Original Article

Gender-related differences in recovery of locomotor function after spinal cord injury in mice

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Study design: In order to study the role of gender in recovery, we induced a thoracic compression spinal cord injury (SCI) separately in 2-month-old male and female C57Bl/6 mice.
Objectives: We intended to assess effects of gender on recovery of hindlimb motor function and to correlate these with histomorphologic profiles of injured spinal cord tissue.
Methods: Locomotor function was evaluated by three means: a modified locomotor scoring system for rodents, beam walking and computerized activity meter. Histology was analyzed by comparison of hematoxylin and eosin-stained perfused specimens.
Results: Locomotor scores were 2.2 ± 0.9 on day 1 in male mice, while, in contrast, they were significantly higher, 7.3 ± 1.7, in females ($P < 0.02$). On day 14 Basso, Beattie and Bresnahan scores were 9.5 ± 2.2 in male mice and 16.0 ± 2.2 in females ($P < 0.03$). Terminal histology showed that the spinal cord architecture was relatively better preserved in female mice and that the extent of necrosis and infiltration of inflammatory cells was less compared to males.
Setting: Neurobiology Research Laboratory of University of Kansas Medical School in US Department of Veterans Affairs Medical Center, Kansas City, Missouri.
Conclusion: We found that the severity of the initial injury as well as the ultimate recovery of motor function after SCI is significantly influenced by gender, being remarkably better in females. The mechanism(s) of neuroprotection in females, although not yet elucidated, may be associated with the effects of estrogen on pathophysiological processes (blood flow, leukocyte migration inhibition, antioxidant properties, and inhibition of apoptosis).
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Introduction

Animal and human studies have shown that the response to CNS injury is different in females and males.[1–4] Hall et al[2] showed that 24 h after incomplete ischemia produced by temporary unilateral carotid occlusion, male gerbils demonstrated significantly more neuronal loss in the cerebral cortex and the CA1 of the hippocampus than did females. Alkayed et al[5] reported that using a model of temporary middle cerebral artery

(MCA) occlusion both Wistar and hypertensive female rats had smaller infarcts in the cortex and caudate-putamen than did males of the same strain. Similarly, a higher survival rate was found in females than in males after hypoxic brain injury in mice.[6] Data from human studies also show gender-related differences. For example, the incidence of ischemic stroke is lower in premenopausal women compared to men.[7,8] Similarly, after a closed head injury, 100% of female rats, as compared to 75% of the males, survived.[3] Roof et al[9] reported that male rats were significantly impaired in their performance on the Morris water maze task after

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electrolytic lesion of cortex, while lesioned females performed the task as well as did noninjured controls.

Data regarding gender differences after spinal cord injury (SCI) are more limited. However, using a moderate degree ($5 \, gm/mm^2$) of compression SCI, Hauben et al[4] showed that adult female mice recovered significantly better than did their male littermates. In a recent study, 17β-estradiol (100 mg/kg) 1 h pre- or post-injury improved motor function and reduced the size of lesion in male rats after mild SCI.[10] This latter study reinforces the concept that estrogen-like hormones may have neuroprotective roles in the CNS.[10]

We have been using a compression SCI model to study the pathophysiology of primary and secondary injury.[11–16] Previously, we have shown that in this model there is a necrotizing lesion at the site of compression after moderate injury, which causes atrophy of the spinal cord at 14 days postinjury.[17] Morphometry of hematoxylin and eosin (H&E) and luxol fast blue (LFB)-stained sections as well as microtubule-associated protein 2 (MAP-2) immunocytochemical staining showed that both gray and white matter were reduced.[12,17] In the current study, we compared male and female young mice after a moderate compression-induced SCI.[12,17–20]

Methods

Male ($n = 11$) and female ($n = 9$) mice (C57BL/6) were used in this study. Food and water were provided *ad libitum* before and after the injury. The mice were kept at a temperature of 20°C controlled by a thermostat and exposed to alternate light and dark periods of 12 h. All experiments were conducted with the approval of the Animal Care and Use Committees of both the Kansas City Veterans Affairs and University of Kansas Medical Centers according to VA and NIH guidelines.

