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Adaptations of the Motor System in Animal Models of Spinal Cord Injury and Disuse

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1. Introduction

More than 1.3 million patients are currently living with a spinal cord injury (SCI) in North America (Reeve Foundation). There is no cure for SCI although recent advances in acute care interventions (*e.g.*, removal of bone fragments, decompression, anti-inflammatory drugs) have increased survival and reduced neurological dysfunctions (Baptiste & Fehlings, 2007). Accordingly with the *American Spinal Injury Association* (ASIA) guideline, tetraplegic (cervical lesions) and paraplegic (thoracic lesions or below) patients are classified either as ASIA-A, ASIA-B, ASIA-C or ASIA-D (see Table 1). Quadriplegia also called tetraplegia is when a person has a SCI within the cervical area which results in paralysis of all four limbs. In addition to the arms and legs being paralyzed, the abdominal and chest muscles will also be affected which result in weakened breathing and the inability to properly cough and clear the chest. Paraplegia is when the level of injury occurs at the thoracic level or lower. Although they typically experience leg movement and abdomen problems, paraplegics can use their arms and hands.

ASIA-A	No voluntary motor control and no sensation below injury level
ASIA-B	No voluntary motor control, some sensations below injury level
ASIA-C	Some motor control (< grade 3) and some sensations below injury level
ASIA-D	Some motor control (> grade 3) and some sensations below injury level
ASIA-E	Normal voluntary motor control and sensation below injury level

Table 1. Classification of SCI severity

Advanced rehabilitation 'activity-based' strategies such as body weight-supported treadmill training or BWSTT (leg movements generated passively by manual assistance from therapists) and functional electrical stimulation (FES)-biking are increasingly used especially with motor-incomplete (ASIA-C and ASIA-D) patients. Indeed, given that spared descending pathways exist and, thus, some voluntary motor control remains in these subclasses of patients, it becomes possible to further increase voluntary ambulation using BWSTT training (Dobkin et al., 2006; Hicks & Ginis, 2008). However, motor system, metabolic outcomes or health benefits associated with these approaches remain unclear (Hicks & Ginis, 2008; Duffell et al., 2009). In turn, chronic SCI patients classified as motor-complete (ASIA-A & ASIA-B) generally experience greater health problems often referred to as 'secondary complications' that are

associated with significant changes of the motor, locomotor, skeletal, cardiovascular, circulatory and hematologic problems (Huang & DeVivo, 1990; Bauman, 1999; Riegger et al., 2009; Rouleau et al., 2010,2011; Spungen, 2003). No safe, effective and regulatory agency-approved treatments against these chronic problems exist yet.

In the last few years, great therapeutic hopes for motor-complete SCI patients (ASIA-A and ASIA-B) have emerged from physical activity-based studies performed in adult complete paraplegic cats showing that basic locomotor movements (i.e., hindlimb stepping) can be restored partially with regular treadmill training, weight support, passively generated movement and administration (*i.t.* or *i.p.*) of drugs such as clonidine, an alpha-2 noradrenergic agonist (Barbeau et al., 1993; Chau et al., 1998). Regular assisted training combined with clonidine and a few other monoaminergic drugs have even induced, in some cases, episodes of overground walking with Canadian crutches in previously wheelchair-bound SCI patients (Barbeau et al., 1998). Clear evidence suggests that clonidine can, in fact, facilitate walking through reflex-mediated actions by decreasing spinal reflexes and hence spasticity and clonus (Waindberg et al., 1990; Remy-Neris et al., 1999). Unfortunately, at doses used for locomotor enhancement in some paraplegic patients, clonidine was also found to induce severe side effects even if given *i.t.* (i.e., bradycardia, sedation, hypotension - pers.com. Dr. Hugues Barbeau). It had therefore become imperative to identify other pharmacological strategies and compounds that could safely and more specifically enhance locomotor function recovery or reduce motor system changes and problems in chronic and, if possible, in motor-complete SCI patients (i.e., for whom BWSTT or other comparable approach does not yield beneficial effects). The identification of therapeutic approaches aimed at reducing or preventing motor system alterations in SCI or comparable chronic conditions (e.g., burn patients, AIDS patient with cachexia, etc.) would benefit both patients and health care systems for which associated costs are significant (approximately $100,000 - 400,000 per year/patient, Table 2).

Initial hospitalization	$140,000
1st year paraplegics	$152,000
1st year tetraplegics	$417,000
Averaged life time paraplegics	$428,000
Averaged life time tetraplegics	$1,350,000

Table 2. Costs of SCI in U.S. dollars (source: http://www.sci-info-pages.com/facts.html)

Although, these therapeutic approaches may not be designed to repair or cure SCI, they would nonetheless contribute at preventing (in acutely injured patients), reducing or reversing (in chronic SCI patients) secondary complications associated with motor system changes and significantly reduced physical activity (see also *section 2.6*).

2. Motor system changes associated with spinal cord injury and disuse

The motor system may be divided into several organs and structures. There is the central nervous system (CNS) that comprises the brain and the spinal cord. Its one hundred billion neurons are involved in motor and sensory functions (Kandel et al., 2000). The brain consists of the pyramidal and extrapyramidal system specifically associated with voluntary motor control. These brain structures constitute the main command centres that control voluntary

muscular contraction. Most of their neuronal commands are sent to neurons and motoneurons located in the spinal cord where sensory motor integration and final motor commands sent to muscle are organized for proper induction of coordinated movements.
In contrast, locomotion and other rhythmic and partially involuntary motor behaviours are largely controlled by signals and neuronal commands generated in the brainstem and spinal cord. In fact, complex neuronal circuits located in these non-cortical areas of the CNS are known to be capable of generating motor functions even in absence of descending inputs from cortical areas and other brain regions (Guertin & Steuer, 2009; Guertin, 2010). In fact, locomotion, micturition, ejaculation, scratching, erection, and respiration are amongst the motor behaviours that are mainly controlled by spinal cord and brainstem circuits (see Fig.1).

