Methylprednisolone fails to improve functional and histological outcome following spinal cord injury in rats

UNIVERSITÀ DEGLI STUDI DI TORINO

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Ab s t r a c t

Currently, methylprednisolone sodium succinate (MPSS) is the standard treatment following acute spinal cord injury (SCI) as a consequence of the results obtained from the National Acute Spinal Cord Injury Studies. However, many have questioned the efficacy of MPSS because of its marginal effects. Additionally there has been criticism of both study design and statistical interpretation. The functional consequences of experimental SCI have been assessed in many ways. The purpose of this investigation was to determine the effects of MPSS vs. saline solution (SS) following moderate T10 contusion injury in rat. Functional recovery was evaluated using the 21-point Basso, Beattie and Bresnahan (BBB) locomotor recovery scale, the inclined plane, the beam walk, footprint analysis and the horizontal ladder. To optimize the precision and accuracy of functional results we examined the locomotion on a treadmill using three-dimensional (3D) analysis. Stereology was used to estimate the amount of damaged tissue. The results of the traditional functional methods showed that administration of the NASCIS dosage of MPSS following acute spinal cord contusion did not lead to any significant differences in the functional recovery of MPSS- vs. SS-treated animals. More importantly, the results of the 3D kinematic showed that the MPSS administration did not affect the flexion/extension of the hip, knee and ankle joints during the step cycle. Finally, stereological results revealed no statistically significant differences between the two experimental groups. Altogether, our results support data previously reported by several authors, suggesting that MPSS does not lead to improved functional outcome following experimental acute SCI.

Introduction

Traumatic spinal cord injury (SCI) is one of the leading causes of disabilities in young adults. Currently, the synthetic glucocorticoster-oid methylprednisolone sodium succinate (MPSS) is the mainstay of therapy following acute SCI. Since the published results from the landmark National Acute Spinal Cord Injury Studies (NASCIS) II trial during the early 90 s, MPSS has become widely used in the treatment of SCI. MPSS must be given within the first 8 h after SCI as a bolus and continued as a constant rate infusion for at least 23 h (Bracken et al., 1990, 1992). However, many clinicians now question the use of an MPSS protocol because of conflicting results of experimental studies
(Koyanagi and Tator, 1997; Yoon et al., 1999) combined with the relatively small neurological improvements seen in humans (Short et al., 2000; Sayer et al., 2006).

While there is a wide range of insults that result in SCI, more than 80% are characterized by severe spinal cord contusion rather than transection of the spinal cord, even when there are massive vertebral injuries (Metz et al., 2000). The development and use of models that aim to mimic the type of SCI that is observed clinically and which specific tracts are lesioned, are crucial to understand the basic biology of SCI and developing effective therapeutic strategies (Adams et al., 2007). At present, we owe much of our understanding regarding the morphology and pathology of SCI in humans to spinal cord contusion in rodent models (Gruner, 1992; Kwon et al., 2002).

In light of this controversy, we investigated the effects of MPSS administration after contusion injury

using a wide variety of behavioral and morphological assessments to measure recovery.
Adequate evaluation of the efficacy of therapeutic interventions following spinal injury is critical in order to distinguish among spontaneous recovery, compensatory strategies, and recovery of behavioral function dependent on the therapeutic measure (Muir and Webb, 2000). Therefore, much effort has been focused on developing several methods to analyze locomotion in rats after experimental SCI, especially at thoracic levels. So far, most of SCI studies used solely the 21-point Basso, Beattie and Bresnahan (BBB) locomotor recovery scale (Basso et al., 1995) for the evaluation of functional recovery. It is now well accepted that while the BBB locomotor scale covers a broad range of functional recovery, it can be less sensitive at specific levels of recovery, partly due to the ordinal nature of the scale. Another limitation in the BBB score is the correct assessment of forelimb–hindlimb coordination, even when two highly experienced observers are involved (Koopmans et al., 2005).