Controlled compression SCI

We used a mouse SCI compression model as described earlier.[12,17] The animals were anesthetized with ketamine (75 mg/kg) and acepromazine (2.5 mg/kg). A laminectomy at thoracic (T)10 vertebra was performed leaving the dura intact. The animals were then placed in a stereotaxic apparatus and two adjustable forceps were applied to the spinous processes and tissue around one vertebra proximal and distal to the laminectomy in order to stabilize the spinal cord. The compression device consisted of a 2×1 mm rectangular plastic plate at the end of a 4-cm long rod. The upper part of the rod was hollow to fit a round plastic platform on which different weights could be placed. A steel clamp attached to an arm of a stereotaxic device held the middle part of the rod. In this fashion we were able to adjust the compression plate at a desired location over the spinal cord with the help of the micromanipulators of the stereotaxic device. To produce moderate SCI, we used $5 \, g/mm^2$ compression. By gently releasing the clamp holding the rod, the weight was allowed to compress the spinal cord for 5 min via the longitudinally oriented plastic plate. This was then carefully removed and the skin was sutured. The mice were kept under a heating lamp until they regained consciousness.

Recovery of function

'Basso, Beattie and Bresnahan' locomotor rating scale We used several methods to assess recovery of hindlimb motor function. The principal one was the 'BBB' locomotor rating scale for mice, an acronym for the investigators (Basso, Beattie and Bresnahan) who developed this system now in widespread use for rodent models of SCI.[21] In the BBB, a digital system resembling the scoring used by spinal cord physicians for human SCI victims and established by the American Spinal Injury Association ('ASIA score'), wherein 2–3 independent and 'blinded' (as to treatment) observers score the animal's hindlimb function and a value of 0 represents paralysis and 21 is equivalent to complete recovery.[22] BBB hindlimb motor function evaluation was performed before injury and on days 1, 4, 7, 10 and 14 postinjury and entered into a computer program designed to analyze the data.[23]

Beam walking In addition to BBB, the mice were also assessed using their ability to walk on 50 cm long steel beams with decreasing widths from 2 to 0.5 cm in order to evaluate their fine motor function as described previously.[19] The mice were only tested on beams if they did not show obvious motor function deficits during open field testing. The mice were allowed to walk on the bars, and the narrowest bar they were able to walk on without any slips in at least two trials was recorded. If the mice were not able to walk on the 2-cm bar they were scored 0. If the mice were able to walk on the 2-cm bar, they were scored 1; if 1.5-cm bar, then 2; if 1-cm bar, then 3; if 0.7-cm bar, then 4; and if 0.5-cm, then 5.

Computerized locomotor activity test On day 14, the animals were also tested in a force plate actometer.[24] For this quantitative assessment of hindlimb function, animals were placed in a 23-cm-high Plexiglas chamber with a force-sensing floor 28×28 cm as previously reported.[24] Their activity was monitored for 10 min in a dark room and distance traveled was recorded on a computer.

Perfusion and fixation

At 14 days after compression of the spinal cord, the mice were reanesthetized as described above. The chest was opened and a stainless-steel 21 gauge cannula was inserted into the left ventricle. The right atrium was then opened to permit exsanguinations and the mice were perfused by standard techniques.[17] Following fixation, the vertebral columns were excised and placed in the same fixative for 24 h. The spinal cords were then dissected out and 2 mm thick transverse sections were

cut at the site of compression. Samples were also taken from the proximal and distal peri-injury zones, which corresponded to the position of the proximal and distal ends of the compression plate. The tissue was dehydrated overnight and embedded in paraffin for subsequent studies.

Statistics

Factorial analysis of variance and Fisher's PLSD for *post hoc* testing (Statview, Abacus Concepts, Inc., Berkeley, CA, USA) were applied to compare the means of various groups. A difference with a *P*-value of <0.05 was considered statistically significant. The values in the figures and text are given as the mean$\pm$SEM. Any statistically significant difference between females and males is denoted by (*).

Results

After SCI, two males and one female developed autophagia and were euthanized. None of the animals developed obvious infections.

Motor performance tests

Locomotor rating score (BBB) At 1day after compression SCI, all mice showed significant motor deficit of the hindlimbs. Both males and females gradually recovered during the observation period, although motor function was significantly better throughout the observation period in the female mice (Figure 1). The injury resulted in paraplegia of the hindlimbs in male mice on day 1 after injury. The BBB scores were 2.2 ± 0.9 on day one in male mice. In contrast, BBB scores were 7.3 ± 1.7 in females ($P<0.02$). The BBB scores were 5.9 ± 2.1 (extensive movement of two joints) on day 7 in males and 13.9 ± 2.6 (weight supported stepping and frequent forelimb hindlimb coordination) in females ($P<0.03$). The BBB scores were 9.5 ± 2.2 on day 14 in male mice but were 16.0 ± 2.2 in female mice ($P<0.03$). Thus, male mice had some weight support on day 14 while females had consistent plantar stepping and forelimb–hindlimb coordination, indicating improved outcomes.

