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Original Article

Sex influences on sensory responses following spinothalamic tract injury in rats

Fatemeh Abbaszadeh [1,2], Mina Afhami [1], Elham Saghaei [4], Kobra Naseri [4], Majid Hassanpour-ezatti [2], Masoumeh Jorjani [3,4*]

1. Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2. Department of Biology, Faculty of Basic Science, Shahed University, Tehran, Iran
3. Neurobiology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Department of Pharmacology, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ABSTRACT

Introduction: There is some evidence of significant differences in the recovery after spinal cord injury (SCI) between males and females. In this study, we investigated the sensory function and involvement of astrocytes in the sex differences of central pain syndrome in the unilateral spinothalamic tract (STT) injury model in rats.

Methods: Rats were divided into two groups: SCI and Sham groups received a unilateral electrolytic lesion on STT at T8-T9 and a control sham surgery respectively. After recovery from surgery, the sensory function was monitored for 28 days using tail flick and von Frey filament tests. The glial fibrillary acidic protein (GFAP) level was also measured by Western blot at the same time points.

Results: Mechanical hypersensitivity was increased from days 3 to 28 post-injury in male rats ($P<0.001$), but no significant change was observed in females. In the tail flick model, male rats had significantly elevated thermal withdrawal latency on day3 after STT lesion, while females showed a reduction in latency ($P<0.001$). Sex differences in GFAP level were observed during 4 weeks of study after injury. Results in the first week showed that GFAP level decreased in females, but the marked elevation was observed from days 7 to 28 in males ($P<0.05$).

Conclusion: This study revealed the sex differences in sensory dysfunction and the related astrocyte reactivity after SCI. It suggests a need for more studies using both sexes to fully explore the influence of sex on the recovery of sensory impairments post-SCI.

Keywords:
Sex differences
Spinal cord injury
Sensory hypersensitivity
Astrocyte reactivity
Rat

Introduction

Spinal cord injury (SCI) is a complicated disorder resulting in loss of motor and sensory functions in patients. SCI consists of two defined phases: a primary phase which is attributable to the cord injury and a secondary phase which is caused by the cascade of systemic and local neurochemical and pathophysiological changes following SCI (Oyinbo, 2011; Tran et al., 2018). These secondary responses stimulate central nervous system areas that are involved in the sensory processing of pain, and consequently, patients experience a type of neuropathic pain called central pain syndrome (CPS). Several

* Corresponding author: Masoumeh Jorjani, msjorjani@sbmu.ac.ir
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studies have shown significant differences in recovery and post-injury consequences between males and females (Sipski et al., 2004). Measurements of allodynia and hyperalgesia, two aspects of CPS, are performed to evaluate the progress of SCI recovery (Yezierski, 2000). The underlying mechanism of CPS has not been fully clarified. By introducing an animal model for studying the CPS, Wang et al. (2008) showed that partial differentiation of relay cells in the ventral posterolateral nucleus of the thalamus augments neural excitability and this hyperexcitability is responsible for post-injury pain syndrome (Wang and Thompson, 2008). The recovery and repair of spinal cord lesions have been linked to the production of glial cells during CPS and a role for reactive astrocytes. In the nervous system, astrocytes increase the stability of injury sites, restrict the development of injury, accelerate blood-brain barrier repair, reduce inflammatory cells and play a neuroprotective role (Gaudet and Fonken, 2018; Sribnick et al., 2005). Dee spite these advantages, the role of astrocytes in SCI-related chronic pain should not be ignored (Gao and Ji, 2010). Animal studies have demonstrated an association between glial activation and neuropathic pain development after peripheral nerve damage (Kim et al., 2021). Also, a correlation between glial fibrillary acidic protein (GFAP) level and below-level neuropathic pain severity after SCI has been reported (Detloff et al., 2008). There are also some reports that sex steroidal hormones accelerate recovery after spinal cord injuries (Sengelaub et al., 2018). Also, Parducz *et al.* reported that sex hormones modify the morphology, size, synaptic density, and function of neuronal cells as well as the morphology of glial cells in the central nervous system (Parducz et al., 2006). The potencial of estradiol to stimulate funcł tional and morphological changes in glial cells suggests a new molecular mechanism for sex differences in central pain (Arevalo et al., 2010).

