ORIGINAL ARTICLE

Non-assisted treadmill training does not improve motor recovery and body composition in spinal cord-transected mice

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Study design: Experiments in a mouse model of complete paraplegia.

Objectives: To evaluate the effect of non-assisted treadmill training on motor recovery and body composition in completely spinal cord-transected mice.

Settings: Laval University Medical Center, Neuroscience Unit, Quebec City, Quebec, Canada.

Methods: Following a complete low-thoracic (Th9/10) spinal transection (Tx), mice were divided into two groups that were either untrained or trained with no assistance. Training consisted of placing the mice during 15 min with no further intervention (that is no tail pinching or body weight support) on a motorized treadmill (8–10 cm s⁻¹) five times per week for 5 weeks. Locomotor performances were assessed weekly in both groups using two complementary locomotor rating scales. After 5 weeks, all mice were killed and adipose tissue, *soleus*, and *extensor digitorum longus* muscles were dissected for analyses.

Results: No significant difference in locomotor performances or in muscle fibre type conversion was found between trained and untrained mice. In contrast, body weight, adipose tissue, whole muscle, and individual fibre cross-sectional area (CSA) values were significantly lower in trained compared with untrained animals.

Conclusions: Non-assisted treadmill training in these conditions did not improve motor performances and contributed to further accentuate body composition changes post-Tx, suggesting that assistance provided manually, robotically, or pharmacologically may be key to spinal learning and recovery of locomotor function and body composition.

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Introduction

It has been well established that adult paraplegic animals can recover some locomotor functions using regular treadmill training (TT) typically assisted with sensory stimulation and/or pharmacological aids.^{1,2} In fact, even without pharmacological aids, regular TT assisted with a weight-supporting harness and/or tail or sexual organ pinching can enable the expression of involuntary 'reflex' stepping in low-thoracically spinal cord-transected (Tx) animals.^{3–5} However, without assisted training, some spontaneously occurring small amplitude hindlimb movements were also shown to progressively occur a few weeks post-Tx in untrained Tx mice,^{6–8} suggesting that basic spontaneous sublesional plasticity and spinal learning events may occur even in the absence of assisted training. Untrained Tx mice were also found to

undergo significant changes in body composition—for example, body weight (–24%) and *soleus* fibre properties (–32% in mass and maximal force) within a few days post-Tx,⁹ whereas assisted TT was found to partially prevent muscular property changes in Tx cats and rats.^{10,11} All and all, it remains unclear the extent to which TT, in its most basic form (that is without assistance), may improve locomotor performances and prevent or restore body composition changes post-Tx. In this study, we assessed and compared locomotor performances and body composition (body weight, region-specific adipose tissue mass, muscle/fibre cross-sectional area (CSA), and phenotype) between untrained Tx mice and non-assisted trained Tx mice.

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Materials and methods

Animal model and surgical procedures

All experimental procedures were conducted in accordance with the Canadian Council for Animal Care guidelines and

accepted by the Laval University Animal Care and Use Committee. A total of 22 male CD1 mice (Charles River Canada, St-Constant, QC, Canada) initially weighing 30–35 g were used for this study. Preoperative cares included administration of lactate-Ringer's solution (1 ml, s.c.), an analgesic (0.1 mg kg⁻¹ buprenorphine, s.c.), and an antibiotic (5 mg kg⁻¹ enrofloxacin, s.c.). All surgical procedures were performed under aseptic conditions. Mice were anesthetized with 2.5% isoflurane. A small incision was made on their back to expose the mid-to-low thoracic vertebrae. The spinal cord was then completely transected intervertebrally with microscissors inserted between the 9th and 10th thoracic vertebrae.^{6–8} To ensure that complete Tx was achieved, the inner vertebral walls were explored and entirely scraped with small scissors tips. The opened skin area was sutured and animals were placed for a few hours on heating pads. Mice were left in their cage with food and water *ad libitum* during 3 days after surgery. Postoperative cares included administration of lactate-Ringer's solution (2 × 1 ml per day, s.c.), buprenorphine (2 × 0.1 mg kg⁻¹ per day, s.c.), and enrofloxacin (5 mg kg⁻¹ per day, s.c.). Bladders were manually emptied twice daily for the first week and once a day throughout the study. Complete Tx was confirmed by (1) initial full paralysis of the hindlimbs, (2) postmortem visual and microscopic examination of the spinal cord lesion, and (3) histological examination of coronal or midsagittal spinal cord sections stained with luxol fast blue/cresyl violet for myelinated axons and Nissl substance, respectively.