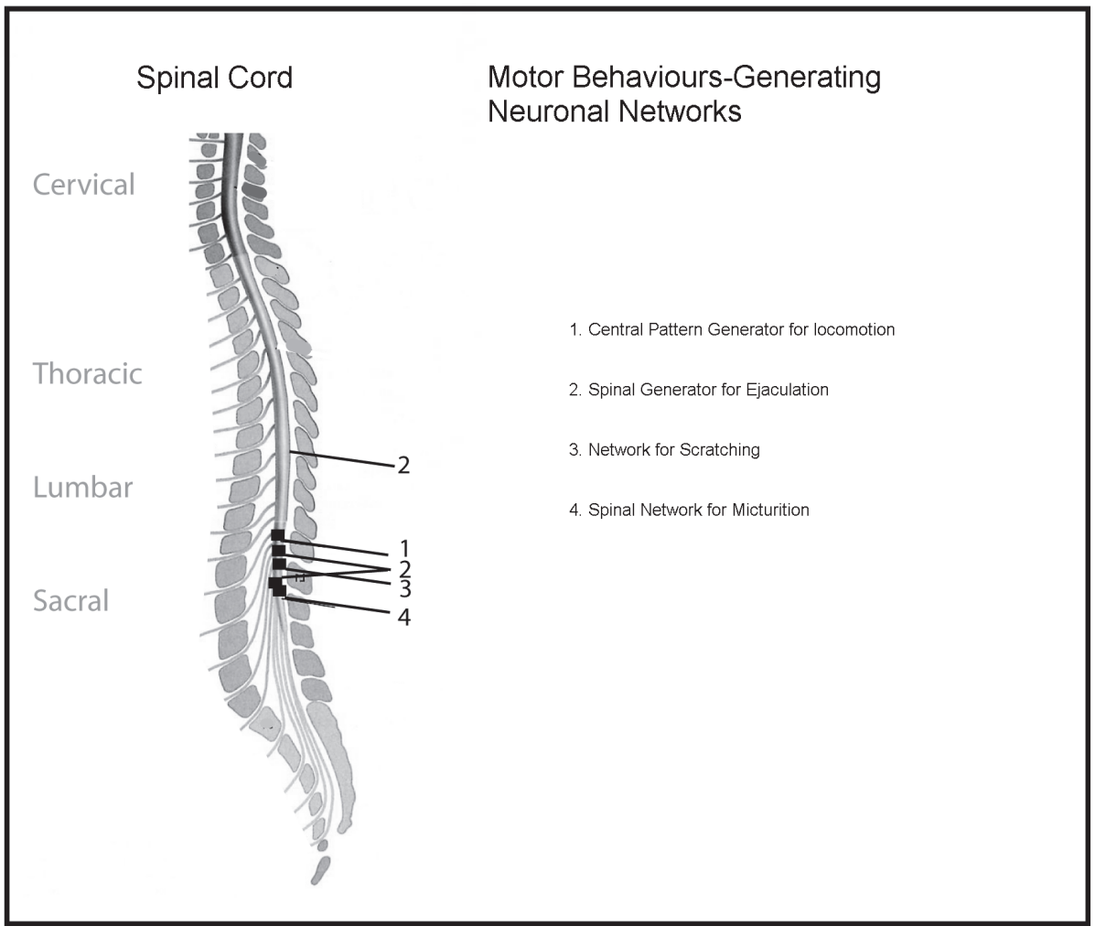

Fig. 1. Neuronal networks in the spinal cord that control, brain-independently, complex motor behaviours. Respiration (not shown here) is also largely controlled by non-cortical structures including the brainstem (e.g., Pre-Bötzinger complex).

Thus, the CNS controls either directly or indirectly the muscular systems. Although some types of muscles such as the cardiac and smooth muscles are considered controlled by the autonomic nervous system and hormones, the striated skeletal muscle system is directly controlled by the CNS. This is the main reason why after SCI, an immediate and irreversible

loss of sensory and voluntary motor control is found. This said, increasing evidence suggests that functions controlled mainly by the spinal cord can nonetheless be elicited despite SCI using specific pharmacological or electrical approaches (see *section 2.6*).
In humans, the striated skeletal muscle system comprises approximately 650 muscles. It is formed by different fiber types and properties including slow-twitch fibers (type I) and relatively fast to very fast-twitch fibers (IIa, IIb and IIx)(Table 3). The main action of skeletal muscles in motor control is to allow movement execution. Almost all skeletal muscles either originate or insert on the skeleton. When a muscle moves a portion of the skeleton, that movement results into flexion, extension, adduction, abduction, etc. (Martini & Nath, 2011).
The human skeleton consists of both fused and individual bones supported by ligaments, tendons, muscles and cartilage. Among several functions, it primarily serves as a scaffold for movements controlled by the CNS and muscles as mentioned earlier. The biggest bone in the body is the femur which is also the main skeletal structure affected after chronic SCI, disuse or immobilization. Finally, energy and other metabolic processes involved in motor control and movements largely depend upon the integrity of the circulatory and hematologic systems – i.e., distribution of erythrocytes and oxygen to muscles.

Type I	Slow twitch, high fatigue resistant, high oxidative, low glycolytic
Type IIa	Moderately fast twitch, fairly high fatigue resistant, high oxidative, high glycolytic
Type IIx	Fast twitch, intermediate fatigue resistant, intermediate oxidative, high glycolytic
Type IIb	Very fast twitch, low fatigue resistant, low oxidative, high glycolytic

Table 3. Muscle fiber types and main properties

All in all, the main components of the motor system described above are changed and altered specifically in patients with complete and motor-complete SCI as well as in patients suffering of chronic disuse and immobilization (burn patients, AIDS patients, some patients with cardiac or pulmonary problems)(Huang & DeVivo, 1990; Bauman, 1999; Riegger et al., 2009; Rouleau et al., 2010,2011; Spungen, 2003; Lainscak et al., 2007).

2.1 Spinal cord-transected murine model of complete paraplegia

In brief, all experimental procedures were conducted in accordance with the Canadian Council on Animal Care guidelines. Mice were generally housed 4-5 animals per cage in a controlled-temperature environment (22 ± 3°C), maintained under a 12h light:dark cycle with free access to water and food. Before surgery, pre-operative care was provided 30 minutes prior to anesthesia. It included subcutaneous injections of 1.0 ml of lactate-Ringer's solution, 0.1 mg/kg of buprenorphine, and 5 mg/kg of Baytril, an antibiotic. Initially, complete anesthesia was conducted using 2.5% isoflurane in a cage of induction. Anesthetized animals were then shaved dorsally (2-cm) from the mid-dorsal area to the neck. Then, each animal was maintained under complete anesthesia using a specially adapted facial mask delivering directly 2.5% isoflurane to the animal. The shaved area was cleaned with 70 % (*v/v*) isopropyl alcohol and, then with 10% (*v/v*) povidone-iodine solution whereas eyes are protected from dryness using ocular lubricant. The first skin incision was made using fine scissors over 2 cm along the midline from the mid-dorsal area to the neck. Fat tissues (interscapularis fat) were cut and removed to expose the high-thoracic segments.

The latissimus dorsi fascia was cut bilaterally to expose the vertebral column between the 4th and 6th thoracic vertebrae. Curved forceps were then used to tightly hold that area of the vertebral column that was cleaned from fascia and muscles to improve the grip. Forceps were also used to gently lift that part of the vertebral column which, once bent upward, eased the transection of the intervertebral ligaments between the 9th and the 10th vertebrae. This last part was critical to offer an open access for insertion of extra fine microscissors between the 9th and 10th thoracic vertebrae for the complete transection of the spinal cord. Then, the inner vertebral walls were explored and entirely, but delicately, scraped three or four times with fine scissors tips in order to sever any small fibres which had not been previously cut. It is important to scrape carefully to avoid severing the intervertebral ligaments located ventrally (i.e., if severed, it may lead to a dislocation of the vertebral column and corresponding bleeding). Throughout the transection procedures, bleeding although minor was controlled by applying pressure with cotton tips. The interscapularis fat was carefully replaced and the opened skin area was closed using 3 or 4 Michel suture clips. Michel suture clips are generally faster to install and are normally associated with less infection problems than typical suture threads. This overall surgical procedure was conducted under aseptic conditions using only perfectly cleaned materials and surgical tools – materials were previously autoclaved and tools were continuously sterilized throughout the procedure using a portable quartz beads-sterilizer).

Once the surgical procedures completed, anesthesia was interrupted and mice were placed in a large cage equipped with a heating pad placed underneath. It is critically important to use only minimal heating intensity (35°C) to avoid rapid dehydration, heat shock and death during the recovery period. Generally, the animals recovered completely within 15 min although we normally left them on the heating pad overnight with free access to food and water. The recovery procedure was found to be critical to ensure a high percentage of survival post-surgery (typically around 95% if everything is performed as described). The next day, the animals were replaced in their initial cage with their initial cage mates in order to reduce potential aggressions and fights.

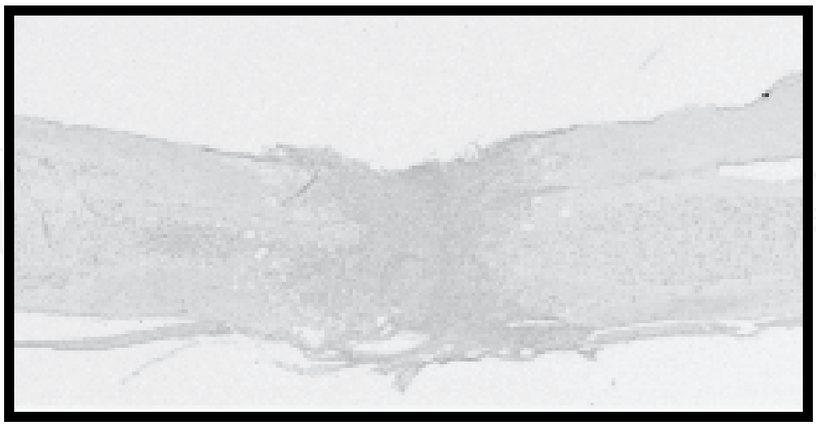

Fig. 2. Spinal cord histology. Luxol blue and Cresyl violet staining of a longitudinal section of the spinal cord from a non-laminectomized spinal cord-transected mouse one week post-surgery.