In this study, we investigated the sensory function and the involvement of astrocytes (GFAP levels) in the sex differences in CPS following unilateral electrolytic lesion on the spinothalamic tract (STT) in rats.

Materials and methods

Animals and Experimental groups

Adult male and female Sprague-Dawley rats (n= 88, 220–250 g) were kept in separate cages according to the sex and groups, on a 12-h light/dark cycle with food and water ad libitum. The experiments were approved by the ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.NRC.REC.1398.28). Male and female rats were divided into two groups. Sham control group received only laminectomy and SCI group received lesions on STT. The number of rats in each group was 7.

Surgery

STT lesion was induced by the method described by Wang and Thompson (2008) with a modification. Rats were anesthetized with a mixture of ketamine/xylazine (60:5 mg/kg, intraperitoneal). After laminectomy on T8–9 vertebras, the dura was opened with iris scissors. The right STT pathway was injured using a tungsten microelectrode (1 MΩ). It was placed in the coordinates of 0.5 – 0.7 mm lateral to the midline and 1.6 –1.9 mm deep in the spinal cord for 90 s (Naseri et al., 2013).

Behavioral assessment

Heat hyperalgesia

Thermal pain was measured by the tail flick test in all groups. Tail flick latency time (TFL) was measured as the time between the first heat exposure and the time of tail withdrawal. The cut-off time was considered 10 seconds. Each rat was examined three times with intervals of at least one minute. The mean of the three trials was reported as the TFL for each rat.

Mechanical allodynia

Mechanical pain was evaluated using the method described by Ren with calibrated Von Frey filaments that were applied to the hind paw (Ren, 1999). Briefly, the rats were habituated to a plexiglass box without any restriction for 10 min. Stimuli were applied to the dorsal surface of the second and third toes of both hind paws by a set of calibrated Von Frey filaments. Each filament was tested five times at one-minute intervals. An ascending series of the filaments (6, 8, 10, 15, 26, and 60 g) were then used depending on the withdrawal response to the starting filament. The minimum force of the filaments that produced a response was considered a paw withdrawal threshold (PWT).

Western blotting

Western blotting was performed on T8–9 spinal segments at the lesion site (n=3). Spinal tissues were ho-

mogenized with lysis buffer (NaCl 150 mM, Tris/HCL 50 mM, Triton X-100, SDS 0.1%, EDTA 1 mM, sodium deoxycholate 0.25%, and protease inhibitor cocktail) and the supernatant was collected after centrifugation at 12000 rpm for 10 min. The protein concentration was evaluated by Bradford assay. An equivalent protein sample was run on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 2% skim milk for 75 min and incubated with anti-GFAP (1:1000, Cell signaling) and anti-Beta actin (1:1000, Cell signaling) overnight at 4°C. These membranes were further incubated with HRP conjugated secondary antibody (1:10000, Cell signaling) for 90 min, developed with a chemiluminescence kit (ECL solution), and exposed to X-ray films. The intensity of specific bands was quantified by densitometry with Image J software. Beta-actin was used as internal standard control.

Statistical analysis

Statistical analysis was done using GraphPad Prism, Version 6. Data are presented as the mean ± SEM and differences among groups were assessed by the two-way ANOVA ordinary tests followed by Bonferroni's post-hoc analysis. Statistical significance was set when $P< 0.05$.