In addition to BBB scoring, we included other traditional methods such as the inclined plane, the beam walk, footprint analysis and the horizontal ladder. Because the main limitation of these endpoint measures is that they provide no information of how the task is being performed, we obtained continuous kinematic measures in order to provide a precise means of assessing neural control over motor output. We obtained highly accurate continuous kinematic measure-ments throughout the step cycle using a three-dimensional (3D) motion analysis methodology. Finally, the Cavalieri stereological method was used to obtain an estimation of various quantitative parameters of the lesion size.
Materials and methods

Experimental animals

Forty Wistar adult female rats (Harlan, Barcelona, Spain) weighing approximately 200 g were used in this study. Data were collected from a total of thirty six animals (see Training procedure). All animals were kept in ventilated, humidity and temperature-controlled rooms with a 12/12-h light/dark cycle. The animals were housed on sawdust and received food pellets and water ad lib. All procedures were performed with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Surgical procedure

The animals were randomly and blindly divided into 3 groups. In the methylprednisolone group (MPSS group; n = 15) an intraper-itoneal bolus of MPSS (30 mg/kg) was administered 10 min after SCI, and then it was infused at a rate of 5.4 mg/kg/h for an additional 23 h, using an osmotic mini-pump (model 2001D, Durect Corp., Cupertino, CA, USA). After overnight equilibration in sterile 0.9% saline at 37 °C, the pumps were implanted intraperitoneally and removed after 2 days under isoflurane anesthesia (b5 min). The MPSS dosage was chosen based on the NASCIS II protocol and on successful spinal cord injury studies in the rat model. In the vehicle group (SS group; n = 15), the animals received, an equivalent volume of a saline solution, intraperitoneally. They were also infused at a rate of 5.4 mg/kg/h for an additional 23 h, using the same osmotic pumps. Animals subjected to identical surgical procedures, without impaction (Sham group; n = 4), served as sham-operated controls.

Rats were anesthetized with ketamine (80 mg/kg) and medeto-midine (0.2 mg/kg). A midline incision exposed the spinal column at the T8–T11 level, and the paravertebral muscles were dissected bilaterally to visualize the transverse apophyses. Care was taken to perform a laminectomy at the T10 vertebral level that was only slightly larger than the 2.5 mm impactor tip. The animals in the MPSS and SS groups then received a 200-kdyn contusion with the Infinite Horizon spinal cord injury device (Precision Systems and Instrumen-tation, Lexington, KY, USA). The surgical procedure was performed with the aid of an operating microscope (M680, Leica, Wetzlar, Germany). The wound was subsequently irrigated with saline solution, the muscle, the fascia and the skin were reapproximated with absorbable sutures. Body temperature was maintained with a heating pad at 37±0.5 °C during the surgery and postoperative period. An ophthalmic gel (Lacryvisc, Alcon, Lisbon, Portugal) was applied to prevent drying of the eyes.

After surgery, 10 ml of 0.9% sodium chloride and 30 mg/kg of sulfadiazine and trimethoprim were injected subcutaneously. Access to food was facilitated by placing softened food pellets directly in the bottom of each cage. The state of hydration and gastrointestinal function were monitored daily. Lactated Ringer's solution was injected subcutaneously when necessary. Rats were weighed daily for the first 7 days postsurgery and then weighed weekly. Postoper-ative care included the manual expression of bladders twice a day until bladder function returned, as well as injections of sulfadiazine and trimethoprim twice a day for up to 1 week.

Training procedure

Two weeks before the collection of data, rats were trained to walk on a horizontal ladder and on a horizontal runway which were 1-m long and elevated 1 m above the floor. Training was considered successful when the animal was able to complete these tasks without hesitation. Additionally, each animal was first trained daily to walk consistently on a treadmill at a speed of 30–40 cm/s, within a Plexiglas enclosure, 53 cm×10 cm×14 cm, with a removable top (Letica, Scientific Instrument, Barcelona, Spain). During two weeks ten minute long training sessions were given once a day with mild intensities of footshock initially used as negative reinforcement to improve performance. In our training protocol when the rats failed to walk regularly and spontaneously after three sessions, they were eliminated from further study. We attempted to minimize stress by minimizing noise levels and handling the rats gently to obtain locomotion under normal conditions.