Beam walking Beam walking tests fine motor function of the animals. Only one male and one female were able to walk on a 2 cm bar 14 days after moderate compression SCI and were scored 1.

Actometer test Females and males walked 55.4 ± 2.4 and 49.5 ± 3.3 m, respectively, during the observation period. Although the means showed a trend towards significance indicating that females displayed greater activity after SCI than males, they were not statistically significant.

Histology

At 14 days after moderate injury there were many inflammatory cells, mostly macrophages and lympho-
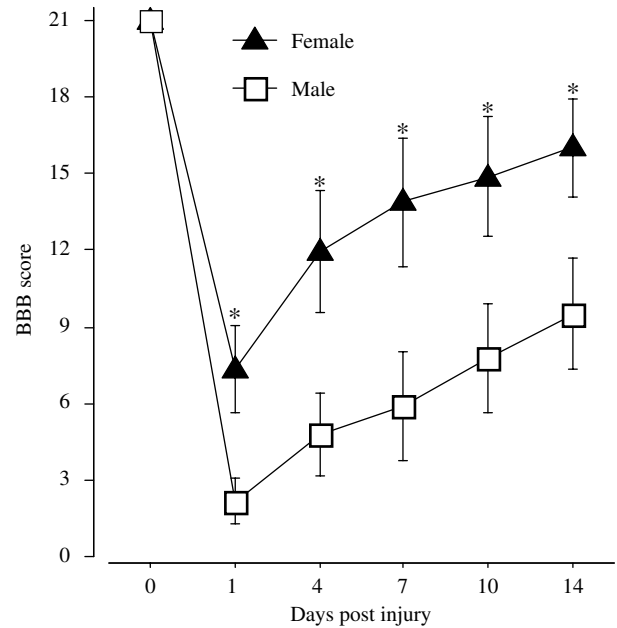
Figure 1 Open field hindlimb motor function (BBB) was scored before injury and on postinjury days 1, 4, 7, 10 and 14 after SCI. (*) Indicates significant difference between female and male mice

cytes, in the injury zone in female mice (Figure 2a–c). After moderate injury in males (Figure 2d–f) the architecture of the spinal cord was distorted. However, the subpial regions of the white matter were, to some extent, spared.

Discussion

This study shows novel findings that the severity of even the initial (day 1) injury is gender specific in mice. It also confirms previous studies in rats using the same compression SCI model that recovery of motor function after SCI is better in females. Evidence is emerging that the CNS responds differently to injury in males and females, and that gender-specific differences in pathophysiological processes exist. This information is important in order to formulate new therapeutic strategies for victims of human SCI. There may be a number of possible mechanisms regarding the observed difference in magnitude of both the initial injury and recovery thereafter.