Results

Behavioral results

Thermal pain threshold was almost the same in male and female rats in both intact (day 0) and sham groups. After the STT lesion, we observed a decrease in the thermal pain threshold in SCI female group rats compared with the female sham group, also, an increase in pain

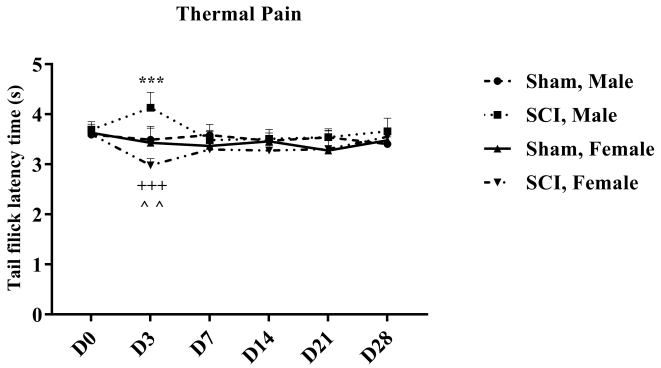

FIGURE 1. Sex differences in thermal pain during 4 weeks post-surgery in STT injured rats. Data are mean ± SEM, (^{+++}P<0.001 SCI, male vs. SCI, female; $^{***}P$<0.001 SCI, male vs. sham, male; $^{\wedge\wedge\wedge}P$<0.001 SCI, female vs. sham, female), two-way ANOVA followed by Bonferroni posttest. n=7, SCI: spinal cord injury, STT: spinothalamic tract.

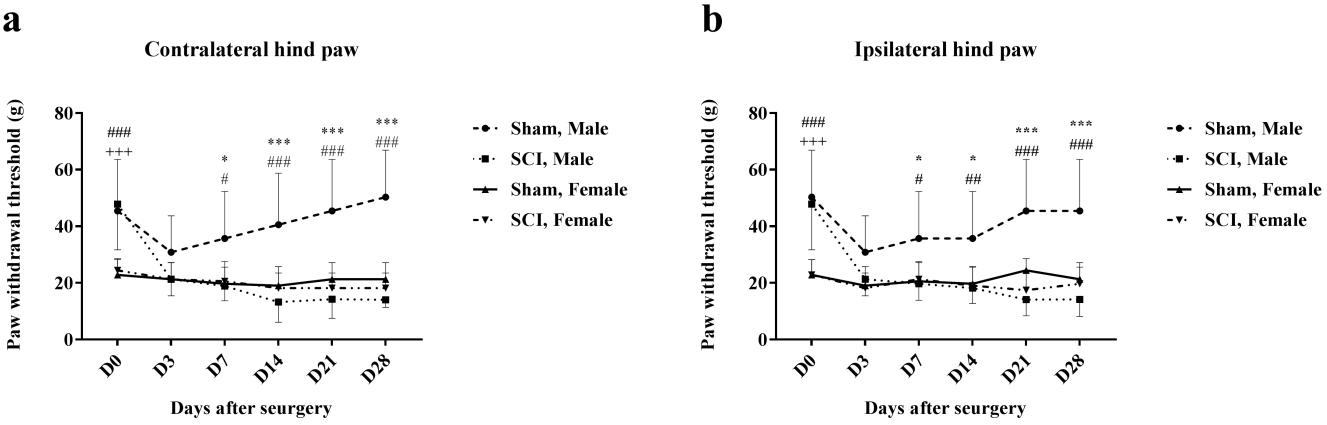

FIGURE 2. Sex differences in mechanical pain during 4 weeks post-surgery in STT injured rats. ((a) Contralateral (b) Ipsilateral hind paw). Data are mean ± SEM and present paw withdrawal threshold ipsilateral and contralateral to injury side (^{+++}P<0.001 SCI, male vs. SCI, female; $^{*}P$<0.05, $^{***}P$<0.001 SCI, male vs. sham, male; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 sham, male vs. sham, female). Two-way ANOVA followed by Bonferroni posttest. n=7, SCI: spinal cord injury, STT: spinothalamic tract.