Functional assessment

All tests were performed before spinal injury and for 7 weeks post-injury. Using the BBB locomotor rating scale and the inclined plane, injured rats were assessed 2 days after surgery, and then once a week. For analysis of the beam walk and the horizontal ladder the animals were assessed 2 weeks postoperatively and then once a week. Additionally, the footprint analysis was assessed 3 weeks postoper-atively, and then once a week until the end of the study. Kinematic data was collected at week 3 postoperatively and at the end of the experiment.

The BBB scale was described by Basso et al. (1995). In this scale, specific components of functional behavior such as the limb movement, paw placement/position, stepping, coordination, toe clearance and tail position are analyzed. A score of 0 was given if there was no spontaneous hindlimb movement, a score of 21 indicated normal locomotion. The locomotor activity of individual animals was evaluated in an open field, which was a 100×100-cm transparent Plexiglas box, with walls of 11-cm during 4 min by two experienced examiners.

The beam walk test was performed according to the descriptions of von Euler et al. (1996). Seven beams of different widths were used as narrow pathways, with the following score: (1.7-cm=7; 2.7-cm=6; 3.7-cm=5; 4.7-cm=4; 5.7-cm=3; 6.7-cm=2; 7.7-cm=1). Rats were placed on the widest beam and the ability to cross the horizontal beam without foot slips within two trials was observed. The procedure was repeated on successively narrower beams. The narrowest beam a rat could walk was recorded.

In the horizontal ladder task, rats crossed a ladder constructed using 5-mm rungs separated 2.5-cm apart. Every animal had to cross the ladder with 25 rungs four times and the total number of foot falls was recorded (Webb and Muir, 2003).

Animals were also subjected to the inclined plane test, defined as the largest angle at which the rat could maintain a stable position for 5 s (Rivlin and Tator, 1977). The inclined plane was a 28×30-cm floor with a 20×25-cm grooved (grooves 1-mm deep and 5 mm apart) rubber surface.

Footprint analysis was modified from de Medinaceli et al. (1982). Animals were tested in a confined walkway measuring 42-cm long and 8.2-cm wide with a dark shelter at the end. A white paper was placed

on the floor of the rat walking corridor. The hind paws of the rats were pressed down onto a finger paint-soaked sponge, and they were then allowed to walk down the corridor leaving its hind footprints on the paper. The base of support was determined by measuring the core to core distance of the central pads of the hind paws.

To collect the 3D kinematic data the hair was clipped around the left hindlimb, to improve the visual image obtained for analysis. Hemispheric markers, obtained from a plastic sphere cut into two parts and covered by adhesive infrared-reflective paper (3M Scot-chlite, Minnesota, USA) with a diameter of 2 mm were placed on the skin over five anatomic landmarks on the lateral side of the left hindlimb: the iliac crest, the greater trochanter, the knee joint, the lateral malleolus, and the fifth metatarsal head. The same operator performed all marker placements to avoid inter-tester variability. The retroflective passive markers were used together with infrared illumination produced by an array of light-emitting diodes (Miniflood 100, Derwent Systems Lda, Cramlington, Northumberland, UK) mounted on top of each camera. Three CCD cameras (Redlake PCI 500S, San Diego, USA) were used to record the position of these markers. Kinematic data were collected at a sampling rate of 125 Hz. The magnification of these cameras was calibrated to cover a 20 cm length of the treadmill apparatus, and allowed the recording of five consecutive steps. Data were recorded while the rats walked at a speed of 40 cm/s, which is within the normal walking velocity, where they typically utilize a lateral sequence walk. The images were acquired using the software Midas2.0 (Xcitex, Cambridge, USA). The image dimension was 480×420 pixels, codified in 256 gray levels. Cameras were strategically placed around the left hindlimb to minimize marker occlusion, maximize resolution and to improve the accuracy of the 3D reconstruction process. The precise procedure for camera calibration and for the 3D reconstruction process has been described in detail previously (Couto et al., 2008). The hindlimb joint angles were measured at the flexor side of each joint. The knee position was computed indirectly as recently proposed (Couto et al., 2008).

