Revised systemic administration of morphine promotes recovery of locomotor function after spinal cord injury in rats

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Abstract: Targeting activation of astrocytes during early stages of spinal cord injury (SCI) has been viewed as a potential strategy to improve neurological functions. Repeated systemic administration of morphine (RSAM) can induce activation of astrocytes in the spinal cord. Moreover, previous studies also revealed neuroprotective effects of morphine. Therefore, we investigated the effect of RSAM during an acute stage of SCI on neurological outcome in rats. RSAM significantly improved the Basso, Beattie, and Bresnahan score from 7 d after SCI and the inclined plane score from 14 d after SCI. However, the differences in thermal nociceptive thresholds and tactile sensory thresholds between the 2 groups were not statistically significant. RSAM increased expression levels of spinal IL-17, and decreased plasma levels of IL-17 at 1 d and 3 d after SCI. Furthermore, RSAM promoted activation of astrocytes around the damaged area, which prevented the spreading of damage into the surrounding tissue. At 28 d after SCI, RSAM preserved a greater area of white matter. These observations suggest that RSAM during an acute stage of SCI promotes recovery of locomotor function without promoting development of hyperalgesia and allodynia.

Keywords: Morphine, spinal cord injury, functional recovery, inflammation, astrocytes

Introduction

Spinal cord injury (SCI) is a devastating neurological condition. SCI consists of primary mechanical injury and secondary injury. Secondary injury, which lasts from minutes to weeks, is a destructive cascade of events and causes degeneration of neurons and axons unaffected or partially affected by the primary injury [1]. Activation of astrocytes after SCI has traditionally been viewed as detrimental because of their role in inhibiting axonal growth [2]. However, accumulating evidence indicates that activation of astrocytes plays an important role in repairing the initial injury, and preventing secondary injury as well as consequent loss of function [3]. At present, targeting activation of astrocytes and upregulating the beneficial effects of activated astrocytes during an early stage of SCI is viewed as a potential strategy for improving neurological functions [4].

Morphine is an opioid and is commonly administered for treatment of chronic pain after SCI. It has been shown that repeated systemic administration of morphine (RSAM) induces activation of astrocytes with increased expression of glial fibrillary acidic protein (GFAP) in the spinal cord [5]. Moreover, morphine exerts immunomodulatory and anti-inflammatory effects [6]. Previous in vitro and in vivo studies also revealed the neuroprotective effects of morphine [7]. However, it is unclear whether RSAM at an acute stage of SCI can produce beneficial effects on repair of injury. Therefore, we investigated the effect of RSAM at an acute stage of SCI on neurological outcome in rats with compressive SCI.

Materials and methods

Subjects

Adult male Sprague-Dawley rats weighing 260-280 g were obtained from the Animal Experiment Center of Harbin Medical University. The rats were housed individually in barrier
cages in a temperature and humidity controlled room with a 12:12 h light-dark cycle. Standard rat chow and water were available ad libitum. The study was approved by the Institutional Animal Care and Use Committee of Harbin Medical University. All procedures met the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Groups

Rats were randomly allocated into 2 groups (n = 28 in each group): vehicle-control group and morphine-treated group. Following compressive SCI, the rats received subcutaneous injections of sterile saline (1 mL/kg) or morphine hydrochloride (10 mg/kg, Northeast Pharmaceutical Group, Shenyang, China) daily for 7 consecutive days. The first injection was administered at 2 h post injury.

Surgery

The rat model of compressive SCI was established according to a previous report [8]. Briefly, fasted rats were anesthetized with intraperitoneal administration of sodium pentobarbital (50 mg/kg). A dorsal laminectomy at the 10th thoracic vertebral level was performed to expose the spinal cord, leaving the dura intact. The spinal cord was compressed from both lateral sides for 20 s using a microvessel clip with a closing force of 8 g. The skin and muscle incisions were then closed with 4-0 silk sutures. Gelfoam was applied to control bleeding as needed.

Post-operative care

The SCI rats were given 2 mL of sterile saline intraperitoneally to compensate for fluid loss and placed on a thermostatically controlled blanket to maintain body temperature until they revived. For the first 24 h after surgery, room temperature was maintained at 25-27°C. Bladders were manually expressed twice a day until spontaneous voiding returned.