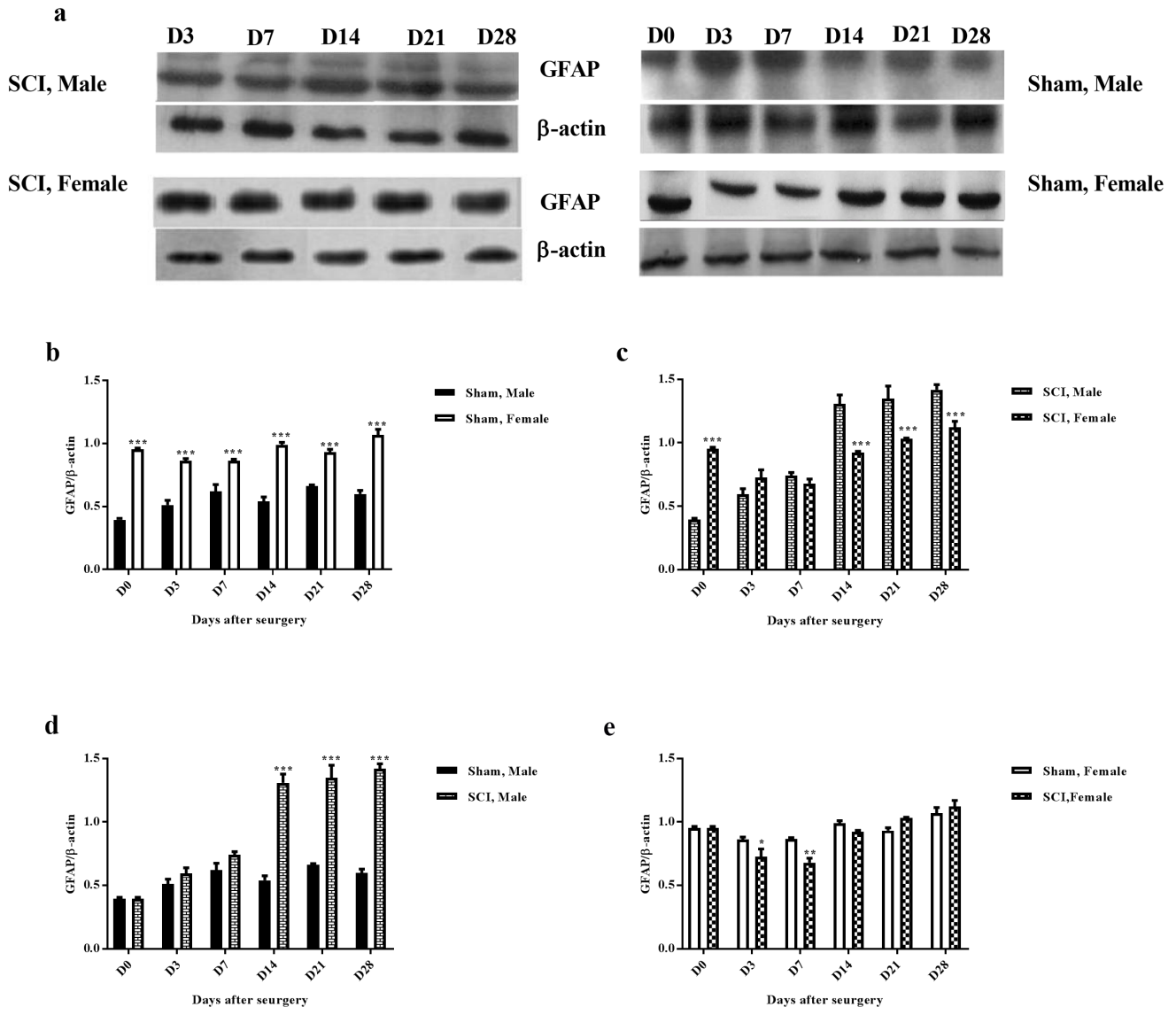

FIGURE 3. Effects of unilateral lesion of STT on the spinal levels of GFAP protein in male and female rats. Western blot band of GFAP. β-actin is used as the loading control. The optical density ratio of GFAP (b); sham male vs. female rats (c); SCI male vs. female rats (d); sham male vs. male SCI rats (e); and sham female vs. female SCI rats. Data are mean ± SEM, (+++P<0.001 SCI, male vs. SCI, female; ***P<0.001 SCI, male vs. sham, male; ^P<0.05, ^^P<0.01 SCI, female vs. sham, female; ###P<0.001 sham, male vs. sham, female). Two-way ANOVA followed by Bonferroni posttest. n=3, SCI: spinal cord injury, STT: spinothalamic tract.

threshold in the SCI male group compared with sham male rats was detected only on day3. These changes were statistically significant (P<0.001, Figure1).