Histological and stereological analysis

At the time of withdrawal, animals were anesthetized with ketamine (80 mg/kg) and medetomidine (0.2 mg/kg) and perfused through the heart with 300 ml of phosphate buffered solution (PBS) followed by 300 ml of 4% paraformaldehyde in cold 0.1 M PBS, pH 7.4. The T10 level of the spinal cord was then exposed from a dorsal approach and a 2-cm long segment centred on the lesion point (i.e. 1 cm upstream and 1 cm downstream to it) was withdrawn and fixed in 4% paraformaldehyde for 4 h. Specimens were then washed in phosphate buffer saline (PBS) and routinely embedded in paraffin. Blocks were then serially cut (at 10-µm nominal thickness) perpen-dicular to the main spinal cord axis.

Twenty slides located at about 1-mm distance from each other were then processed for silver staining (Bio-Optica, Milan, Italy). For stereological assessment, design-based sampling (Geuna and Varejão, 2008) and the Cavalieri method (Geuna, 2000; Aslan et al., 2006) were used to estimate the following parameters: lesion length, lesion volume, area of damaged tissue at the lesion epicentre, total cross-

sectional area of the spinal cord at the lesion epicentre and the rate between area of damage and total spinal cord area at the lesion epicentre (defined as the section where the largest area of tissue damage was detected). All stereological estimations were carried out on a workstation equipped with a DM400 microscope with DFC320 digital camera, a IM50 image manager system (Leica Microsystems, Wetzlar, Germany) and a Reconstruct© software developed by J.C. Fiala from the Department of Biology of Boston University (Fiala, 2005).

Numerical and statistical analysis

The step cycle, which is the basic unit of measurement in gait analysis, was split into two parts, the stance and the swing phase. The stance phase was defined as the part of the step cycle that begins as soon as the foot contacts the ground or the treadmill belt, and terminates when the foot starts its forward movement. In accordance with our previous work, we subdivided the stance phase in the rat into three major components: (1) weight acceptance, occupying the first 20% of the stance phase; (2) mid-stance, occurring between 20 and 40% of the stance phase; and (3) push-off, for the last 60% of the stance phase (Varejão et al., 2002). The swing phase was considered to begin at the onset of forward movement and to terminate as the foot strikes the ground or the treadmill belt. This phase was divided into two periods according to the traditional Philippson (1905) scheme: the first period where the hindlimb retracts to the body (F), and the second one of extension (E1), during which the knee and ankle extend in preparation for foot placement. For each step, the duration of the stance and swing phases was normalized. Cubic-spline interpolation was applied to the original data about the angular position of the hip, knee and ankle to obtain 101 samples per step cycle regardless of their duration. This numerical treatment was performed with Matlab computational software (The MathWorks Inc., Natick, MA, USA).

3D joint angular displacements were calculated for the hip, knee and ankle joints. In addition, the following gait parameters were included: extension before toe-off and foot progression. Extension before toe-off was quantified by measuring the angle of a connection from the hip to the fifth metatarsal head to a perpendicular line from the hip (Fouad et al., 2000). Foot progression was measured as the angle in degrees between the line of progression and the foot segment (reference line from the lateral malleolus to the fifth metatarsal head) (Couto et al., 2008).

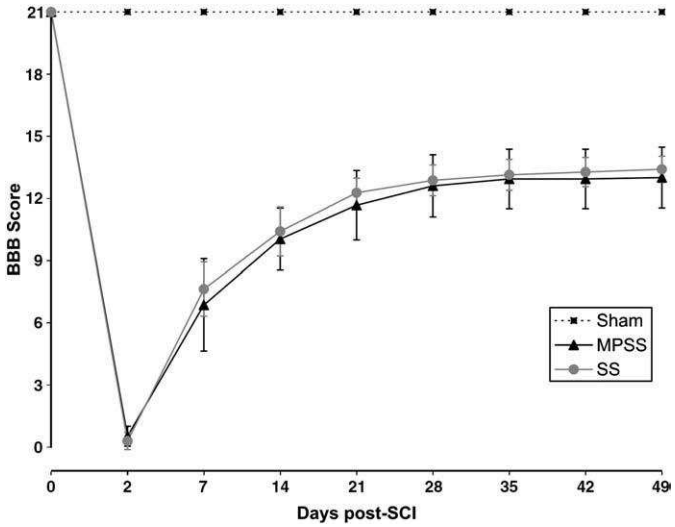

Fig. 1. Time course of motor recovery showing Basso, Beattie, and Bresnahan (BBB) scores. All values are given as means±standard deviations. No significant differences at i
ndividual time points were noted between MPSS (black) and SS (gray)
groups.