Survival analysis and behavioral assessments

Twelve rats in each group were used for survival analysis and behavioral assessments for a study period of 28 d. One examiner who was blinded to the experimental procedures performed all behavioral assessments.

Overall locomotor recovery was evaluated using the Basso, Beattie, and Bresnahan (BBB) score [9], which is based on observations of hind limb joint movements, stepping, coordination, trunk stability, and tail position. The rats were allowed to move freely for 4 min in an open field with a nonskid floor. Hind limb locomotion was then scored from 0 to 21 points (complete hind limb paralysis to normal locomotion). This assessment was performed prior to surgery to exclude rats with abnormal locomotion, and then on 1, 7, 14, 21, and 28 d after SCI.

The inclined plane score [10] was used to assess equilibrium and residual strength of fore and hind limbs. This test requires a rat to maintain its position on a movable plane with a rough surface, which can be adjusted by 5° increments to a maximum angle of 90°. The maximum angle at which the rat could support its weight for 5 s was recorded as the inclined plane score. This test was administered prior to surgery to obtain baseline data, and then on 1, 7, 14, 21, and 28 d after SCI.

The tail-flick test [11] was employed to examine thermal nociceptive thresholds prior to surgery and 28 d after SCI. The rats were gently restrained and the distal one-third of the tail was immersed in water maintained at 52 ± 0.5°C. The nociceptive endpoint was the characteristic withdrawal of the tail from the warm water. An average of 3 consecutive trials separated by 5 min intervals was designated the nociceptive threshold.

At the same time points when thermal nociceptive thresholds were measured, tactile sensory thresholds were determined by Von Frey filaments, which were applied to the hind paw plantar surface using a modified version of the up-down method [12]. The rats were placed in individual Plexiglas chambers with a wire mesh floor and allowed to acclimate for 15 min before testing. Ten filament applications were used, and approximately 30-60 s separated each touch. The tactile sensory threshold was defined as the lowest gram force needed to produce hind paw withdrawal during at least 50% of its applications.

Assay of cytokines

At 1 d and 3 d after SCI, 12 rats in each group (n = 6 per time point) were killed with an overdose injection of sodium pentobarbital. A blood sample and a 1-cm segment of spinal cord tissue centered on the injury site were collected.
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Table 1. Changes of weight in the 2 groups

<table>
<thead>
<tr>
<th>Time</th>
<th>Vehicle-control (n = 8, g)</th>
<th>Morphine-treated (n = 9, g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior to surgery</td>
<td>281.59 ± 6.38</td>
<td>286.92 ± 5.29</td>
</tr>
<tr>
<td>7 days</td>
<td>299.68 ± 18.48</td>
<td>280.91 ± 11.99</td>
</tr>
<tr>
<td>14 days</td>
<td>336.61 ± 29.55</td>
<td>329.67 ± 21.70</td>
</tr>
<tr>
<td>21 days</td>
<td>390.45 ± 25.73</td>
<td>382.34 ± 24.26</td>
</tr>
<tr>
<td>28 days</td>
<td>418.09 ± 40.36</td>
<td>416.46 ± 39.42</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD.

To determine the levels of pro-inflammatory cytokines, the levels of IL-6 and IL-17 in spinal tissue homogenates and plasma were analyzed using ELISA kits (BlueGene Biotech, Shanghai, China) according to the manufacturer’s instructions.

Immunofluorescent labeling of GFAP

At 7 d and 28 d after SCI, 8 rats in each group (n = 4 per time point) were killed with an overdose injection of sodium pentobarbital. The spinal cord that included the lesion center was quickly removed and frozen. Transverse sections through the lesion epicenter were sliced with a cryostat to a thickness of 5 μm. The sections were washed 3 times with PBS. They were then incubated at 37°C with goat serum (BOSTER, Wuhan, China) for 45 min. After washing 3 times with PBS, they were then incubated overnight at 4°C with a primary antibody raised against GFAP (mouse monoclonal, 1:100, Millipore, Billerica, MA). The sections were then washed 3 times with PBS, incubated with a secondary fluorescent antibody (goat anti-mouse, 1:100, ZSGB-BIO, Beijing, China) for 1 h at room temperature and examined by fluorescence microscopy.