Postoperative care, provided a few hours after surgery as well as every day for the next 4 days, included injections of lactate-Ringer's solution (2 x 1 ml/day, s.c.), buprenorphine (2 x

0.1 mg/kg/day, s.c.), and Baytril (5 mg/kg/day). Bladders were also manually emptied twice a day until a spontaneous return of some micturition reflexes. For voiding, the bladder was gently squeezed between the thumb (side of the bladder) and two fingers (e.g., the index and one other finger placed the other side of the bladder). This maneuver requires time and experience. In male mice, it was specifically challenging since, in addition, penises have to be maintained against a paper towel throughout the maneuver to improve successful voiding (i.e., it appeared to contribute, perhaps via capillary action, to urine expulsion outside the urinary tract). The belly and sexual organ were cleaned daily using paper towels and chlorhexidine gluconate solution (0.05 % *v/v*) to prevent urinary infection. Normally, with these procedures, mice that survived the firsts 24 hours, remained relatively healthy for a long period of time (i.e., several months). Finally, Michel suture clips were removed after 10 or 14 days post-surgery. Cages were cleaned regularly (ideally, cages needed to be changed every 3 or 4 days) and mice were cleaned, as described above, on a daily basis to prevent urinary tract infection. All in all, once anesthetized, this surgical procedure took no longer than five minutes whereas another 5-10 minutes was typically required for animals to recover from anesthesia.

This approach led to complete paraplegia (Figs. 2 & 3) – an immediate and irreversible loss of sensory and voluntary motor control below injury level (low-thoracic level). Although, it is possible to maintain these animals relatively healthy for severaonmonths post-spinal transection, a number of neuronal, muscular, skeletal, vascular, and hematologic changes were rapidly displayed. A detailed characterization of these changes is presented in the following subsections.

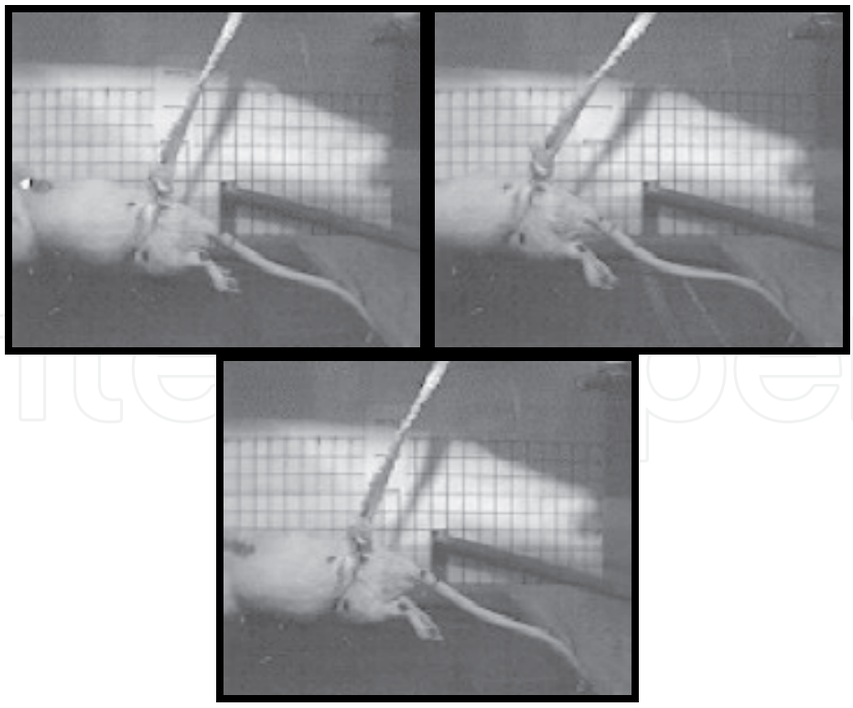

Fig. 3. Video images of a paraplegic mouse placed on a treadmill. A complete loss of hindlimb movement is encountered immediately following the spinal cord transection.

2.2 Disuse-related bone loss, biomechanical property changes and factures

Nearly all SCI individuals experience a drastic loss of bone mineral content (up to 30% at the femoral level) leading to a marked increase of fracture incidence within one year after injury (Ragnarsson & Sell, 1981; Garland et al., 1992; Wilmet et al., 1995; Lazo et al., 2001; Sabo et al., 2001). Although, the basic mechanisms underlying osteoporosis in post-menopausal women have been extensively studied, those involved in chronic immobilization and disuse have received considerably less attention. In animal models of disuse, traditionally in rats, hindlimb immobilization has been found to induce a drastic and sudden loss of femoral bone tissue suggesting that different mechanisms may be involved in disuse vs. estrogen-deficiency/aging-related osteoporosis (Bagi & Miller, 1994). For instance, a 10-30% decrease of cancellous bone has been reported within only a few weeks in the ipsilateral femur of rats that had their hindlimbs immobilized with a cast or an elastic bandage (Ito et al., 1994; Ma et al., 1995; Mosekilde et al., 2000). Comparable changes have been found in other models of disuse such as in tail-suspended rats (Wronski et al., 1989). Some of these disuse-related changes are believed to be mediated by both an increase of osteoclastic bone resorption and a decrease of osteoblastic bone formation (Rantakokko et al., 1999). On the other hand, growing evidence suggests that several factors other than mechanical unloading *per se* can influence the combination of cellular and molecular mechanisms underlying disuse-related bone loss. For instance, in the case of disuse induced by a lesion of the sciatic nerve, the loss of bone tissue in rats is caused partly by a disruption of the neurogenic innervation of the bone marrow (Zeng et al., 1996). Moreover, differential tissue- and biomarker-specific changes have been reported in the tail-suspension vs. sciatic nerve lesion models (Hanson et al., 2005). In the case of microgravity, bone tissue changes have been attributed mainly to a marked decrease of osteblast formation in young adult rats (Matsumoto et al., 1998). Taken together, those data suggest that the combination of various factors specific to each model and condition of disuse may dictate, to some extent, the different sets of molecular mechanisms involved in demineralization and bone loss.

Here, we characterized some of the main structural and functional adaptive changes occurring specifically within a few weeks in adult spinal cord transected mice. In brief, within a few weeks post-transection, paraplegic mice were weighed, sacrificed and the femoral bones dissected and cleaned of soft tissue. The femurs were wrapped in saline-soaked gauze and frozen at -20 degrees C in sealed vials until testing. For histomorphometry, the left femoral bones were fixed with paraformaldehyde, decalcified, paraffin embedded and stained with acid fuchine using the Masson's trichrome procedures. Histomorphometric analyses were performed with a NOVA Prime, Biioquant's image analysis system (R&M Biometric, Nashville, TN) for primary bone morphometric parameters. Three bone slices at the metaphyseal level were analyzed. For densitometry, measurements were made with the rigth femoral bones of sham and paraplegic mice. Bone mineral content (BMC, g) from the femora of each animal was assessed using dual-energy X-ray absoptiometry (DEXA, model Piximus II, Lunar Corporation, Madison WI, for details, see Kolta S, De Vernejoul M.C. et al. 2003). Bone mineral density (BMD, g/cm^2) was calculated as BMC divided by projected bone area. Each femur was scanned separately for whole bone analysis. For biomechanical assessment, on the day of testing, the femur was slowly (4 hours) tawed at room temperature. They were placed horizontally on the three-point bending device (MTS, Eden Prairie, MN). The mechanical resistance to failure was tested using a servo-controlled electromechanical system (Intron, Instron, Canton, MA). The crosshead speed for all tests was 10 mm/sec until the femur fractured. Displacement and

load values were acquired at 100 Hz, recorded and stored on PC. Off-line data analyses were performed to calculate maximal strength (N), stiffness (slope of the linear part of the curve to failure, N/cm), and elasticity deformation (N). Bones were kept wet throughout testing and used for histomorphometrical testing (proximal end).
All histomorphometric measurements and analyses were made from the metaphyseal area of the left femora. The cancellous bone volume was found to decrease by 25.2% in paraplegic mice (within 1 month post-transection) compared with control (non-paraplegic). The average trabecular bone thickness was found to decrease by 10.65%. The thickness was initially of 25.55 micron in the control groups and of only 22.83 micron in the paraplegic group. The number of trabecular bone areas decreased rapidly also after injury. In the control group, the average trabecular number was 3.38 nbr/mm2 whereas in the paraplegic group, it decreased to only 2.89 nbr/mm2 representing a 14.50% decrease. On the other hand, the trabecular separation, defined as the space between trabecular bone areas, increased after injury. In fact, on average, the trabecular separation increased by 24.03% within 1 month post-SCI (Picard et al., 2008).
The bone mineral density (BMD) of the left femora measured by dual-energy X-ray absorptiometry (DEXA) significantly changed after injury. The BMD was just below 0.09 g/cm2 in control and of 0.0731 in paraplegic mice. Bone mineral content (BMC) also proportionally decreased after injury (see *sections 2.6* and *2.7* for further details).
The maximum force in N required for the crosshead to fracture the right femora at the mid-diaphyseal level was decreased by 13% on average within a few weeks post-transection (Fig.4D). The stiffness in N/mm was also reduced after injury with average values of 57.23 and 51.08 in control and paraplegic groups, respectively, representing a 10.8% decrease (Fig.4B). The elastic force decreased also by approximately 15% in early spinal transected mice compared with control (Fig.4C).

