NeuN-stained sections of tissue samples obtained from the spinal cords of rats in the (A) sham control group, (B) SCI only group, and (C) SCI + CSC group (A-C: 400×). Compared to sham controls, the arrows in (A-C) indicate NeuN immunoreactivity (exhibited neuronal loss in SCI group). CSC treatment preserved neurons after SCI. (D) Vertical bars indicate the mean (± SEM) number of NeuN-stained cells per tissue section in each group (n = 3). *, $P < 0.05$ and ***, $P < 0.001$ indicate the level of statistical significance.

4. Discussion

SCI can induce the activation of immunity-related cells in the spinal cord's parenchyma and the activation of astrocytes and microglia (Davies et al., 1996; Hung et al., 2005). Reactivated astrocytes produce inflammatory cytokines and chemokines, which trigger microglial activation (favoring the detrimental M1 over the protective M2 phenotype) (Liu et al., 2011; Luo et al., 2002). Injury-activated astrocytes and microglia release considerable nitric oxide and ROS quantities, which amplify the inflammatory cascade (Li et al., 2007). Profound inflammatory responses can eventually lead to mitochondrial dysfunction and the enhanced production of ROS and nitric oxygen species (NOS) (Lucas et al., 2006; van Horssen et al., 2019), which can, in turn, contribute to neuronal apoptosis (Xu et al., 2006) and potential neurological deficits (Hurlbert, 2006). Thus, the therapeutic targeting of acute SCI can help to mitigate secondary insults to the spinal cord.

Therapeutic hypothermia has been shown to exert neuroprotective effects in the central nervous system (CNS) in response to injuries, such as TBI and SCI (Bernard and Buist, 2003). Animal studies have demonstrated that physiological ATP concentrations can be maintained by decreasing cellular consumption or increasing production through glycolysis, thereby preserving the CNS's neurological function (Arrica and Bissonnette, 2007). In cases of hypoxic-ischemic brain insults, therapeutic hypothermia has been shown to attenuate secondary damage by decreasing proinflammatory responses, such as neutrophil and microglia stimulation. Therapeutic hypothermia has also been shown to decrease the generation of free radicals (which would otherwise lead to oxidative stress) and extracellular glutamate levels (which would otherwise lead to excitotoxicity) (Hachimi-Idrissi et al., 2004). Finally, therapeutic hypothermia has been shown to mediate secondary insults related to SCI, including the release of cytochrome-3 and the activation of caspase-3 and calpain (Ohmura et al., 2005).

One secondary injury related to SCI involves the hypertrophic formation of astrocytes and increased GFAP expression, which can, in turn, lead to glial scarring and a corresponding decrease in axonal and neuronal regeneration (Morino et al., 2003; Stichel and Müller, 1998). Glia-mediated neuroinflammation following acute SCI can lead to mitochondrial dysfunction, cellular apoptosis, and eventual neuron loss (Scholpa and Schnellmann, 2017). Ameliorating injury-induced astrocyte activation has been shown