Assessment of white matter sparing

At 28 d after SCI, the remaining rats in the 2 groups were deeply anesthetized and transcardially perfused with saline followed by 4% paraformaldehyde. The spinal cord that included the lesion center was removed and postfixed in 4% paraformaldehyde overnight. The tissue was then embedded in paraffin and transverse sections through the lesion epicenter were stained with fast green FCF. The detailed procedures are as follow: (1) sections were dewaxed, (2) processed with 95% alcohol for 1 min, (3) incubated at 37°C with fast green alcohol solution for 30 min, (4) washed 2 times with 95% alcohol for 10 s (5) washed 2 times with distilled water for 15 s, (6) separated 2 times with 0.3% lithium carbonate for 10 s, (7) washed 2 times with distilled water for 15 s, (8) counterstained nuclei with nuclear fast red for 30 min, (9) washed with distilled water, dehydrated in graded alcohol, and processed with xylene. Image-Pro Plus analysis software (Media Cybernetics, Silver Spring, Maryland, USA) was used to determine the area of white matter. Proportional white matter sparing was calculated as white matter area/total cross sectional area.

Statistical analysis

The number of rats in each group was selected based on our preliminary experiments. Data are presented as mean ± SD and all analyses were performed using SAS 9.1.3 software (SAS Institute, Cary, North Carolina, USA). Kaplan-Meier analysis was used to analyze survival rates of the 2 groups. Comparisons between 2 group means were made using the Student’s t test. Repeated measures data were compared using a mixed-effect model followed by Bonferroni’s tests. A P value of < 0.05 was considered significant.

Results

Survival analysis

During the 28-d observation period, 4 rats in the vehicle-control group died and 3 rats in the morphine-treated group died. Survival analysis did not reveal a statistical difference between the 2 groups (P > 0.05). Nevertheless, no rats in the morphine-treated group died during the first week after SCI, and the death time of rats in the morphine-treated group was relatively delayed.

Weight gain

The rats in the morphine-treated group lost weight during the first 7 d after SCI. However, weight gain at 28 d did not differ significantly between the 2 groups (P > 0.05) (Table 1).

RSAM improved locomotor recovery without promoting development of thermal hyperalgesia and mechanical allodynia

Prior to surgery, all rats exhibited normal locomotor function. At 1 d after SCI, both groups had a BBB score of zero, which indicates a com-
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Complete loss of locomotion. In addition, SCI produced a significant decrease in the inclined plane score. As time passed, the BBB and inclined plane scores of both groups gradually increased. However, the rats in the morphine-treated group exhibited a significantly higher BBB score 7 d after SCI (9.56 ± 2.51 vs. 5.88 ± 1.25, P < 0.01) and greater improvement for capacity on the inclined plane 14 d after SCI (56.11 ± 6.51 vs. 50.00 ± 3.78, P < 0.01). The improvement in locomotor recovery in the morphine-treated group was sustained until 28 d after SCI (BBB score, 19.44 ± 2.30 vs. 15.13 ± 3.23, P < 0.01; inclined plane score, 62.78 ± 6.18 vs. 55.63 ± 3.20, P < 0.01) (Figure 1A, 1B).

As shown in Figure 1C and 1D, all rats showed a decrease in thermal nociceptive threshold (vehicle-control group, 4.24 ± 0.98 vs. 5.29 ± 0.36, P < 0.01; morphine-treated group, 4.32 ± 0.41 vs. 5.23 ± 0.43, P < 0.05; data obtained 28 d after SCI compared with those prior to surgery). In addition, all rats showed a rise in tactile sensory threshold at 28 d after SCI (vehicle-control group, 34.38 ± 22.69 vs. 9.50 ± 3.63, P < 0.01; morphine-treated group, 28.56 ± 19.22 vs. 9.11 ± 3.59, P < 0.05; data obtained 28 d after SCI compared with those prior to surgery). However, the difference between the 2 groups was not statistically significant (P > 0.05).

