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Original Article

Observation of locomotor functional recovery in adult complete spinal rats with BWSTT using semiquantitative and qualitative methods

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Study design: Experimental rat model of spinal cord transection.
Setting: China rehabilitation research center.
Objective: To investigate locomotor functional recovery in spinal rats with BWSTT using semiquantitative and qualitative methods.
Methods: Five-day postoperative (dpo), adult female complete spinal rats (at T_8 level) received 40 days of body weight-supported treadmill training (BWSTT). Signs of functional recovery were examined with average combined scores (ACOS) and Basso Beattie and Bresnahan (BBB) scales at different time points.
Results: At 1-dpo, none of the spinal rats exhibited hindlimb movements. The spinal rats displayed functional progress with time, but the rare could recover to full weight-bearing hindlimb at 45-dpo. BBB and ACOS scores in the BWSTT group obtained better scores than those in the spinal cord injury (SCI) group at 30- and 45-dpo. Furthermore, all BBB and ACOS scores of spinal rats reached statistical significance between 7- and 30-dpo, and between 15- and 30-dpo. However, only ACOS but not BBB scores in the SCI and BWSTT groups showed statistics differences between 15- and 45-dpo, and between 30- and 45-dpo. The Spearman correlation coefficients of BBB and ACOS scores were 0.913 and 0.972 for the SCI and BWSTT groups, respectively.
Conclusions: The results confirmed the existence of partial spontaneous hindlimb functional recovery in adult chronically spinal cord-transected rats, and that BWSTT can improve motor performance. In addition, our study suggests that qualitative and semiquantiative methods are strongly correlated with locomotor recovery in spinal rats, and the latter may be more sensitive in reflecting minor variance at different time points.
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Keywords: spinal cord injury; locomotion; functional recovery; assessment

Introduction

Animal models play a critical role in the development of experimental therapies for spinal cord injury (SCI). Rat model, as the most consistently studied and standardized model, has received much attention by scientists for the assessment of locomotive performance and to determine the functional consequences of the initial injury, spontaneous function recovery and the efficacy of therapeutic strategies.[1–3] Currently, a number of assessment methods, such as BBB locomotor rating scale,[4,5] modified Tarlov scoring system,[6] grid-walk test,[7] narrow beam test[8] and inclined plane test,[9] have been developed to evaluate the movement recovery after SCI. However, it is believed that these qualitative methods have been designed to assess mainly fine locomotor skills owing to descending fiber regeneration, which results in apparently lack of spontaneous recovery in spinal rodents.[10,11] Ballermann *et al*[12] also found that spinal cord-injured rats developed alternative locomotor patterns following SCI that cannot be discriminated by the use of qualitative visually based analysis. Thus, the quantitative methods to assess motor function after SCI are needed. Guertin proposed a new semiquantitative assessment method, the average combined scores (ACOS) that counts basic locomotor-like movements owing to spinal reorganization at the sublesional level. The author claims that this semiquantitative method could be better for the assessment of spinal cord recovery in adult mice.[10]

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Several studies have reported considerable native locomotive abilities of the completely transected spinal cord in animals (so-called spinal animal),[13–15] and that a period of gravity-assisted treadmill training of the hindlimbs or pharmacological interventions, such as serotonergic agonists treatment, can promote spontaneously functional recovery.[16–17] Therefore, in this study, we used the BBB locomotor rating scale, the most commonly applied qualitative method, and the ACOS semiquantitative method, to observe the characteristic of hindlimb movement recovery in adult spinal rats with body weight-supported treadmill training (BWSTT).

Materials and methods

Animals and grouping

Twenty-six adult female Wistar rats aged 3 months (weight 250–300 g) underwent narcosis (Nembutal 40 mg/kg intraperitoneally). After dissection of soft tissues, laminectomy at the level of thoracic vertebrae 7–9 was performed on the dorsal side of the spinal cord. Twenty rats underwent complete midthoracic spinal cord transection at the T_8 level with a thin scalpel, leading to complete separation of the two stumps. Bleeding was stopped and wound was sutured. Animals were placed in an incubator (37.5°C) until fully recovered from the anesthesia and then each rat was housed individually. On 1–5-day post operative (dpo), rats were injected with gentamicin twice daily (40 mg/kg intramuscular). The urinary bladders were emptied by pressing the abdominal wall until automatic bladder function was recovered. For the sham group ($n=6$), wound was immediately sutured without receiving any treatment. All animals were kept with free access to food and water.