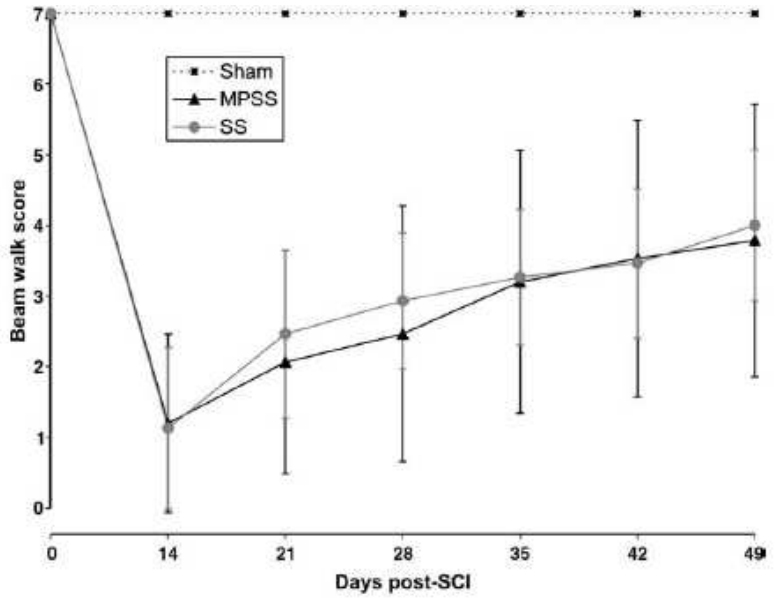

Fig. 2. Time course of motor recovery showing beam walk performance using seven wooden planks of different widths (7.7–1.7 cm) which were 1 m long. All values are given as means±standard deviations. No significant differences at individual time points were noted between MPSS (black) and SS (gray) groups.

Mean±standard deviation (S.D.) values for all of the measured variables are reported. Statistical comparisons between treatment groups were made using the Mann–Whitney U test. The statistical significance was set at the level of P b 0.05. These statistical tests were performed using SPSS computational software (Statistical Package for the Social Sciences Inc., Chicago, USA).

Stereological estimates on lesion extent were subjected to one-way analysis of variance (ANOVA) test using the software "Statistica per discipline bio-mediche" (McGraw-Hill, Milano, Italia). The statistical significance was set at the level of P b 0.05.

Results

All of the contused animals exhibited signs of paraplegia, followed by significant improvement over the subsequent 7 weeks. The voiding of the bladder returned within the first week post-injury. At week 3 postoperatively they could perform weight-supported plantar steps in the treadmill at a speed of 40 cm/s. Laminectomy alone (Sham group) did not affect the neurologic function as shown in the functional assessment.

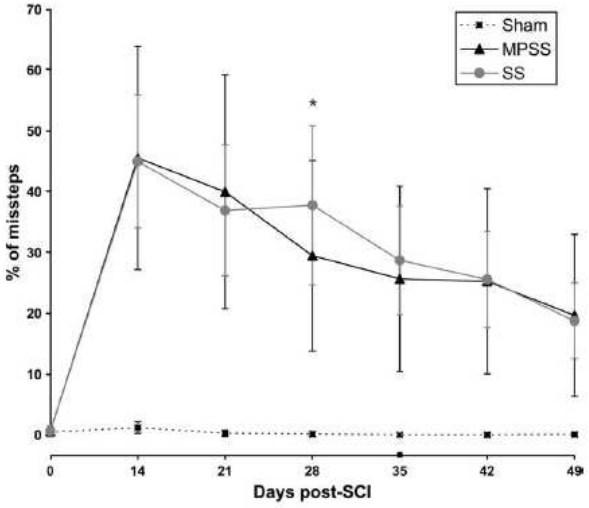

Fig. 3. The horizontal ladder tests the ability of the animals to cross a 1 m long runway with round bars 2.5 cm apart. All values are given as means±standard deviations. Statistically significant difference (P b 0.044), was noted between MPSS (black) and SS (gray) groups on day 28.