Training procedures

Animals were randomly divided into untrained Tx mice (control, *N* = 12) and non-assisted TT Tx mice (*N* = 10). TT began on the third day after surgery (to allow a few days of recovery after surgery) and ended after 5 weeks. Trained mice were simply placed for 15 min on a motorized treadmill belt moving at 8–10 cm s⁻¹ without any other forms of assistance or stimulation. A harness placed around the torso and attached ahead was used to maintain the animals on the treadmill belt. No weight support assistance was provided by the harness.

Assessment of locomotor function recovery

On the third day, first, second, third, fourth, and fifth week post-Tx, hindlimb motor and locomotor movements were assessed using two complementary methods. Each assessment session was performed on the treadmill and lasted no more than 2 min to minimize potential training-induced effects of the assessment *per se* in the untrained group. Hindlimb movements were assessed 'live' using a qualitative motor scale, referred to as AOB, which has been specifically developed for complete Tx rodents. In brief, the scale consists of 22 scores: 0—no movement; 1—weak limb jerks; 2—weak rhythmic movements with no bilateral alternation; 3—large rhythmic movements with no bilateral alternation; 4—weak rhythmic movements with occasional bilateral alternation; 5—large rhythmic movements with occasional bilateral alternation. Higher levels of recovery are described in detail in the original article.⁵ We also used a semi-

quantitative assay called Average Combined Score (ACOS) that has been developed in our laboratory for 'live' semi-quantitative assessment of hindlimb movements in Tx rodents.^{6–8} ACOS is designed to assess non-locomotor movements (NLM, number min⁻¹), locomotor-like movements (LM, number min⁻¹), and amplitude arithmetically combined as follows: [NLM + (2 × LM)] × amplitude. One NLM was defined as a non-bilaterally alternating movement including jerks, fast-paw shaking, and twitches. One LM was defined as a flexion followed by an extension (or *vice versa*) occurring bilaterally in alternation, not necessarily including weight-bearing capabilities. We did not consider LMs or NLMs induced during bowel movements to avoid non-related afferent (sacral) stimulation-induced movements.¹² Amplitude was characterized by assigning one of the three values; 0—no movement; 1—movements considered to be less than half the range of motion of a normal step; 2—movements considered to be greater than half the range of motion of a normal step.