Rats in the BWSTT group ($n=10$) received body weight-supported treadmill (band velocity $\sim$7–10.5 m/min) training at 5-dpo. Training was manually performed, a plantar position stepping on a rodent treadmill, in episodes of 30 min/day, 5 days/week. The rat was supported over the treadmill (direct current motor-driven) with the help of a well-adjusted harness via a pulley system. Body weight support was monitored with a special weigher equipment, beginning at 50% and then decreasing to 25%. For the SCI group ($n=10$), animals did not receive training after spinalization.

To confirm complete spinalization before postmortem, a proximal retransection was performed at 45-dpo. After anesthesia with ether vapor, the spinal cords of animals in the SCI and BWSTT groups were completely retransected at the T_6 level. After 2 h, their motor behavior was observed again. If the motor behavior was disturbed, the rat would be discarded from the study. It is based on the theory of 'locomotor behavior of complete spinal rats should not be disturbed if the recovery of locomotion was not attributed to some spontaneous regeneration of axons through the lesion'. Then all animals were deeply anesthetized with sodium pentobarbital and the original lesion spinal cords were removed for histological evaluation. Only those complete spinal animals were kept for further analysis.

The animals were cared for and surgically handled in accordance with the China Animal Community Council Directive.

Evaluation of locomotor recovery

The locomotor activities of rats were recorded by semiquantiative and qualitative methods before operation and at 1- 7- 15- 30- and 45-dpo. It was undertaken by two trained observers under blind conditions, using the BBB and ACOS scales. As reported previously, the BBB locomotor rating was described qualitatively with a 21-point scale by Basso *et al*.[4] According to the ACOS scale reported by Guertin,[10] the hindlimb movements during 1 min were counted in two different categories, total number of nonbilaterally alternating movements (NBA) and bilaterally alternating movements (BA). Amplitude of BA was assigned into three values: 0 – no movement; 1 – less than half range of motion and 2–more than half range of motion. ACOS rate was obtained as follows: $[\mathrm{NBA} + (\mathrm{BA} \times 2) \times \mathrm{Amplitude}]$.

Statistical analysis

Scores of each animal obtained from locomotor rating scales were averaged for each pool of rats (sham, $n=6$; SCI, $n=8$ and BWSTT, $n=8$). Results were expressed as mean$\pm$SEM. The results were analyzed by SPSS 11.5. Statistical analyses were divided into two parts: (i) one, where different groups were compared against each other at each time point, using a one-way ANOVA test with repeated measures. Bonferroni test (equal variances assumed) or Dunnett's T3 test (equal variances not assumed) was used as a *post hoc* test to determine the significance of the differences between SCI and BWSTT groups and (ii) the second part where comparisons at different time points in the SCI and BWSTT groups were analyzed using paired-samples *t*-test with two-sided probabilities. BBB and ACOS correlation was evaluated using the Spearman test. A $P<0.05$ was chosen as the significance level.

Results

General state

All animals in the sham group survived. Two rats in both the SCI and BWSTT groups died. The causes of death included anesthetic accident and urinary infection. The survival rate was 84.65%. In both the SCI and BWSTT groups, at 45-dpo the 16 survived rats were confirmed completely spinalized through proximal spinal cord retransection, postmortem examination and histological evaluation.

Rats in the sham group kept normal locomotor activities during the experiment. In contrast, spinalization resulted in complete hindlimb paralysis on animals of the SCI and BWSTT groups. Nevertheless, animals in

these two groups began to show signs of recovery at 15-dpo. Although motor performances of rats in the BWSTT group were better than those in the SCI group, none of the animals could recover to full weight bearing with hindlimb.