2.3 Muscular atrophy, muscle fiber-type conversion, and strength loss

It is well-documented in various rat models that the contractile properties of slow twitch muscles change into more fast-like muscles after chronic spinalization (Roy et al., 1991; Talmadge, 2000). Hindlimb extensor muscles such as soleus (SOL) typically exhibit extended atrophy (e.g., up to 50%) and type I to type II muscle fiber conversion following spinalization in rats (Krikorian et al., 1982; Lieber et al., 1986 a,b; Midrio et al., 1988; Talmadge et al., 1995). Contraction and relaxation times as well as maximal tetanic force (Po) and maximal twitch force (Pt) have also been found to be importantly decreased in rat SOL several months after spinalization (Davey et al., 1981; Talmadge et al., 2002).
Evidence from other models of inactivity and immobilization suggests that some of these changes, in fact, are induced very early after inactivity and reduced muscular activity and loading. For example, a 10% loss of body weight (Pierotti et al., 1990) accompanied by a 40-50% decrease of SOL mass, TPT and 1/2 RT (Frenette et al., 2002) and a rapid reduction in slow myofibril proteins (Thomason et al., 1987) have been reported after 1-2 weeks of hindlimb suspension in rats. Comparable results have been found within less than 2 weeks in rats after spinal cord isolation (i.e., de-afferented and spinalized, Grossman et al., 1998) or in microgravity (Fitts et al., 2001). In addition, a 40% reduction of SOL cross sectional area has been found only 10 days post-spinal cord transection in rats (Dupont-Versteegden et al., 1999). The possibility that other early changes may occur after spinal cord transection is largely unexplored.

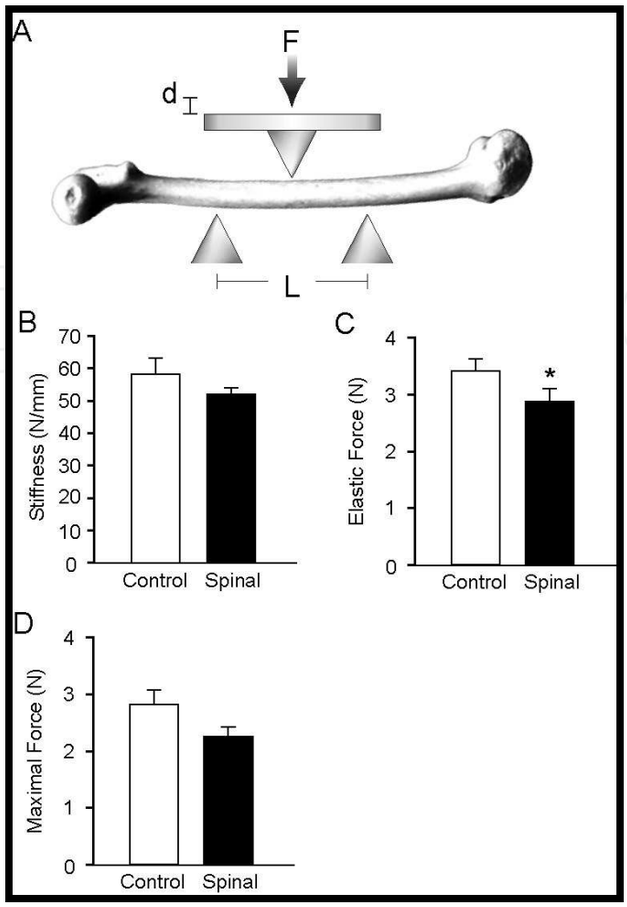

Fig. 4. Bone mechanical properties. Two-point bending test (A) revealed decreased femoral stiffness (B), elasticity (C) and maximal force (D) in untrained spinal transected mice (spinal, black) versus control (intact animals, white)(unpublished data).

Here, we characterized some of the earliest adaptations in gross anatomy and muscle properties at only 7 days following spinal cord transection in adult mice (Landry et al., 2004). In brief, whole body weight was measured daily during the first week post-spinalization. After dissection of SOL for functional tests in vitro (see section below), animals were sacrificed with pentobarbital overdose. Forelimbs and hindlimbs were surgically removed just below the shoulder and the hip joints respectively. Paws as well as all parts of the pectoral and back muscles attached to the forelimbs were removed. Tests included weight measurement of the left forelimb and hindlimb as well as of the right SOL. To further assess muscle atrophy, limbs were weighed in air and in water to measure volume changes. Volume was calculated as follows with a volumic mass of 0.998 for water at room temperature (22°C):

$$\text{Volume} = (\text{weight in air} - \text{weight in water}) \times (\text{volumic mass of water})$$

For measurement of contractile properties, we anesthetized animals with pentobarbital sodium (50 mg/kg). The right SOL was carefully dissected and incubated in fully oxygenated Krebs-Ringer bicarbonate buffer solution maintained at 25°C and supplemented

with glucose (2 mg/ml). *In vitro* measurement of muscle contractile properties was performed as described elsewhere (Côté et al., 1997). In brief, one tendon was attached to a rigid support at the bottom of the bath, and the other end was connected to an isometric force transducer (Grass FT-03) through a stainless steel hook. An initial resting period of 15 min was allowed before testing. Muscles were carefully stretched to their optimal length, defined as the length at which maximal isometric twitch tension is produced. One single twitch contraction was elicited and the following measurements were obtained: maximum twitch tension (Pt), time-to-peak tension (TPT), and one-half relaxation time (1/2 RT). After measurement of twitch parameters, muscles were stimulated for 1 s at frequencies of 10, 20, 35, 50, 80, and 100 Hz to determine maximal tetanic tension (Po, N/cm^2). The value used for muscle density was 1.062 g/cm (Koh & Brooks, 2001) and the ratio of fiber length to muscle length used was 0.71 (Brooks & Faulkner, 1988).

We reported that paraplegic mice at 7 days post-surgery encountered a drastic loss in body weight (Landry et al., 2004). On average, a 24% decrease in weight was found at 7 days post-spinalization. A similar loss was found in another group of paraplegic mice that received instead daily injection of lactate-Ringer's solution (2 ml/day, s.c.) during the first week post-spinalization suggesting that dehydration did not contribute to weight loss.

The specific weight of individual body parts was also examined in paraplegic mice. In intact mice, the average weight of forelimbs and hindlimbs was 436 and 1239 mg respectively. At 7 days post-spinalization, hindlimb weight decreased by 28% compared to intact mice. Interestingly, a 21% reduction in the forelimbs of paraplegic mice was also observed during the same period of time. Relative to body weight, the loss observed in hindlimbs was greater than the one in forelimbs. Similar reductions in volume were found respectively in hindlimbs and forelimbs.

Regarding properties, for soleus mass displayed significantly lower values (-32%) in untrained paraplegic mice at 7 days post-spinalization compared with intact animals. A 33% decrease of Po was measured at 7 days post-spinalization. The absolute tension generated at different frequencies of stimulation showed mainly that SOL force was reduced in paraplegic mice compared to control at stimulation frequencies above 35 Hz. On the other hand, maximal tension was reached at lower stimulation frequencies for paraplegics compared to control.

Our data showed also in soleus a change toward faster-type properties in the first few days post-immobilization (transection).The surprising initial and rapid conversion to slower contractile properties at 7 days post-spinalization is further supported by changes found in contraction and relaxation times (TPT and 1/2 RT respectively). TPT became slower (i.e., increased time of contraction) by 21% at 7 days compared to control. Similar changes were observed with 1/2 RT which became slower (i.e., increased time of relaxation) by 48% at 7 days post-spinalisation.