In intact (day 0) and sham female rats mechanical pain was lower than in males in both ipsi and contralateral hind paws during 4 weeks. STT lesion caused a sharp decrease in PWT in both paws of male rats compared to day 0. This difference was significant between the sham and SCI male groups on days 7, 14, 21, and 28. In the next weeks of the study, PWT in both paws significantly decreased after STT injury in males but not females (P<0.001, Figure 2a, b).

Western blot analysis

The spinal levels of GFAP were evaluated in sham and SCI groups in males and females on days 0, 3, 7, 14, 21, and 28 (Figure 3a). The basal level of GFAP expression in sham female rats was higher than in male rats on day 0 (P<0.001, Figure 3b). An increase in the expression level of GFAP was observed in male rats after STT injury, this increase was significant between male SCI and sham group (P<0.001, Figure 3d) and between injured male and female rats (P<0.01, P<0.001, Figure 3c). We observed a decreased level of GFAP expression after STT injury in female rats on days 3 and 7 (compared to

sham), this decrease was significant between female SCI and female sham group ($P<0.05$, $P<0.01$, Figure 3e).

Discussion

The sexual difference has been reported in pain perception following SCI. Numerous reports indicate the role of sex steroids in various models of pain. Also according to the studies, sex steroids play important role in lesion repair and protection in SCI. (Bartley and Fillingim, 2013; Shiao and Lee-Kubli, 2018). Studies on the effect of gender on SCI outcomes and underlying mechanisms help us to identify new therapeutic targets for SCI patients. Based on our results, thermal pain was almost the same in intact male and female rats. These findings are similar to some previous reports (Mousavi et al., 2007), but different from other reports in basic (Kuo et al., 2010) or clinical (Fillingim et al., 2009) studies. We observed a significant hypoalgesia on day 3 in male rats but a decrease in thermal pain threshold in females. This response in males probably originated from the analgesic effect of testosterone (Nag and Mokha, 2009). A review of sex-related pain in humans showed that women are more sensitive than men to pain modalities, such as thermal pain which may be influenced by sex hormones, the endogenous opioid system, and genetic predisposition (Bartley and Fillingim, 2016). Our findings confirmed the previous reports about the lower mechanical pain threshold in female rats rather than males. The higher GFAP levels and activity of astrocytes in the spinal cord of intact females compared to male rats are likely to make this difference. Considering the role of astrocytes in the induction of chronic pain as well as the production and release of cytokines and inflammatory mediators (Gao and Ji, 2010; Ji et al., 2019), we can explain the lower mechanical pain threshold in females compared to male rats.

Clinical studies have also shown that women have lower pain thresholds, higher pain ratings, and less tolerance to a range of painful stimuli. In addition, some evidence in support of the effect of sex hormones on pain processing implies that females' sensitivity to painful stimuli is greater than males in the deep tissue pain model, although there are controversial findings on this subject (Traub and Ji, 2013). During the first few days of surgery, in the very short term, a decrease in pain threshold was observed in the sham group, possibly due to the removal of the spinal cord and surrounding tissue. This result was discussed. From day 3 after injury, the pain threshold progressively until a threshold similar to day 0 is achieved.