BBB locomotion score

Rats in the sham group exhibited normal hindlimb function over the observation period (BBB score 21) as well as scores observed in all animals before operation (Figure 1). At 1-dpo, spinal rats from the SCI and BWSTT groups displayed flaccid paralysis (BBB score 0). At 7-and 15-dpo, a part of spinal rats showed articular movement (BBB score 0–3), and no differences were observed between the SCI and BWSTT groups. There was a partial functional recovery at 30- and 45-dpo, nevertheless BBB scores still did not surpass the score 10. BBB scores of the SCI group (mean $\pm$ SEM: 30 days, 1.875 ± 0.74252; 45 days, 2.125 ± 0.87500) were lower than those of the BWSTT group (mean $\pm$ SEM: 30 days, 6.125 ± 1.32877; 45 days, 6.500 ± 1.28174). A statistical significance exists between these two groups ($P_{30d} = 0.049$, $P_{45d} = 0.043$). Compared with preoperation, the SCI and BWSTT groups exhibit high significances in BBB scores ($P < 0.01$) at each time point after spinalization. Moreover, a statistical significance was found in the SCI and BWSTT groups at the next different points: between 1- and 30-dpo ($P_{SCI} = 0.040$, $P_{BWSTT} = 0.002$), between 1- and 45-dpo ($P_{SCI} = 0.046$, $P_{BWSTT} = 0.001$), between 7- and 30-dpo ($P_{SCI} = 0.048$, $P_{BWSTT} = 0.003$) and between 15- and 30-dpo ($P_{SCI} = 0.048$, $P_{BWSTT} = 0.003$). In the BWSTT group, statistical differences were observed between 1- and 15-dpo ($P = 0.020$), between 7- and 45-dpo ($P = 0.002$) and between 15- and 45-dpo ($P = 0.001$). No differences were found at other time points ($P > 0.05$).

ACOS score

The numbers of rats displaying NBA and BA are illustrated in Table 1. Before intervention, no significant differences with the ACOS scale were found in the three groups (Figure 2). At 1-dpo, rats in the sham group (mean $\pm$ SEM: 296.625 ± 5.30477) showed a transient decrease in ACOS score, in contrast to spinal rats where an obvious reduction was observed (mean $\pm$ SEM: 0 ± 0). There were no significant differences in the ACOS scores at 7- and 15-dpo between the BWSTT and SCI groups. However, at 30- and 45-dpo, ACOS scores in the BWSTT group (mean $\pm$ SEM: 30 days, 64.625 ± 15.95970; 45 days, 77.875 ± 17.80995) were greater than those in the SCI group (mean $\pm$ SEM: 30 days, 14.125 ± 5.04422; 45 days, 18.250 ± 6.52673), and it reached statistical significance ($P_{30d} = 0.044$, $P_{45d} = 0.034$). When we compared the ACOS scores at each point after spinalization with those observed before spinalization, high significances ($P < 0.01$) were observed. In the SCI and BWSTT groups, statistical significance was reached between 1- and 30-dpo

Table 1 Number of rats displaying NBA and BA at different time in each group

	Sham group (n = 6)		*SCI group* (n = 8)		*BWSTT group* (n = 8)	
	NBA	*BA*	*NBA*	*BA*	*NBA*	*BA*
Before operation	6	6	8	8	8	8
dpo-1	6	6	0	0	0	0
dpo-7	6	6	3	0	5	0
dpo-15	6	6	3	0	6	2
dpo-30	6	6	6	3	7	5
dpo-45	6	6	6	3	7	6

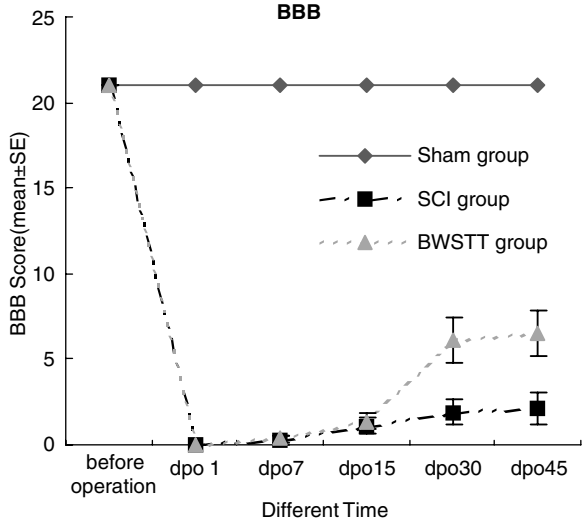

Figure 1 The BBB locomotor rating scale: data presented were the average scores at different time for each group (sham: $n = 6$; SCI: $n = 8$; and BWSTT: $n = 8$ rats)