As mentioned above, it is well-known that there is an important shift in fiber phenotype distribution a few weeks post-SCI even more so in soleus. Generally, slow fibers tend to change for a faster phenotype after 2 weeks post-spinal cord transection. After spinal cord transection, 50-55% of the slow type fibers showed important fiber type conversion, shifting to a hybrid isoform (faster phenotype) whereas fiber type conversion was not observed in another hindlimb muscle, EDL, often classified as a purely fast-twitch muscle (Table 4).

Fiber type %	Non-TX	TX untrained
EDL type II	98.7 ± 0.3	98.7 ± 0.6
EDL hybrid	1.3 ± 0.3	1.3 ± 0.6
SOL type I	54.6 ± 2.6	2.9 ± 1.5
SOL type II	45.4 ± 2.6	46.5 ± 2.5
SOL hybrid	0 ± 0	50.7 ± 3.3

Table 4. Fiber type conversion in normal (non-TX) and untrained paraplegic (TX untrained)(unpublished data).

2.4 Circulatory and hematologic changes associated with increased risks of blood cloth formation and deep vein thrombosis

Among the cardiovascular and pulmonary problems associated with SCI, deep venous thrombosis (DVT) is one of the most serious complications in patients that survive to the accident. Indeed, DVT constitutes the third most common cause of death in SCI patients (Waring & Karunas, 1991; DeVivo, 1999) and, despite prophylaxic methods (e.g. anticoagulant administration), a significant proportion of SCI patients will develop a pulmonary embolism caused by DVT (Deep et al., 2001).

Complete paraplegic and tetraplegic individuals are particularly vulnerable given that spasticity, typically found in incomplete SCI patients, may decrease the risks of DVT formation (Green et al., 2003). Generally, DVT formation is attributed to a combination of factors including also venous stasis, venous injury, and hypercoagulability. In turn, these factors facilitate platelet, LDL-cholesterol, and leukocyte adhesion, procoagulant system activation, and hence, thrombin generation. Although, few animal models of DVT and/or pulmonary embolism exist (Frisbie, 2005), none have been developed to study these complications after SCI which may explain why the specific mechanisms of DVT formation in paralytics remain poorly understood.

Here, we characterized, in spinal cord transected (Tx) mice, some of the physiological changes occurring after SCI that could possibly contribute to DVT formation (Rouleau & Guertin, 2007; Rouleau et al., 2007). Specifically, we characterized also alterations of deep vein diameter in the hindlimbs of Tx mice because venous distensibility and capacity changes may participate to DVT formation (Miranda & Hassouna, 2000). We took advantage of this experimental model to measure with great precision (µm), using *in vivo* fluorescence confocal microscopy, changes in diameter of the femoral and saphenous veins. All tests were performed weekly during one month post-Tx since risks of DVT in patients have been reported to increase by several folds specifically during the first few weeks after SCI (DeVivo et al., 1999).

In brief, we put the tail on a heated cushion to dilate the tail vein 10 min before injection. Then 200 µl of 5 mg/ml fluorescein isothiocyanate-dextran (FD-40) (Sigma, St-Louis, MO) dilute in injectable endotoxin-free dPBS (Sigma), was injected intravenously into the tail vein. Animals were killed by CO_2 asphyxiation around 10 min after injection. The skin was cut to access to the femoral and saphenous veins. Microscope observation and measurement were performed with an Olympus BX61WI confocal system and analysed with Fluoview 300 (Carsen group, Markhan, Canada).

For hematologic data, peripheral blood was collected at various times post-transection by cardiac puncture. Each blood sample was analyzed for platelet quantification with a CELL-DYN 3700® automatic blood cell analyzer (CD3700)(Abbott Laboratories, North Chicago, IL).

We found by measuring deep vein diameter using *in vivo* fluorescent confocal microscopy techniques that the femoral and saphenous veins drastically increased in size after SCI compared with intact mice. This is illustrated in Fig. 5 showing typical examples from a control (left panel) and from a paraplegic mouse at 3 week post-TX (right panel). We can clearly distinguish that the femoral vein drastically increased in size post-transection compared with control. In fact, average values calculated for the femoral vein revealed, for control animals, an average diameter of 319 μm augmented to 458 μm at 3 weeks post-surgery (Fig. 5). Comparable increases of saphenous vein diameter were found after spinal transection (338 μm in control vs 433 in paraplegics)

The hematologic data revealed mild anemia that occurs as early as at 7 days post-transection. Specifically, average counts of erythrocytes (10.11 x 10^{12} /L in control mice) decreased to values ranging from 9.91 to 9.54 x 10^{12} /L in paraplegic mice. Hemoglobin concentrations were decreased from 164.9 ± 2.8 g/L in controls to 153.3 g/L in paraplegic mice. Decreased hematocrit levels were also found in paraplegic mice (range from 0.46 ± 0.01 to 0.44 ± 0.01 L/L) compared with controls (0.48 ± 0.01 L/L, Fig. 1*C).* In turn, platelet counts remained unchanged after spinal transection with levels of 16.76 ± 0.80 x 10^{11} /L in controls and 17.73 ± 0.75 in paraplegic mice (Rouleau et al., 2007).

2.5 Complex spinal cord network that controls locomotor rhythm generation

The Central Pattern Generator (CPG) for locomotion is a network of neurons located in the lumbar area of the spinal cord that is capable of producing the basic commands for stepping even when isolated from supraspinal and sensory inputs (Grillner & Zangger, 1979, see also Guertin, 2010). Early evidence of a CPG emerged a century ago from the pioneer work of Sherrington (1910) and Brown (1914). In the 70s, low-thoracic spinalized rabbits and cats were used to show that an endogenous release of 5-HT induced by 5-HTP can generate fictive locomotor-like rhythms in the spinal cord (recorded with electroneurograms) of acute spinal cord-transected animals (Viala & Buser, 1971) or increase extensor muscle activity in regularly treadmill-trained and sensory-stimulated spinal animals (Barbeau & Rossignol, 1990, 1991). A clear demonstration of its existence was provided in 1979 by Grillner who could induce, with L-DOPA, locomotor-like neural activity in the motor nerves of completely de-afferented, curarized, and spinal cord-transected cats (Grillner & Zangger, 1979). In rats, the CPG was found, with activity-dependent labeling (e.g., c-fos), to be located mainly in rostral segments of the lumbar spinal cord (Cina & Hochman, 2000). Comparable results were found in mice where CPG activity was found to originate from lumbar segments with critical elements in L1-L2 (Nishimaru et al., 2000).

In the 80s and 90s, *in vitro* isolated spinal cord preparations were extensively used to study the pharmacological control of CPG neurons at the system and cellular levels. Initially discovered in lampreys, bath application of N-methyl-D-asparate (NMDA) was found to induce rhythmic activity (recorded from ventral roots) that shared locomotor characteristics – called 'fictive locomotion'. This provided evidence that even a perfectly isolated CPG can be activated with drugs. Then, neonatal rat and mouse spinal cord isolated preparations were developed and used also to study *in vitro* drug-induced CPG-mediated locomotor-like neurographic activity. These studies have essentially revealed that bath-applied combinations of drugs such as NMDA, 5-HT and DA can best induce robust fictive locomotor-like rhythms in the mammalian isolated spinal cord (Cazalets et al., 1992;

Kjaerulff & Kiehn, 1994). Although, these studies have revealed that several families of drugs need to be combined for enhanced CPG activation, most of the compounds used *in vitro* were synthetic neurotransmitters (e.g. 5-HT and DA) which, unfortunately, do not constitute good candidates for drug treatments because of poor selectivity (e.g. activation of all receptor subtypes) and incapacity to cross the BBB.

In humans, evidence of a CPG was provided after showing that 'automatic' (involuntary) stepping-like movements could be triggered spontaneously under certain conditions or by epidural stimulation at the L2 level in SCI patients confined to a bed (Dimitrijevic et al., 1998). Although a completely isolated CPG can produce locomotor rhythms, sensory inputs (*i.e.* muscle proprioception, vision, etc.) were found to provide useful feedback signals to the CPG that can re-enforce muscle contraction and adapt stepping to external disturbances (Rossignol & Dubuc, 1994). However, none of these studies have identified a full CPG-activating drug that can, upon systemic administration, potently elicit acutely powerful weight-bearing stepping in complete SCI animals with no other stimulation/assistance (e.g., non-therapetically relevant tail pinching or other sensory stimulation).