Muscle immunohistochemistry

On killing, left *soleus* and *extensor digitorum longus* (EDL) muscles were dissected, frozen in melting isopentane, and stored at -80 °C until further use. Serial cross-sections of 12 µm-thick from the muscle mid-portion were cut with a cryostat maintained at -20 °C (2800E Frigocut, Leica Instruments, Germany) and mounted on Superfrost plus glass slides (VWR Canlab, Mississauga, ON, Canada). For individual fibre labelling, Myosin Heavy Chain slow or fast primary antibodies were used (MHCs or MHCf, specific for MHC isoform type I and type II, respectively) (Vector Laboratories, Burlingame, CA, USA). First, cross-sections were incubated 1 h in a blocking solution containing 10% rabbit serum, 1% triton X-100, and 0.1 M phosphate-buffered saline (PBS). Cross-sections were then washed in PBS 0.1 M and incubated for 2 h in a solution containing MHCs or MHCf primary antibodies (dilution 1/50 in 0.1 M PBS containing 1% rabbit serum and 1% triton X-100). Sections were rinsed in PBS 0.1 M before incubation with a goat anti-mouse IgG(H+L) Alexa Fluor 488 secondary antibodies (Molecular Probes, Eugene, OR, USA) (dilution 1/500 in 0.1 M PBS containing 1% rabbit serum and 1% triton X-100). Slides were then rinsed in PBS 0.1 M and mounted with PBS-Glycerol (50–50). Some sections were treated as above, excepted that the primary antibodies were omitted from the incubation solution as control. Immunofluorescence labelling was visualized on an Olympus BX61WI confocal microscope with a 10 × water-immersion objective. Images were captured using FluoView 300 (Olympus Canada Inc., Markham, ON, Canada) and analysed with ImageJ (ImageJ 1.40, Research Services Branch, NIH, Bethesda, MD, USA). Analyses consisted of determining *soleus* and *EDL* muscle fibre type composition, whole muscle CSA, and fibre CSA for fibre type I, II, and I + II (type I + II is a hybrid fibre isoform, labelled with both MHCs and MHCf antibodies). For the latter analysis, 50 fibres/isoform/muscle were averaged. In cases where the number of fibres was <50 fibres/isoform/muscle, CSA was calculated using all available fibres.

Data analyses

Comparisons between untrained and trained Tx mice for locomotor recovery levels and body weight were assessed using a two-way analysis of variance followed by a Bonferroni *post hoc*. Differences in adipose tissues, muscle, and fibre CSA as well as fibre type relative distribution were examined using an unpaired Student *t*-test. Results are reported as mean $\pm$ s.e.m.

Results

Hindlimb movement recovery assessed with the AOB and ACOS rating scale

Occasional hindlimb movements of small amplitude (NLMs and LMs with no weight-bearing capabilities) have already been shown to progressively occur spontaneously in untrained Tx mice.^{6–8} Here, Tx mice from the untrained group displayed hindlimb movements corresponding to AOB scores no greater than '1' (Figure 1a). Similar results were found in the trained mice. For both groups, two-way analysis of variance revealed a significant increase in score ($P < 0.001$). However, no difference between groups were found throughout the 5 weeks ($P = 0.723$). Similarly, from the third day through the fifth week, scores from ACOS locomotor rating scale showed a moderate but significant increase (Figure 1b, from 0 at 3 days post-Tx to <4 at 5 weeks post-Tx, Figure 1b, $P = 0.002$). Again, no difference between the untrained and trained Tx mice was observed ($P = 0.321$). Note that no LM was found throughout this study (that is only NLMs mainly constituted of small amplitude unilateral flexions were observed).

Body weight and site-specific adipose tissue

Body weight was measured before surgery and before each assessment session (Figure 2). During the first week,

Figure 1 Hindlimb locomotor movements assessed with the Antri-Orsal-Barthe (AOB) (a) scale and Average Combined Scores (ACOS) (b). On the third day, none of the groups showed hindlimb movements. Both AOB and ACOS score showed moderate, but significant increase in score over time. However, no difference between groups was found.

comparable data were found in untrained and trained Tx mice, which displayed significant body weight losses ($P < 0.001$). Subsequently, between the second and the fifth week, untrained Tx mice progressively recovered some weight (~ 1 g per week), whereas at 2 weeks post-Tx, trained Tx mice continued losing weight ($75.1 \pm 1.2\%$), before a progressive regain. Region-specific adipose tissue masses were examined postmortem in all animals. Tissues from the interscapular, subcutaneous abdominal, and intraperitoneal regions were collected (Figure 3). When combined, total

Figure 2 Body weight values in untrained and trained spinal cord Tx mice. Values are reported as percentages of presurgery values. On the first week, values ranged between $76.8 \pm 1.2\%$ and $78.0 \pm 1.4\%$. On the fifth week, weight from untrained and trained mice reached $85.2 \pm 0.8\%$ and $80.4 \pm 1.9\%$, respectively. $**P < 0.01$, $***P < 0.001$.