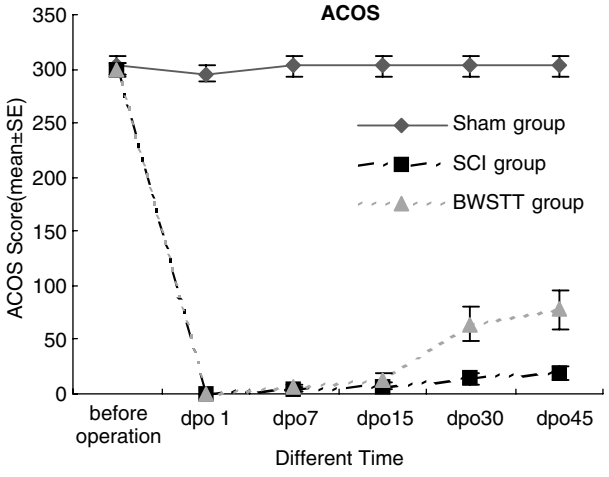

Figure 2 ACOS = NBA + (BA × 2) × Amplitude; data presented were the average scores at different time for each group (sham: $n = 6$; SCI: $n = 8$; BWSTT: $n = 8$ rats)

($P_{SCI} = 0.027$, $P_{BWSTT} = 0.004$); between 1- and 45-dpo ($P_{SCI} = 0.027$, $P_{BWSTT} = 0.003$); between 7- and 30-dpo ($P_{SCI} = 0.020$, $P_{BWSTT} = 0.005$); between 7- and 45-dpo ($P_{SCI} = 0.022$, $P_{BWSTT} = 0.003$); between 15- and 30-dpo ($P_{SCI} = 0.021$, $P_{BWSTT} = 0.0013$); between 15- and 45-dpo ($P_{SCI} = 0.023$, $P_{BWSTT} = 0.004$) and between 30- and 45-dpo ($P_{SCI} = 0.032$, $P_{BWSTT} = 0.007$). In addition, there was a statistical significance in ACOS scores between 7- and 15-dpo in the BWSTT group ($P = 0.049$).

Correlation of BBB and ACOS scores

The Spearman correlation coefficients of BBB and ACOS scores were 0.913 for the SCI group and 0.972 for the BWSTT group, respectively.

Discussion

Spontaneous locomotor recovery of hindlimb after SCI

Our results confirm the signs of partial hindlimb movement recovery in adult rats after 2 weeks of complete transection of the spinal cord. The BWSTT approach would improve motor performance. Similar results have been observed previously.[16,18] We also found that the locomotor functions of spinal rats with or without BWSTT were all increased over time. It is believed that locomotor recovery may be attributed to the evolving autonomy of spinal segmental excitability below the site of the lesion, which is called central pattern generators (CPG). As time after injury progresses, spinal networks might readapt to the new situation in which no descending control is provided.[18,19] Edgerton *et al*[16] described that a hindlimb locomotor pattern close to normal can be obtained by training after spinalization in adult cats. Our data showed that the spinal rats with training also acquired higher BBB scores and ACOS scores than the spinal rats without training. This could be partially attributable to the fact that training may induce an activity-dependent tuning of the inhibitory synaptic strengths in the network that may compensate for the absence of these substances to trigger activity in the spinal networks, such as monoamines.[19,20] However, in our study, rare animals could recover to full weight bearing with hindlimb. Therefore, further studies of spinal reorganization and plasticity after SCI could be necessary for the development of rehabilitation therapies.

Originally, we planned to use two qualitative methods, the BBB scale and the Combined Behavioral Score (CBS),[6] to assess simultaneously locomotor recovery. Nevertheless, this latter method was discarded because motor score scale is very rough and several items (eg swimming and righting) lack diversity in spinal cord-transected rats. Strikingly, using CBS we found an interesting phenomenon that restoration of withdrawal reflex after complete spinal transection coincided with restoration of locomotor recovery. Three rats in the SCI group and one rat in the BWSTT group without withdrawal reflex acquired 0 score by the BBB scale at 45-dpo. Those rats with hyperactive withdrawal reflex obtained better locomotor recovery. This finding supported the view that some of the spinal cord networks that control stepping seem to share the same interneurons that are involved in flexion reflexes.[21,22] Several studies have shown that the restoration of spinal cord reflexes would provide a measure of the functional restoration of the spinal cord networks responsible for generating stepping after a complete spinal cord transection in adult rats.[21] In intact animals, ipsilateral and crossed spinal reflexes conveyed by polysynaptic circuits do play an important role during walking.[21] In fact, we found that recovery was paralleled by crossed withdrawal reflex, that is, reflex to withdraw not only the ipsilateral hindlimb but also the contralateral hindlimb by flexion in response to extension, pain and pressure. Valero-Cabre *et al*[23] observed that ipsilateral reflex components mediated by thick A-fibers (H reflex and C1) but not by thin fibers (C2 and C3) remained present after injury, showing long-lasting facilitation whereas contralateral reflex components were abolished after injury and showed limited recovery, by electrophysiological evaluations in control rats and spinal cord transection rats. Nevertheless, all animals with higher locomotor scores in the BWSTT group presented a crossed withdrawal reflex in the present study. It is reasonable to believe that crossed withdrawal reflex may more suggest the reorganization of spinal circuits after injury correlated with the ability to generate alternation rhythmical locomotor activity. The restoration of hindlimb function recovery when facilitated by treadmill training stimulation coincided with the restoration of crossed withdrawal reflex after complete mid-thoracic spinal transection. Altogether, these observations suggest that BWSTT represents a useful tool for investigating locomotor activity induced by spinal cord reorganization after a SCI and most likely excites a range of reorganization strategies involving spinal cord reflex circuits connecting muscle and skin sensory afferents with intact spinal interneurons and motorneurons.