Changes post-spinal cord transection were also found in sublesionally-located neurons (below injury level). Since most of these changes were found in neurons located in upper lumbar segments of the spinal cord, they were postulated to correspond with changes in CPG neuron candidates. Immediate early genes (IEGs) constitute a large family of genes well-known as early regulators of cell growth, differentiation signals, learning and memory. We reported in low-thoracic spinal cord-transected mice, that IEGs such as *c-fos* and *nor-1* expression respectively increased and decreased within a few days in the segments L1-L2, specifically in the dorsal horn and intermediate zone areas (Landry et al., 2006). Changes in the lumbar spinal cord of rostrally-transected animals were of special interest since some of these segments (e.g., L1-L2 in mice) were shown to contain critical central pattern generator (CPG) elements as mentioned earlier. Given that IEGs are better known for their role in CNS development and plasticity, spontaneous changes of IEG expression (i.e., specifically *c-fos* and *nor-1*) in L1-L2 segments may be considered as among the first sublesional cellular events associated with altered cellular functions and properties post-SCI. This said, some of these changes may be associated also with other phenomena than plasticity or reorganization of spinal motor and locomotor networks. For instance, *c-fos* and nor-1 were used as markers in experimental models of pain and transient global ischemia suggesting a role in several functions (see Landry et al., 2006a).

Other key elements including transmembranal receptors may be considered good candidates for plasticity and reorganization of motor and locomotor networks located sublesionally following a spinal cord-transection (and probably to some extent also after partial injuries). For instance, we found using *in situ* hybridization increased 5-HT_{1A} mRNA levels in L1-L2 segments in 5-HT_7-deficient mice compared with wild-types (Landry et al., 2006b). This was interpreted as evidence suggesting that even greater changes may occur post-trauma in absence of functionally closely-related genes. Results in mice revealed also increased 5-HT_{2A} mRNA levels in lumbar segments (laminae VII, VIII, and IX) several days after a low-thoracic transection (Ung et al., 2009).

All in all, it is unclear how these changes of neuronal properties and gene expression below lesion level may affect functional recovery and, specifically, the development of approach

designed to reactivate behaviours-generating neuronal networks (e.g., CPGs for locomotion, micturition, ejaculation, etc.). Nonetheless, it has been postulated by others that such changes may contribute to increase sublesional network excitability and, thus, may facilitate training-induced learning and rehabilitation.

2.6 Advanced locomotor training induced pharmacologically as a treatment against motor system changes in SCI

Given that no cure exists yet to repair the spinal cord, an interesting avenue to prevent or reduce some of the motor system changes described in previous sections of this chapter may be to pharmacologically induce episodes of locomotion. To achieve this, an alternative strategy could be to develop a CPG-activating drug treatment that could temporarily re-activate this sublesional network in tetraplegic and most paraplegic subjects.

Experiments mainly conducted in my laboratory since 2004 have led to a better understanding of pharmacological CPG activation *in vivo*. In brief, we found in completely low-thoracic spinal cord-transected mice that a few subtypes of blood brain barrier (BBB) permeable molecules can elicit partial CPG-activating effects (i.e., locomotor-like movements or LMs that resemble crawling - successive flexions and extensions coordinated in both hindlimbs without weight bearing)(Guertin, 2004a; Landry & Guertin, 2004; Landry et al., 2006; Lapointe et al., 2009). We subsequently found that drug combinations with some of these compounds including dopaminergic and serotonergic compounds (e.g., DA precursors such as L-DOPA combined with a decarboxylase inhibitor such as carbidopa, and a 5-HT1A receptor agonist such as 8-OH-DPAT or buspirone, etc.), could elicit significantly greater CPG-activating effects including large amplitude LMs with some equilibrium, plantar foot placement and weight bearing capabilities (i.e., real stepping rather than crawling, Guertin 2004b; Lapointe & Guertin 2008; Guertin et al., 2010, Guertin et al., 2011)(Fig.6). As mentioned earlier, this idea that drug combinations can produce apparently full CPG-activating effects was also supported by comparable findings in *in vitro* isolated spinal cord preparations (better and more stable fictive locomotor neuronal activities in isolated spinal cords, e.g., Cazalets et al., 1992; Kjaerulff & Kiehn, 1994; Kiehn & Kjaerulff, 1996; Jiang et al., 1999; Whelan et al., 2000).

This identification of a potent CPG-activating tritherapy (Guertin et al., 2010) recently received support from a special NIH program (Rapid Access to Interventional Development program) to conduct some of the preclinical studies (toxicity and safety pharmacology in rats). It has been determined that a tri-therapy composed of L-DOPA, carbidopa and buspirone is safe and ideally suited for further development at the clinical level (i.e., each drug is already FDA approved for diseases other than SCI and no abnormal pharmacology or toxicology data was found) as a first-in-class CPG activating drug treatment candidate. However, although efficacy in early chronic SCI mice has recently been demonstrated (Guertin et al., 2010; Guertin et al. 2011), it remains unclear how repeated administration over several weeks would affect disuse-related motor system changes.

As mentioned earlier, chronic SCI patients (especially motor-complete also called ASIA-A or ASIA-B patients) experience often life-threatening health problems also referred to as

'secondary complications' including motor system changes reported here also in this paraplegic mouse model. Using combination therapy, we obtained preliminary data suggesting that repeatedly-treated paraplegic mice can partially prevent some pathophysiological motor system changes found after SCI (Guertin et al., 2011).

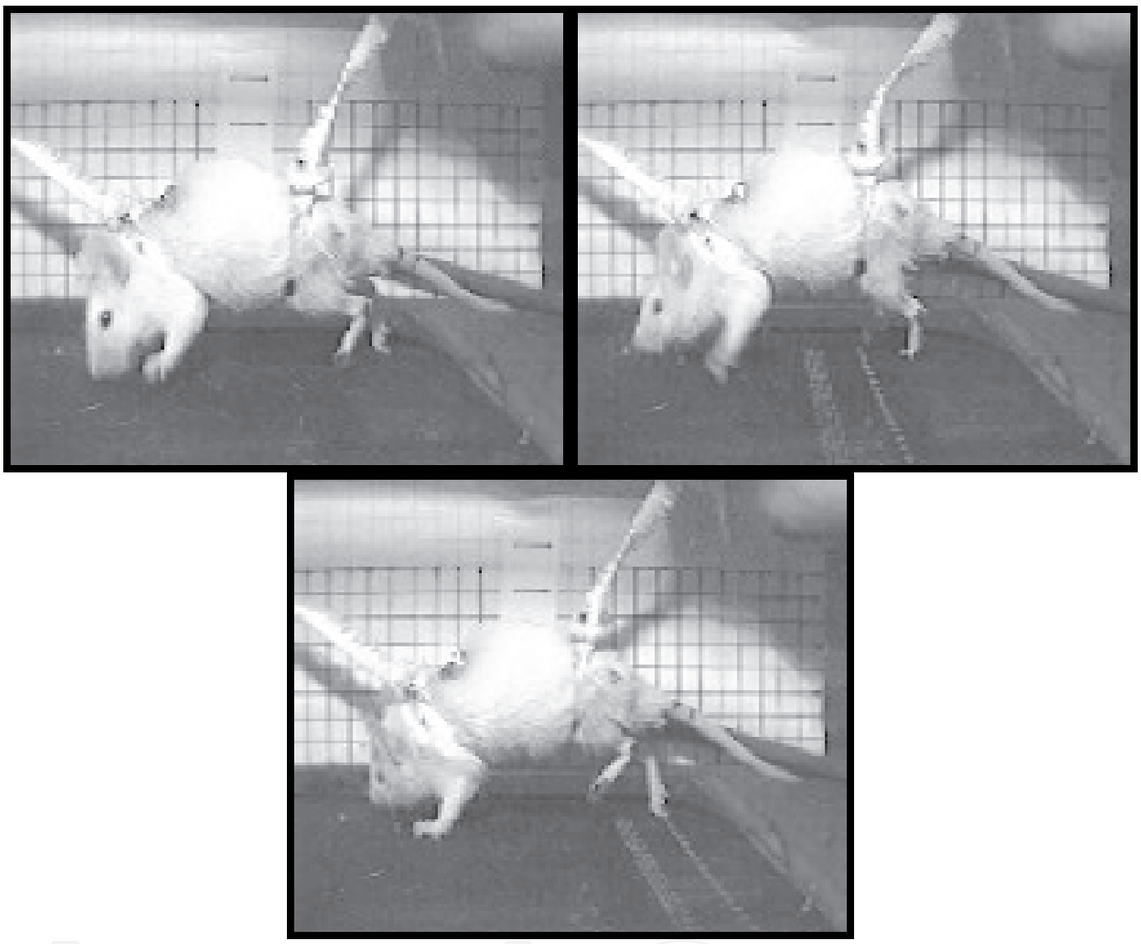

Fig. 5. Video images of a paraplegic mouse placed on a treadmill 15 minutes following administration of a CPG-activation tritherapy. Involuntary movements were generated for approximately 30 to 45 min. Then a complete return to complete paraplegia occurred.