Figure 3 Adipose tissues from the intraperitoneal (b), subcutaneous abdominal (c) and interscapular (d) regions were collected and weighed immediately after sacrifice. Differences between groups for the total adipose tissue changes (a) were attributed mainly to changes in intraperitoneal fat. $*P < 0.05$, $**P < 0.01$.

Figure 4 Whole muscle cross-sectional area (CSA) measurements of (a) soleus and (b) EDL. Specifically, soleus muscles CSA values from the untrained and trained Tx mice reached, 0.56 ± 0.04 and 0.45 ± 0.02 mm², respectively. In EDL, muscle CSA values were of 0.95 ± 0.07 and 0.73 ± 0.07 mm² (untrained and trained mice, respectively), which was significantly different. * $P < 0.05$.

adipose tissue values were found to be 29% lower ($P < 0.05$) in the trained than in the untrained group (Figure 3a). This difference was largely due to changes in intraperitoneal fat tissue, as no difference was observed in subcutaneous abdominal and interscapular fat tissues.

Hindlimb muscle size and composition

Morphometric analyses of soleus (extensor) and EDL (flexor) fibres were performed to assess the effects of non-assisted TT on hindlimb muscle atrophy, composition, and fibre type conversion levels. Both muscles displayed lower CSA values in the trained group compared with the untrained group, although significant differences ($P < 0.05$) were found only with EDL (Figure 4). To assess more specifically individual fibre type distribution (conversion) and CSA, we also studied immunohistochemically identified fibres from the soleus and EDL muscles. It is well known that muscle unloading typically induces fibre phenotype conversion. For both of these muscles, the relative fibre type distribution was comparable ($P > 0.05$) between groups (Figure 5), suggesting that preservation or reversal of fibre phenotype conversion did not occur in non-assisted TT Tx mice. In clear contrast though, large differences between groups were found with respect to muscle fibre CSA. For soleus and EDL, fibres of all phenotypes (type I, II, and hybrid) displayed CSA values in the trained Tx mice that were significantly lower than those in the untrained animals (Figure 6).

Discussion

This study revealed that Tx mice placed on a motorized treadmill with no other form of assistance (non-assisted TT) did not display greater locomotor performances than the untrained Tx mice. In fact, this study clearly showed that this basic form of training, under these conditions (15 min, 5 times per week), negatively affected body composition. Indeed, body weight, adipose tissue, whole muscle, and individual fibre CSA values were lower in trained than

Figure 5 Soleus and EDL individual fibre relative distribution. MHC type I (a), type II (b, d), and type I + II or hybrid (c, e) showed no difference between groups. Untrained and trained groups displayed over twofold higher increases in percentages of type I + II fibres (ranged between $60.3 \pm 6.5\%$ and $69.5 \pm 3.1\%$) than type I (ranged between $4.7 \pm 1.6\%$ and $7.0 \pm 3.0\%$) or type II fibres (ranged between $25.8 \pm 3.1\%$ and $33.5 \pm 5.3\%$). In contrast, for EDL where no type I fibre was found, there were more type II ($\sim 98.5\%$) than type I + II fibres ($\sim 1.5\%$).

untrained Tx mice, indicating greater muscular atrophy and less fat tissue in non-assisted TT Tx mice.