One of these might depend on the neuroprotective and/or neuroregenerative actions of estrogen. Both the brain and spinal cord are capable of undergoing many morphological changes throughout life (neuroplasticity), and gonadal steroids play an important role in many of these processes. Such molecules are implicated in the development of sexually dimorphic structures in the brain, in the control of physiological behaviors and functions and both brain and spinal cord responses to mechanical insults or harmful substances. Neuroprotective effects of estrogen were observed in females after
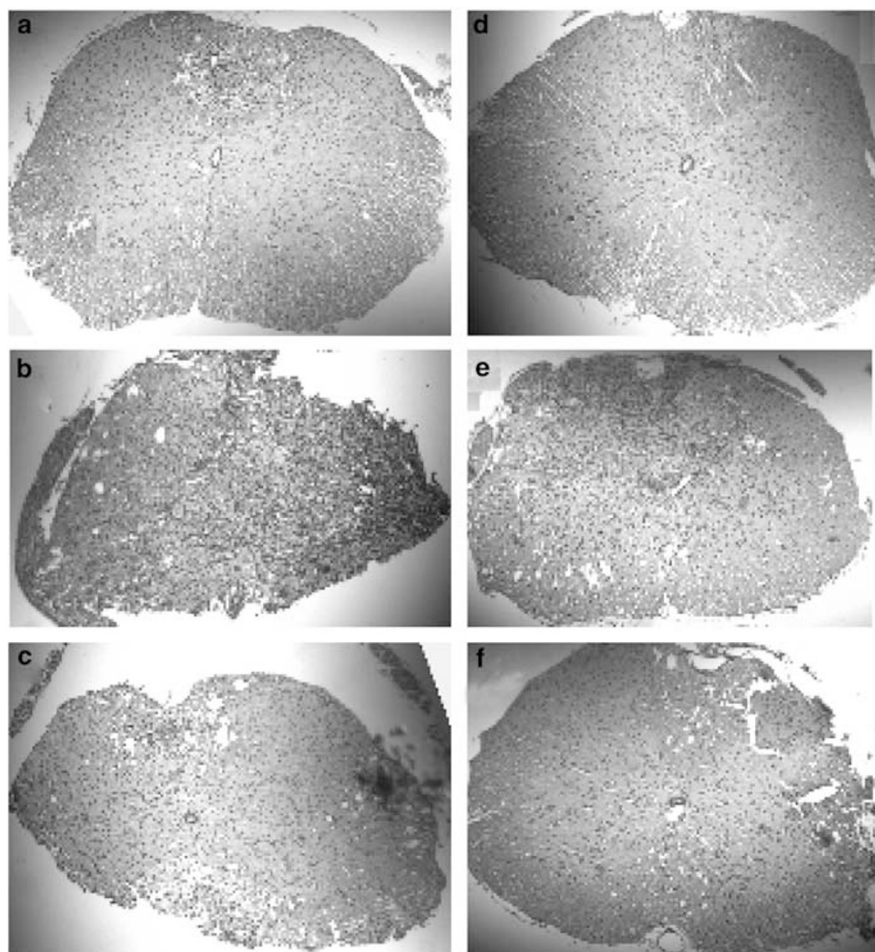
Figure 2 Representative photomicrographs of spinal cords from moderately injured male (left panel, a–c) and female (right panel d–f) mice 14 days following compression SCI. Hematoxylin and eosin staining reveals extensive postinjury tissue damage, vacuolization and gliosis. In males, a large central area of necrosis occurs at the site (epicenter) of injury (**b**) extending also to the rostral (**a**) and caudal (**c**) peri-injury zones. Note the relatively extensive infiltration of inflammatory cells in injured male spinal cord. However, the subpial regions of the white matter were, to some extent, spared. In female mice the architecture of the spinal cord was relatively better preserved. Inflammatory cells and macrophages are clearly seen, especially in the dorsal and central spinal regions at the epicenter (**e**), but these appeared less in females than in males. Again, some inflammatory cells in rostral (**d**) and caudal (**f**) peri-injury zones were observed; however, compared to males the damage was much less extensive

MCA occlusion that were lost in ovarectomized rats.[5] In turn, treatment of ovarectomized rats with 17β-estradiol decreased the ischemic infarct size.[25] One likely consideration is that estrogen provides neuroprotection by improving the blood flow[3] through enhanced production of nitric oxide (NO).[26] However, as we have previously shown that motor function recovery from SCI is better in mice lacking neuronal NO synthase (nNOS)[20] and therefore enhanced production of NO does not seem to be an important mechanism in SCI. It is possible that estrogen may produce vascular relaxation in an NO-independent manner by stimulating smooth muscle Maxi-K channel gates directly.[27]

Leukocyte adhesion and infiltration occurs after SCI and intercellular adhesion molecules, such as ICAM-1 and P-selectin, play important roles in these processes. Estrogen may also improve blood flow after CNS injury by reducing leukocyte adhesion, which occludes small vessels.[28] We have previously shown that mice lacking ICAM-1 and P-selectin have better motor function recovery after SCI and this might be a possible mechanism of neuroprotection by estrogen in SCI.[12,20]

Another possible mechanism of neuroprotection by estrogen might be by virtue of its strong antioxidant properties, resulting in more oxidative damage in males.[2] Improved recovery after SCI in females may also be due to effects of estrogen on anti-apoptotic proteins such as bcl-2. Apoptosis after SCI has been confirmed by many groups including ours.[16,29,30] Pre-treatment of neurons *in vitro* with 17β-estradiol increased bcl-2 levels and enhanced the survival of neurons from excitotoxicity.[31] Moreover, after brain ischemia the bcl-2 signal was higher in females compared to males in the ischemic penumbra.[32]