Subcutaneous administration (several times per week) of a first-generation combination treatment was found, upon each injection (within 15 min), to repeatedly induce temporarily (during approx. 30-45 min) episodes of weight bearing stepping in non-assisted paraplegic mice at least during one month.

Regarding body weight values, combination therapy-treated paraplegic animals progressively displayed a moderate increase in weight suggesting that repeated administration of this combination therapy was well-tolerated (i.e., a loss of weight would have suggested toxic effects and additional health problems). No significant difference was found in bone mineral density (BMD) values in femoral bones of tritherapy-treated vs. placebo-treated paraplegic mice. Post-mortem examination of muscle size (whole surface area and fiber cross-sectional area or CSA) measured from cryostat transverse

sections prepared from two hindlimb muscles, *soleus* (SOL) and *extensor digitorum longus* (EDL), was performed to assess the effect of combination therapy-induced training on muscular atrophy normally found after SCI. We found values corresponding with larger muscles and muscle fibers in the combination therapy-treated compared with the placebo-treated paraplegic animals. Sol values increased by 24% in combination therapy-treated paraplegic mice (0.61 ± 0.05 mm^2) compared with placebo-treated ones (0.49 ± 0.03 mm^2, fig. 3A). Comparable results were found in EDL (combination therapy-treated 0.91 ± 0.03 mm^2 vs. placebo-treated 0.77 ± 0.06 mm^2)(not shown). At the cellular level, comparisons between combination therapy-treated and placebo-treated animals revealed that type I fiber CSA values non-significantly changed whereas type II fiber and intermediate fiber (type I + II labeled) CSA values significantly increased subsequently both by 8% (Fig. 3C, 3D). An analysis of muscle fiber-type ratios (i.e., proportion among all fibers of type I, type II or type I + II fibers) indicated that no significant changes were found between groups. Subpopulations of red blood cell (RBC) constituents were assessed and compared between groups. Levels of RBC, platelet, hemoglobin and hematocrit were significantly increased by 11%, 19%, 10% and 10%, respectively, in combination therapy-treated vs. placebo-treated paraplegic animals.

All in all, these results revealed that pharmacological activation of the CPG four times per week during 1 month can prevent anemia and prevent partially muscle atrophy. Circulatory systems were not further examined in this study. On the other hand, this study showed that bone loss typically occurring post-transection in this animals can not be prevented in these conditions. Altogether, it is suggested that training conditions or treatments may have to be optimized for further physiological effects on all parts of the motor system.

Along this idea, we recently conducted a study where paraplegic animals received an anabolic agent, namely clenbuterol, in addition to tritherapy-induced locomotor training. We found that tritherapy-treated paraplegic mice with or without clenbuterol treatment displayed significant locomotor function recovery during 2 months upon each administration of the CPG-activating therapy (Fig.7). To further characterize movements induced by the tritherapy-training, angular excursion at the hip, knee and ankle, as well as movement amplitude values were analysed. Typical examples of hindlimb kinematics are shown in figure 7. Hip, knee and ankle angular displacement showed similar patterns in intact, tritherapy-trained alone and tritherapy-trained + clenbuterol paraplegic animals. Untrained paraplegic animals displayed a consistent lack of angular excursion at the hip level although some displacements were found at the knee and ankle levels (hip: 85°, knee: 30-47°, ankle: 28-125°). Hindlimb movement amplitude values measured by calculating toe displacement in X and Y axis (step "length" and "height") revealed that intact mice had greater step length values than both tritherapy-trained paraplegic groups. On the other hand, both groups of tritherapy-trained paraplegic animals showed similar step length values which were significantly greater than those in untrained paraplegic mice. The coefficient of variation (CV) was higher in untrained mice. Intact, tritherapy-trained and tritherapy-trained + clenbuterol paraplegic mice showed similar step height values. However, differences were found in the variability of the step height, as shown by CV, where intact animals displayed less variability than the other groups of tritherapy-trained animals. No Y axis movement amplitude was observed in paraplegic untrained mice since no weight-bearing movement are normally expressed spontaneously. Overall, a significant increase in performances over time was observed in tritherapy-trained groups of paraplegic mice movement kinematic values were comparable with those from intact animals.

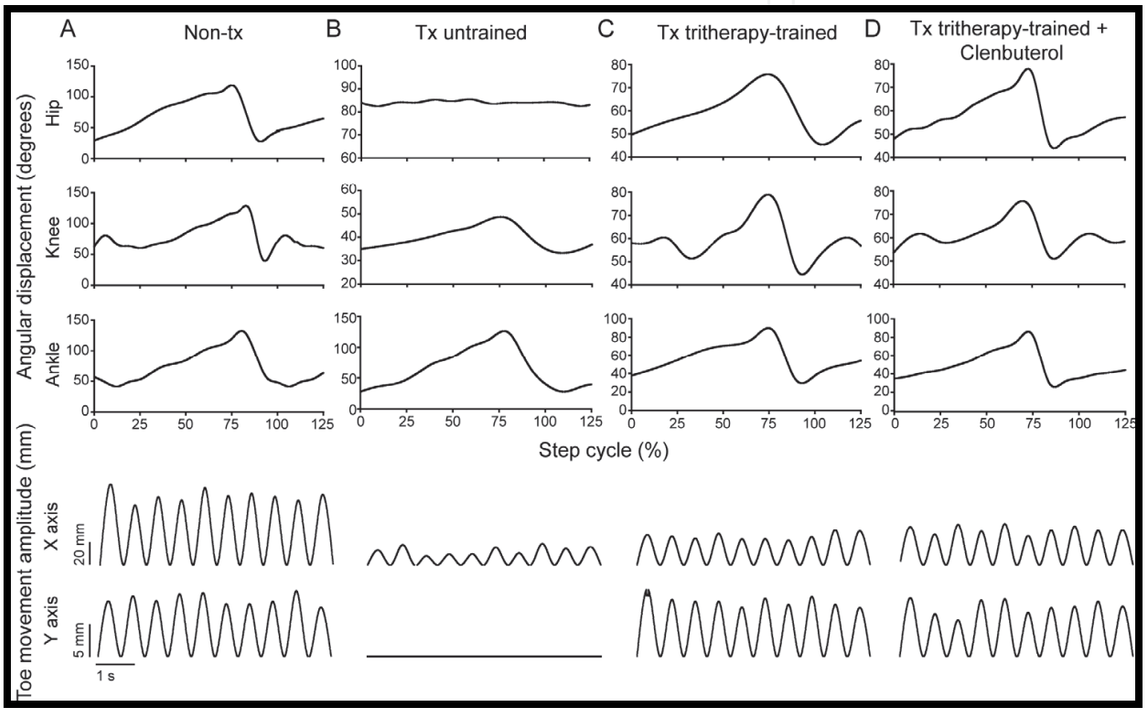

Fig. 6. Kinematic analyses in intact (non-Tx), paraplegic (Tx) untrained, paraplegic tritherapy-trained and tritherapy-trained and treated with clenbuterol. Step parameters from both tritherapy-trained paraplegic mice were similar with those from intact animals suggesting that the tritherapy appropriately restored episodes of locomotor movements.