From day3 after STT injury, we observed a gradual decrease in mechanical pain sensitivity in male rats but not in females. Appropriately, GFAP expression level in the spinal cord of male rats increased following STT but it did not alter in female rats. This indicates the activation of sex-dependent astrocytes in response to injury and a possible role in post-chronic pain (Gao and Ji, 2010; Watson et al., 2014). On the other hand, there is a report that intrathecal injection of Flurocitrate and Fluroacetat as inhibitors of astrocytes activity can reduce neuropathic pain in rats (Okada-Ogawa et al., 2009). The same finding was reported by Gupta *et al* who demonstrated higher mechanical hypersensitivity in male than female animals (Gupta and Hubscher, 2012). This could be explained by the neuroprotective effects of estrogen which are more pronounced in females rather than males. Allodynia is the main feature of CPS induction in STT injury (Wang and Thompson, 2008). In this study, we observed thermal hyperalgesia in females on day 3 and mechanical allodynia in males from day 3 to 28. This confirms previous reports that differential cellular and molecular mechanisms might affect neuropathic pain in different genders (Sorge et al., 2015). Moreover, sensory modalities can be modulated differently. After injury nociceptive afferents respond with more sensitivity to stimuli (Gaudet et al., 2017).

Mechanical allodynia observed in both hind paws after unilateral lesion in male rats resembles clinical findings in many SCI patients who complain of mechanical allodynia in both legs (Chambel et al., 2020; Shiao and Lee-Kubli, 2018). In an animal study, Hains and coworkers also reported that SCI leads to reduced locomotor activity, the development of hind limb mechanical allodynia, and thermal hyperalgesia (Hains et al., 2001). On contrary, Schreiber and coworkers reported that pain has been induced only in the contralateral hind paw after SCI (Schreiber et al., 2008). Two weeks after STT injury, the number and density of astroglia at the lesion site increased. According to our previous findings, the microglial marker is upregulated in the acute phase in parallel with the onset of pain, and the astrocyte marker (GFAP) is upregulated during the late phase. In fact, the astroglial reaction is more persistent than the microglial response after injury, and it is more closely related to

chronic pain (Naseri et al., 2013). Watson *et al.* reported similar findings (Watson et al., 2014).

Previously, we showed that estradiol reduces the increase in microglia at the site of injury as well as related astrogliosis (Naseri K. 2012). Also, we reported that estradiol can reduce abnormal neural hyperexcitability and inhibit glial activity in the ventral posterolateral region (Saghaei et al., 2013). The release of pro-inflammatory cytokines from activated cytokines and hyperexcitability of dorsal horn neurons contribute to the persistence of neuropathic pain (Hulsebosch et al., 2009). Ji *et al* reported that neuropathic pain is caused by astrogliopathy, in which the normal capacity of astrocytes to maintain CNS homeostasis is disrupted. Astrogliopathy leads to abnormal extracellular levels of water, glutamate, and K^+ as well as the secretion of proinflammatory chemokines and cytokines due to membrane leakage caused by CX43. These astrocyte-driven pathologies lead to neuroinflammation, neurotoxicity and neuronal hyperexcitability, and chronic pain. Importantly, astrocytes promote chronic pain conditions through neuron–glial and glia–glial interactions (Ji et al., 2019; Kawasaki et al., 2008). Estradiol (a sex hormone) regulates the function and morphology of astrocytes (Acaz-Fonseca et al., 2014). Estradiol acts directly on astrocytes by the Estrogen Receptor (ER)α, ERβ, and G-protein-coupled estrogen receptor (GPR30/GPER1) receptors. In astrocytes, estradiol increases the production of growth factors and glutamate transporters (GLT1 and GLAST) and blocks nuclear factor-κB (NF-κB) translocation (Hadjimarkou and Vasudevan 2018). In addition, it has been reported that the administration of a single dose of 17β-estradiol ameliorates neuropathic pain, including thermal hyperalgesia, and mechanical allodynia in the late phase after SCI in rats. They suggested an important role for activated glial in the maintenance of chronic central pain after SCI (Hains and Waxman, 2006).

Conclusion

Taken together, our data showed considerable sex differences in sensory responses after STT lesion and during the recovery period in rats, which may be attributed to the differences in sex hormones between males and females. In addition, we think that both neuronal and glial cell activation after STT injury participates in the occurrence of post-SCI symptoms. It is suggested to consider the role of glial cells and their sex-dependent variations in the treatment strategies for SCI.

Conflict of interest

The authors declare that there are no competing financial interests.

Acknowledgments

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