Information regarding the gender difference in SCI is limited when using a compression model of moderate SCI ($5 g/mm^2$) or in mice. In the studies reported by Hauben et al[4] spinal cord contusion in adult female rats resulted in worse recovery (BBB scores) when treatment with dihydrotestosterone occurred prior to SCI than in placebo-treated controls. However, when translated to mice, they showed that this difference in recovery was lost when nude female and male mice were used.[4] They concluded that the lack of sexual dimorphism in functional outcome in the nude mice suggests that the positive effect of estrogen or the negative effect of androgen on recovery is T-cell dependent.[4] Recently, another group examined the protective effect of estrogen on functional recovery after mild weight drop SCI in male rats. They gave 17β-estradiol ($100 mg/kg$) 1 h pre- or post-injury that improved motor function and reduced the size of the lesion. 17β-estradiol also significantly reduced the SCI induced increase in apoptotic cell death and caspase-3 activity. Furthermore, 17β-estradiol significantly increased expression of the anti-apoptotic genes, bcl-2 and bcl-x, after SCI while expression of the pro-apoptotic genes, bad and bax, were not affected by drug treatment.[10]

In our study, we found that gender-specific differences in mice were significant after moderate compression SCI using open field testing (BBB scores). However, we did not find significant differences using the locomotor activity (actometer) test. It is possible that this would also be significant if the cohort size was increased. Except for one male and one female, none of the animals were able to walk on beams. These observations indicate that either the observation period was too short (14 days) or gender-associated neuroprotection affects only gross locomotion and does not influence fine motor functions.

Data regarding differences in SCI and recovery patterns in humans are also limited.[33] However, in humans the male:female ratio of SCI is around 4:1[34] and the most important prognostic variable relating to neurologic recovery in a patient with SCI is the completeness of the lesion.[35] Scivoletto et al[36] evaluated sex-related differences of SCI patients, in a study conducted on 281 patients. They reported that female patients had a lower frequency of traumatic lesions, a lower frequency of complications at admission and a higher frequency of incomplete lesions (ASIA impairment C). However, female patients showed the same neurological and functional recovery as male patients. They concluded that gender does not seem to influence spinal cord rehabilitation outcomes despite the fact that men and women showed significant epidemiological differences.[36]

In a recent retrospective study, Sipski et al[37] assessed the gender differences in neurological and functional outcomes after SCI in humans. They used data from 14,333 patients and utilized the ASIA motor index score as a primary outcome measure. The higher the ASIA score, the better the outcome. The subjects were grouped by the severity of their injury with either complete ($P = 0.035$) or incomplete ($P = 0.031$) injury and were examined from system admission to the 1-year anniversary. Changes in the total ASIA scores were found to be significantly greater for women than men. Functional comparison of men and women, using the Functional Independence Measure (FIM) motor subscale, revealed that although men had higher FIM motor scores at rehabilitation discharge among those with motor-complete injuries, except for those with C1–4 and C6 neurologic levels, women with motor-incomplete high tetraplegia (C1–4 levels) injuries had higher discharge FIM motor scores than did similarly afflicted men. There were no significant differences in FIM motor scores among men and women with other levels of motor incomplete SCI. They concluded that women might have a more 'natural' neurologic recovery from SCI than men. However, for a given level and degree of neurologic injury, men tend to functionally do better than women at the time of discharge from rehabilitation.[37]

Finally, one additional note concerns potential mechanisms of estrogen effects on recovery after SCI. Ketamine reduces excitotoxic damage to the CNS, a fact known for some time.[38,39] Furthermore, estrogen minimizes excitotoxicity.[40] Consequently, our use of ketamine and acepromazine anesthesia for this study may actually underestimate the true gender difference afforded on recovery, and this may impact on potential mechanisms underlying gender-based recovery patterns following SCI.

In summary, from our current mouse study BBB scores were significantly better in females from the outset (day 1) and consistently so over a 14 day period during which both gender groups showed evidence of recovery. However, since female mice started with a less severe injury their BBB scores at the end point of the study were even more significantly higher. These observations suggest that neuroprotection provided by gender-related mechanisms occurs immediately, or nearly so, following impact and that the additional improvement occurring in the weeks following injury may not necessarily be gender related since both males and females showed improvement. Our study indicates that there is both a significant difference in ultimate recovery as well as a limitation of initial injury after SCI in females compared to males as measured by gross locomotion scales. These behavioral differences correlate with histologic changes in the spinal cord. Taken together they also underscore the need to pinpoint precisely what molecular mechanisms may underlie the earliest change detected in the sexual dimorphic response to CNS injury as such information may be clinically useful when exploring various therapeutic possibilities for SCI in humans.

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