Femoral BMD and BMC values were measured in order to address whether tritherapy-training alone or combined with clenbuterol can prevent or at least reduce bone loss normally found in untrained paraplegic mice. However, in all groups of paraplegic animals, important losses were found. Untrained paraplegic mice (BMD: 0.0767 ± 0.0010 g/cm², BMC: 0.0381 ± 0.0008 g) and tritherapy-trained paraplegic animals (BMD: 0.0766 ± 0.0011 g/cm², BMC: 0.0378 ± 0.0009 g) showed comparable values whereas in paraplegic trained + clenbuterol groups, femoral BMD (0.0731 ± 0.0012) and BMC (0.0349 ± 0.0009) further decreased.

Morphometric analyses of soleus and EDL were performed in order to further characterize specific muscular property changes in all groups. Muscle CSA, fiber type-specific CSA and relative distribution values were analysed. For soleus CSA, untrained and tritherapy-trained paraplegic mice had significantly lower muscle CSA values than intact animals and tritherapy-trained + clenbuterol paraplegic groups. However soleus CSA in untrained paraplegic mice was not significantly lower than tritherapy-trained paraplegic animals. For EDL, in contrast with muscle mass changes, CSA values showed statistical differences between groups. Tritherapy-trained + clenbuterol paraplegic mice showed higher EDL CSA values than all the other groups. Untrained and tritherapy-trained paraplegic mice had lower CSA values than intact animals.

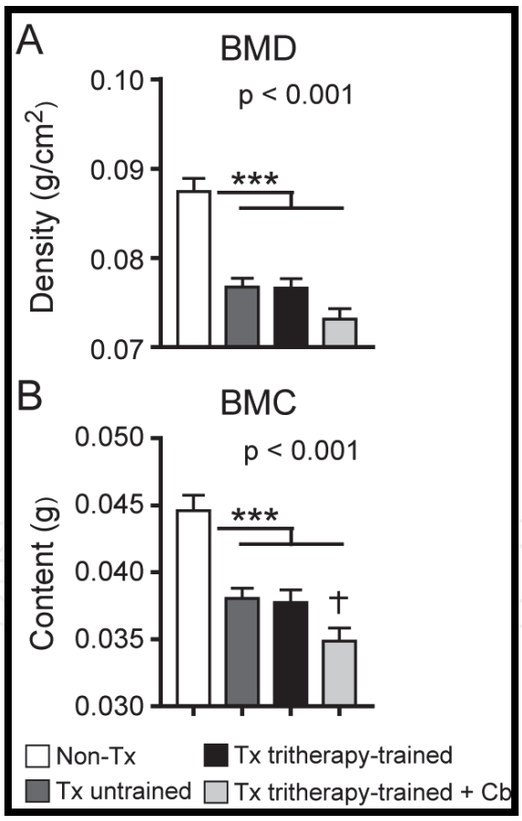

Fig. 7. Femoral BMD and BMC in intact (non-Tx), paraplegic (Tx) untrained, paraplegic tritherapy-trained and tritherapy-trained and treated with clenbuterol. Unfortunately, bone loss was not prevented in both tritherapy-trained paraplegic mice compared with intact animals suggesting that the tritherapy with or without clenbuterol failed to restore bone properties.

Soleus and EDL muscle cross-sectional area				
	Non-Tx	Tx untrained	Tx tritherapy-trained	Tx tritherapy-trained + clen
Soleus	0.85 ± 0.05	0.56 ± 0.05**††	0.64 ± 0.04*††	0.93 ± 0.06
EDL	1.10 ± 0.04	0.95 ± 0.04**††	0.96 ± 0.04**††	1.25 ± 0.05**

Table 5. Soleus and EDL cross-sectional area values in intact (non-Tx), paraplegic (Tx) untrained, paraplegic tritherapy-trained and tritherapy-trained and treated with clenbuterol. Although encouraging anti-atrophying effects were found in tritherapy-treated paraplegic mice, only paraplegic animals that received both clenbuterol + tritherapy displayed a complete restoration of muscle size (even a relative hypertrophic effect was induced compared with intact animals).

More differences were found when analysing individually fiber type-specific CSA values. Specifically, for soleus fiber types, all three fiber types from tritherapy-trained + clenbuterol paraplegic animals displayed larger CSA values than all other groups (type I: 1656.7 ± 80.8 μm^2, type II: 987.2 ± 16.7 μm^2, hybrid: 1145.5 ± 18.0 μm^2). Conversely, untrained paraplegic mice displayed the lowest soleus fiber type CSA of all groups (type I: 783.1 ± 15.1 μm^2, type II: 753.2 ± 9.1 μm^2, hybrid: 750.0 ± 8.1 μm^2). In EDL, type II fiber CSA differences between groups were similar to soleus type II (intact: 1063.5 ± 15.9 μm^2, paraplegic untrained: 908.1 ± 11.4 μm^2, paraplegic trained: 963.4 ± 10.9 μm^2, paraplegic trained + clenbuterol: 11.65.2 ± 17.9 μm^2).

3. Conclusion

These findings provided proof-of-concept data strongly supporting the idea that physical activity can prevent or restore motor system adaptations normally expressed after SCI. However, that study was exploratory and thus, it remains unclear the extent to which physical activity elicited with this pharmacological approach can extensively prevent or reverse secondary complications. Although anemia and partial muscle atrophy were prevented in CPG-activating tritherapy-trained paraplegic mice, addition of anabolic aids such as clenbuterol appeared to synergistically affect positively the motor system in paraplegic mice (complete reversal of atrophy, complete lack of anemia, etc.). Effects on other elements of the motor systems such as blood vessels (e.g., deep vein size) or skeleton remain to be explored or improved. From a scientific perspective, it remains also to be determined clearly what role physical inactivity may play on motor system adaptations post-SCI and corresponding health problems in humans. This said, motor system changes post-SCI obtained in this murine model was found to resemble those typically encountered in patients with SCI or disuse. It may therefore be useful to further study basic cellular mechanisms underlying these changes of the musculoskeletal systems in these conditions. It may also serve to accelerate the development of new therapeutic strategies aimed at reducing or preventing completely all musculoskeletal and biomechanical changes in SCI patients or in patients suffering of disuse or immobilization.

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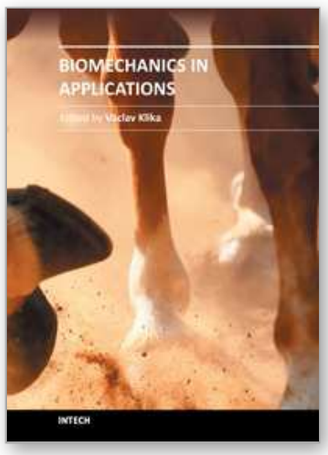

Biomechanics in Applications

Edited by Dr Vaclav Klika

ISBN 978-953-307-969-1
Hard cover, 408 pages
Publisher InTech
Published online 09, September, 2011
Published in print edition September, 2011

During last couple of years there has been an increasing recognition that problems arising in biology or related to medicine really need a multidisciplinary approach. For this reason some special branches of both applied theoretical physics and mathematics have recently emerged such as biomechanics, mechanobiology, mathematical biology, biothermodynamics. The Biomechanics in Application is focusing on experimental praxis and clinical findings. The first section is devoted to Injury and clinical biomechanics including overview of the biomechanics of musculoskeletal injury, distraction osteogenesis in mandible, or consequences of drilling. The next section is on Spine biomechanics with biomechanical models for upper limb after spinal cord injury and an animal model looking at changes occurring as a consequence of spinal cord injury. Section Musculoskeletal Biomechanics includes the chapter which is devoted to dynamical stability of lumbo-pelvi-femoral complex which involves analysis of relationship among appropriate anatomical structures in this region. The fourth section is on Human and Animal Biomechanics with contributions from foot biomechanics and chewing rhythms in mammals, or adaptations of bats. The last section, Sport Biomechanics, is discussing various measurement techniques for assessment and analysis of movement and two applications in swimming.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Pierre A. Guertin (2011). Adaptations of the Motor System in Animal Models of Spinal Cord Injury and Disuse, Biomechanics in Applications, Dr Vaclav Klika (Ed.), ISBN: 978-953-307-969-1, InTech, Available from: http://www.intechopen.com/books/biomechanics-in-applications/adaptations-of-the-motor-system-in-animal-models-of-spinal-cord-injury-and-disuse

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