Effects of TT on locomotor recovery

It has been shown that TT, combined with various forms of stimulation (manual, robotic, and/or pharmacological assistance) leads to some recovery of locomotor functions. In this study, we determined to examine the effect of TT alone (without assistance) on locomotor recovery. This experimental protocol was driven by the idea that progressively occurring motor and locomotor-like movements displayed typically in untrained Tx animals,⁶⁻⁸ if combined with the stimulation provided by the moving treadmill belt, may suffice to further promote locomotor function recovery. However, as shown here, non-assisted TT did not enable the

Figure 6 Soleus and EDL individual fibre cross-sectional area (CSA) values. Immunolabelling for MHC type I (a), type II (b, d), and type I + II or hybrid (c, e). All non-assisted TT mice showed significantly lower fibre CSA than untrained mice. * $P < 0.05$, *** $P < 0.001$.

expression of large amplitude 'reflex' stepping movements in Tx mice, as AOB and ACOS scores remained lower than 1 and 4, respectively (see Figure 1). In fact, involuntary LMs were not even expressed after 5 weeks of training. The lack of differences in hindlimb movement recovery between TT and untrained mice may be supported by the idea that this form of basic training could have been inadequate. This is supported by data from Grau *et al.*¹³ who suggested, using the electric shock model in Tx rats, that appropriate stimulation is key to significant instrumental learning in the spinal cord. Earlier reports showed also, using different modes of training, that spinal learning is task dependent.^{14,15} Furthermore, some data suggest that the spinal cord itself needs to have reached a significant level of excitability for weight-bearing spinal stepping to be expressed.^{16,17} It was also shown that the amount of hindlimb loading (using robotic-controlled weight support assistance) as well as the amount of training (number of

steps) significantly alter stepping quality promoted by training.^{18,19} Other reasons why non-assisted TT mice did not display higher locomotor recovery values than untrained Tx mice may be associated with mild skin irritations that can potentially induce some pain. In Tx rats, there is evidence suggesting that central sensitization triggered by nociceptive stimuli can interfere with instrumental spinal learning of motor task.²⁰ In this study, none of the mice did show signs of hindlimb irritations. However, it cannot be ruled out that pain-related pathways may have been activated by non-visually detectable signs of damage or inflammation. Furthermore, it is intuitive to think that body degradation (decreased weight, muscle CSA, fat tissue) as seen in trained Tx mice is unlikely to promote locomotor function recovery. It may be also that the early timing of implementation for training was inappropriate. Indeed, in traumatic brain injury models, it was found that motor recovery was enhanced when training begins only 2–3 weeks after injury.²¹ As we began training these animals at 3 days post-Tx, this may have contributed to further body degradation as well as to the apparent lack of spinal learning and locomotor function recovery found here. This said, other SCI models reported that training starting immediately after partial SCI contributed to enhanced locomotor function recovery.²²

Effects of TT on body composition

Earlier work from our laboratory has shown important body weight losses after Tx in mice.⁹ One week after Tx, mice generally lose 25% of their body weight followed by a gradual re-increase in weight subsequently. In this study, untrained Tx mice showed comparable weight losses whereas, surprisingly, TT Tx mice underwent greater losses and subsequent lower weight re-increases. Given the results obtained with adipose tissue changes in the trained animals, it is possible to conclude that some of their body weight losses were due to a significant decrease of adipose tissue from the intraperitoneal area. This could have been induced by training, which is known generally to decrease body fat levels.²³ This greater weight loss in trained mice was also associated with muscular atrophy, as TT Tx animals displayed further muscle atrophy than the untrained animals (as seen with *soleus* and *EDL* lower muscle CSA values). This may perhaps be taken as preliminary signs of overtraining or other stress-related factors. However, another factor of stress could have potentially contributed also to weight loss. It is the use of a harness specifically in trained animals to maintain them (rostral-caudal position) on the treadmill. As untrained animals did not need harnesses, we cannot exclude the possibility that harnesses were somehow associated, as other types of contention devices,²⁴ to some stress-related weight loss in the trained TT mice.

Conclusion

This study supports the idea that training-dependent spinal learning and related sublesional plasticity in the lumbar spinal cord of completely low-thoracic spinal cord-transected mammals does not simply depend on

training in its most basic form (non-assisted). In fact, together with results from other studies, our findings strongly suggest that spinal learning and recovery critically depend on various training modalities including type, quality, quantity and, perhaps, onset (post-injury).

Conflict of interest

The authors declare no conflict of interest.

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