RSAM increased expression levels of spinal IL-17 and decreased plasma levels of IL-17

There was no significant difference in the spinal and plasma levels of IL-6 between the 2 groups (P > 0.05) (Figure 2A, 2B). However, the injured spinal cord of the morphine-treated group expressed more IL-17 than that of the vehicle-control group at 1 d and 3 d after SCI (1 d, 27.53 ± 5.68 vs. 13.36 ± 3.46, P < 0.01; 3 d, 35.08 ± 7.74 vs. 24.34 ± 6.21, P < 0.05) (Figure 2C). Especially striking was the observation

Figure 1. Repeated systemic administration of morphine promoted locomotor recovery evaluated by BBB score and inclined plane score, without promoting the development of thermal hyperalgesia and mechanical allodynia. Data are presented as mean ± SD, n = 8 in vehicle-control group, n = 9 in morphine-treated group. **P < 0.01 versus vehicle-control group. #P < 0.05, ##P < 0.01 versus data obtained prior to surgery. SCI = spinal cord injury; BBB score = Basso, Beattie and Bresnahan (BBB) score.
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that the morphine-treated group exhibited significantly lower plasma levels of IL-17 compared with the vehicle-control group at the 2 time points (1 d, 38.25 ± 6.81 vs. 63.29 ± 16.03, P < 0.01; 3 d, 73.47 ± 18.04 vs. 150.40 ± 14.01, P < 0.01) (Figure 2D).

**RSAM promoted activation of astrocytes around the damaged area**

Immunofluorescent labeling of GFAP revealed different astrocyte responses and damage characteristics at 7 d after SCI. In the vehicle-control group, activated astrocytes were distributed diffusely in the damaged area and accompanied by small necrotic areas. In contrast, in the morphine-treated group, activated astrocytes were distributed around the damaged area, forming a clear boundary between damaged tissue and normal or viable spinal tissue. At 28 d after SCI, immunofluorescent labeling of GFAP in the morphine-treated group revealed similar results to those observed 7 d after SCI. However, the vehicle-control group showed extensive spreading of damage into surrounding tissue (Figure 3).

**RSAM decreased loss of white matter at the lesion center**

At 28 d after SCI, transverse sections through the lesion epicenter obtained from the 2 groups showed characteristic necrosis, cavitation, and demyelination. Although the morphine-treated group revealed relatively larger cavities, the lesion area was limited and greater white matter sparing was observed. However, the vehicle-control group showed extremely extensive damage and little white matter sparing. There was a significant difference in the proportional white matter sparing between the 2 groups (11.05 ± 1.77% vs. 5.01 ± 0.36%, P < 0.01) (Figure 4).

Figure 2. Repeated systemic administration of morphine increased the expression levels of spinal IL-17, while decreased plasma levels of IL-17 at 1 d and 3 d after SCI. Data are presented as mean ± SD, n = 6 at each time point. *P < 0.05, **P < 0.01 versus vehicle-control group. SCI = spinal cord injury; IL-6 = interleukin 6; IL-17 = interleukin 17.
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Vehicle-control

Morphine-treated

7 days after SCI

28 days after SCI

A

B

C

D

E

F

G

H
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Discussion

The results of the study showed that RSAM during an acute stage of SCI improved recovery of locomotor function without promoting development of thermal hyperalgesia and mechanical allodynia. Accelerated activation of astrocytes by RSAM seemed to underlie the better recovery of locomotion. In addition, alleviation of a systemic inflammatory response by RSAM may also participate in improving the neurological outcome of SCI.