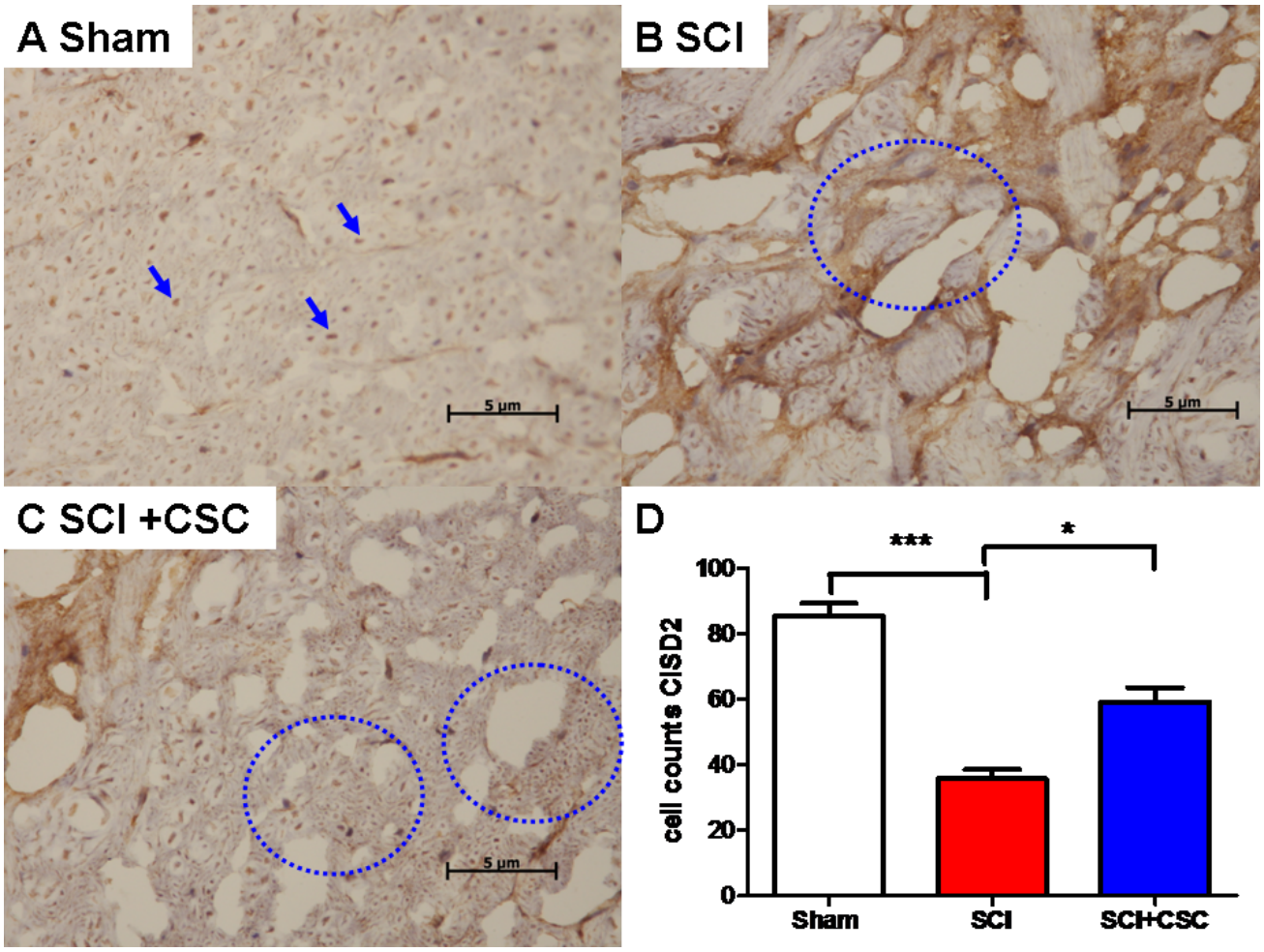

Fig. 6. SCI-induced a decrease in CISD2 expression in the injured spinal cord, the effect of which was attenuated by CSC. Representative photographs of CISD2-stained sections of tissue samples obtained from the spinal cords of rats in the (A) sham control group, (B) SCI only group, and (C) SCI + CSC group (A-C: 400×). (A) The sham group presented a high degree of CISD2 immunoreactivity. (B) CISD2 expression was lower in tissue samples with follicles from the hemisected spinal cord (indicated by a dashed circle). At the injury site of a similar situation in (C) (dashed circle), a densely packed pattern of CISD2 immunoreactivities proved the CISD2-elevating effect of CSC treatment in SCI rats, compared to (B). (D) Vertical bars indicate the mean (± SEM) number of CISD2-stained cells per tissue section in each group (n = 3). *, $P < 0.05$ and ***, $P < 0.001$ indicate the level of statistical significance.

to help prevent pathogenesis in cases of SCI. In the current study, the injury-induced expression of GFAP, caspase-3, and NeuN indicates pathogenesis, including respective astrocytic proliferation, cellular apoptosis, and neuronal damage. We discovered that CSC-induced hypothermia helped attenuate secondary injuries following SCI, including astrocyte-associated inflammatory response, apoptosis, and neuronal deficit.

We found that CSC-induced hypothermia led to the upregulation of CISD2, even though acute SCI tends to downregulate the expression of CISD2. CSC's anti-inflammatory effects can be attributed to the fact that CSC treatment attenuated the downregulation of CISD2 mRNA and proteins. In previous siRNA knockdown models aimed at decreasing CISD2 expression levels, we observed elevated inflammatory responses in SH-SY5Y neuronal-like cells (Lin et al., 2019) and EOC microglia (Lin, 2020). We also confirmed that CISD2 inhibits inflammation via the NFκB pathway (Kung et al., 2020; Lin, 2020). NFκB acts as a regulator of inflammation in mitochondria (Albensi, 2019; Laforge et al., 2016), and NFκB signaling has been implicated in the link between mitochondrial dysfunction and acute SCI. CSC-driven CISD2 augmentation can help overcome the detrimental effects of NFκB signaling, including excessive inflammation and potential apoptosis related to mitochondrial dysfunction. The above mechanisms help explain how CSC treatment attenuates or prevents inflammation, astrocytic activation, apoptosis, and neuronal loss in the tissue surrounding spinal cord injuries. Furthermore, this is the first study to report that CSC-induced therapeutic hypothermia regulates CISD2 expression.

The current study has several limitations, which must be considered in the interpretation of our findings. In the current study, we were limited to only three animals in each group. Many inflammation-related inflammatory factors, such as IL-4, were not addressed, and we did not collect samples at other time points. Subsequent *in vivo* studies will be required to assess CSC treat-

ment's long-term effects on upstream and downstream inflammatory signaling. Note that we did not perform CISD2 knockdown or knockout; therefore, a causal relationship cannot be determined between CISD2 and injury-induced inflammation following CSC.

Nonetheless, the anti-inflammatory effects of CISD2, including the antagonism of PPAR-β, NF-κB, and injury-induced inflammatory mediators, have previously been established in CISD2 knockdown cell models (Kung et al., 2020; Lin, 2020). From this, it is safe to surmise that CSC's effects on CISD2 limit the inflammatory response and reduce the likelihood of apoptosis. Future *in vivo* investigations must elucidate the mechanism(s) underlying CISD2-associated anti-inflammation and glial activation regulation.

5. Conclusions

The results obtained in this study demonstrate that CSC treatment inhibited astrocyte activation, astrocyte-mediated inflammatory responses, cellular apoptosis, and neuronal loss in rats following spinal cord hemisection. These results demonstrate the CISD2-preserving effects of CSC, which could contribute to the prevention of astrocytic activation, astrocyte-mediated neuroinflammation, apoptosis, and neuron loss.

Author contributions

WMK is responsible for the writing of the original draft and formal analysis. CJC was accountable for the resources, supervision, and investigation. TYC conceptualized the study and participated in the experiments and data analysis. MSL acted as the corresponding author and funded, wrote, reviewed, and edited the final draft.

Ethics approval and consent to participate

All rats were treated as per the Experimental Animal Laboratory guidelines under the approval of the Animal Care and Use Committee of Chang Gung University (IACUC Approval No.: CGU14-052).

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

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