We also observed that animals of the BWSTT group with frequency BA in an open-field situation displayed a rhythmic shears gait on the treadmill under partly supported weight. The locomotor pattern reflected the loss of supraspinal control and the increase of spinal excitability after complete SCI in animals. As changes only happened in trained spinal rats, they are consistent with the utilization of BWSTT in facilitating motor learning occurring in the spinal cord.

Behavior evaluation

BBB locomotor rating scale has been proved to be a valid and predictive measure of locomotor recovery as well as a useful method for allowing the communication and standardized comparison of results from different institutions.[4,5] In our study, mean BBB scores in the BWSTT group were higher than those in the SCI group reaching statistical significance at 30- and 45-dpo. Nonetheless, originally the BBB test was designed to

evaluate motor deficits following partial lesions of the spinal cord. This test only takes into account the lumbar spinal cord, which has intrinsic capabilities of generating locomotor activity even when disconnected from supraspinal structures. We observed in this study that at 45-dpo, five rats in the SCI group and four in the BWSTT group reaching lower scores (1–8) were capable of hindlimb joint movements without weight support. No rat in the SCI group and only three in the BWSTT group were rated in the middle part of the scale (9–13) demonstrating varying degrees of hindlimb weight support and forelimb–hindlimb coordination with the highest score 10 (occasionally hindlimb weight support). In our study, no rat reached higher scores (14–21) in which rats show improvements in paw and tail position, toe clearance and trunk stability during a fully supported and coordinated gait.

Initially, the semiquantitative ACOS method was designed to better characterize hindlimb motor and locomotor-like movements in adult mice with complete spinal cord transection.[10] Behavioral tests relying on visual observations in open-field conditions, such as those developed to evaluate locomotion in rats after spinal cord injuries (ie Tarlov test and BBB test), are also used in mice. In this study, we proceeded inversely: we first used the ACOS method to determine functional recovery in adult spinal rats. Like this we can describe the improvement in right–left alternation not only with weak or large amplitude movement, but also with the amount of movement. Our results showed that the ACOS semiquantitative method was highly correlated with the BBB qualitative method for locomotor recovery in spinal rats. Rat ACOS scores in both groups reached statistical significance not only between 15- and 45-dpo but also between 30- and 45-dpo; however, for the BBB scores only the BWSTT group was significantly different between 15 and 45-dpo. So, we believe that the ACOS semiquantitative assessment method may be a more sensitive and discriminative approach to observe differences of locomotor recovery at different time points. However, compared to BBB, the ACOS method is a more complex and time- and energy-consuming test. Finally, it should be noted that, although ACOS semiquantitative method is easy to operate, using video and electromyogram (EMG) recordings to evaluate the locomotor recovery is a more scientific quantitative assessment method.

Both BBB and ACOS scales are strongly correlated with locomotor recovery in spinal rats. The well-known BBB test, as qualitative method example, is a time-saving method to evaluate behavioral locomotor in rats, whereas the ACOS semiquantitative test is more sensitive to reflect minor locomotor recovery in SCI rat at different time points, and more suitable to study rhythmic behaviors in SCI research. It would be accepted as a valid way to document movements associated with the recovery of locomotor function by using the qualitative and semiquantitative method after SCI if necessary.

Altogether, our results demonstrate the benefits of BWSTT to promote the activation of the lumbar CPG for locomotion in adult chronic spinal rats. Both qualitative and semiquantiative methods are sensitive and accurate locomotor assessment methods, and ACOS semiquantiative scale may be more sensitive to reflect minor variance at different time points in spinal rats.

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