RSAM induces activation of astrocytes with increased expression of GFAP in normal spinal cord [5]. However, the impact of RSAM on astrocyte response to SCI is unclear. In our study, we found that RSAM promoted activation of astrocytes around the damaged area, which prevented extension of injury into the adjacent area. As a result, a greater area of white matter was preserved and locomotor function was improved. However, there may be a dose-response relationship. Woller et al. [13, 14] examined the addictive potential of morphine in a rodent model of SCI and found that during an acute stage of SCI, morphine self-administration of approximately 10 mg per day (0.75-1.5 mg per lever press) for 7 d did not affect recovery of locomotor function but did cause significant weight loss. Moreover, self-administration of a higher dose of morphine (3 mg per lever press)
not only reduced weight gain after injury, but also undermined locomotor recovery. The doses of morphine self-administration were far higher than those administered (10 mg/kg) in this study. It is possible that large doses of morphine lead to over-activation of astrocytes and formation of an irreversible glial scar at an early stage. Axon growth is therefore inhibited. Although targeting activation of astrocytes is a potential strategy to improve neurological functions, the degree of astrocyte activation is of great importance for repair and regeneration after SCI. In our study, RSAM of 10 mg/kg per day restricted weight gain only during the first 7 d after SCI. Afterward, body weight increased gradually.

The post-traumatic inflammatory response in spinal cord has been considered one of the possible mechanisms that contribute to secondary SCI, and attenuation of the local inflammatory response has been shown to improve functional recovery [15]. A previous study [16] indicates that intrathecal or intraperitoneal administration of a single dose of morphine 24 h after spinal contusion injury increases expression levels of spinal IL-1β and attenuates locomotor recovery. In this study, we determined the levels of IL-6 and IL-17 in the spinal cord at 1 d and 3 d after SCI. Similar to the study by Hook et al. [16], we observed no difference in spinal IL-6. The reason may be that IL-6 increases 5 to 6 h after injury in rat and human spinal cords, and then declines to baseline 24 h to 48 h after injury [17]. In contrast, we found that RSAM increased expression levels of spinal IL-17. The mechanism underlying the paradoxical results between locomotor recovery and increased spinal IL-17 is complex. It has been reported that pro-inflammatory cytokines are the triggers of activated astrocytes in the acute phase of SCI [18], and activated astrocytes release pro-inflammatory cytokines, which in turn activate more astrocytes [19]. A study by Liberto et al. [20] showed that cytokines might promote neuroprotection and elicit regenerative responses from astrocytes. In addition, Ritz and Hausmann [8] reported that stimulating early cytokine release and astroglial responses significantly improve the functional outcome of SCI. IL-17 is a relatively novel member of the cytokine family and has received less attention in SCI. IL-17 can be produced not only by immunocytes, which invade damaged areas after SCI [21], but also by astrocytes [22]. In our study, the increased spinal IL-17 may have been released mainly by activated astrocytes, and then more astrocytes were activated by IL-17. Apart from a barrier function, activated astrocytes contribute to restoring the extracellular ionic environment [23], reduce excitotoxic death by taking up excess glutamate [24], and produce multiple neurotrophic factors such as nerve growth factor, neurotrophin-3, basic fibroblast growth factor, and ciliary neurotrophic factor [25, 26]. The beneficial effects of activated astrocytes may overcome the detrimental effects of IL-17. Therefore, the local inflammatory response may be only part of a much more complex mechanism for SCI [27], and increased spinal IL-17 does not necessarily predict a bad neurological outcome.

Contrary to increased expression levels of spinal IL-17, we found that RSAM significantly decreased plasma levels of IL-17. The peripheral anti-inflammatory and immunomodulatory effects of morphine have been reported in previous studies [6]. RSAM may also participate in improving the neurological outcome of SCI through alleviating a systemic inflammatory response and improving general status. In contrast to local inflammatory responses in the spinal cord, less attention has focused on the effects of a systemic inflammatory response in secondary SCI. Studies show that a systemic inflammatory response increases the risk of major complications [28], such as pneumonia, cardiac complications, and acute respiratory failure, and should be targeted in the development of new therapeutic strategies to treat SCI [29].

In summary, our results suggest that RSAM during an acute stage of SCI protects injured spinal cord by promoting activation of astrocytes around the damaged area and alleviating a systemic inflammatory response. There are a number of limitations in our study. The major one is that we did not investigate the effects of different doses of morphine and days of morphine use on neurological outcome. In addition, we observed only for a period of 28 d after SCI. If survival analysis were observed for a longer period, differences may emerge. In the future, further study is needed to establish a dose-response relationship and guide the clinical application of morphine.
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Disclosure of conflict of interest

None.

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