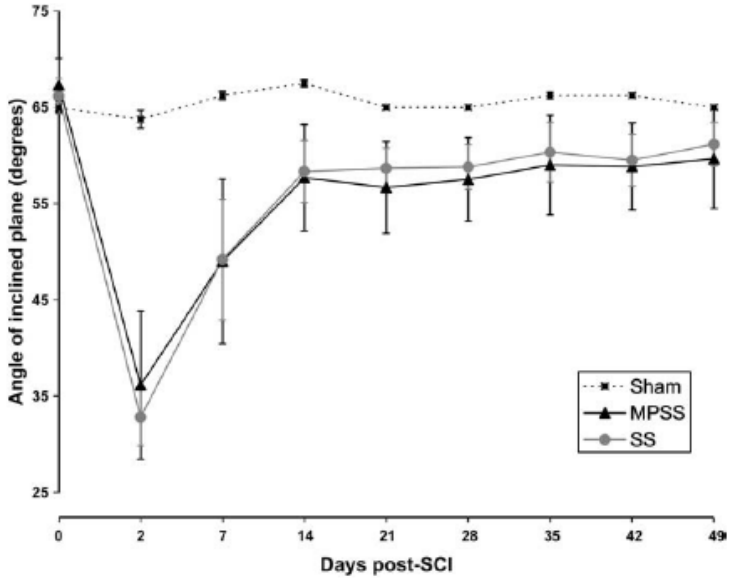

Fig. 4. The inclined plane method measures the maximum inclination of the plane at which a rat could maintain itself for 5 s. All values are given as means±standard deviations. No significant differences at individual time points were noted between MPSS (black) and SS (gray) groups.

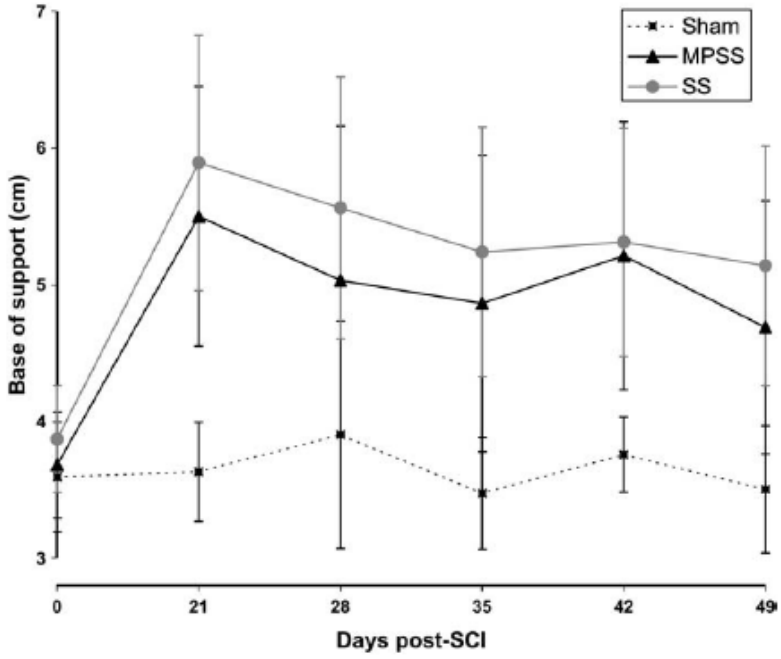

Fig. 5. The base of support was determined by measuring the distance between the central pads of the hindlimbs. All values are given as means±standard deviations. No significant differences at individual time points were noted between MPSS (black) and SS (gray) groups.

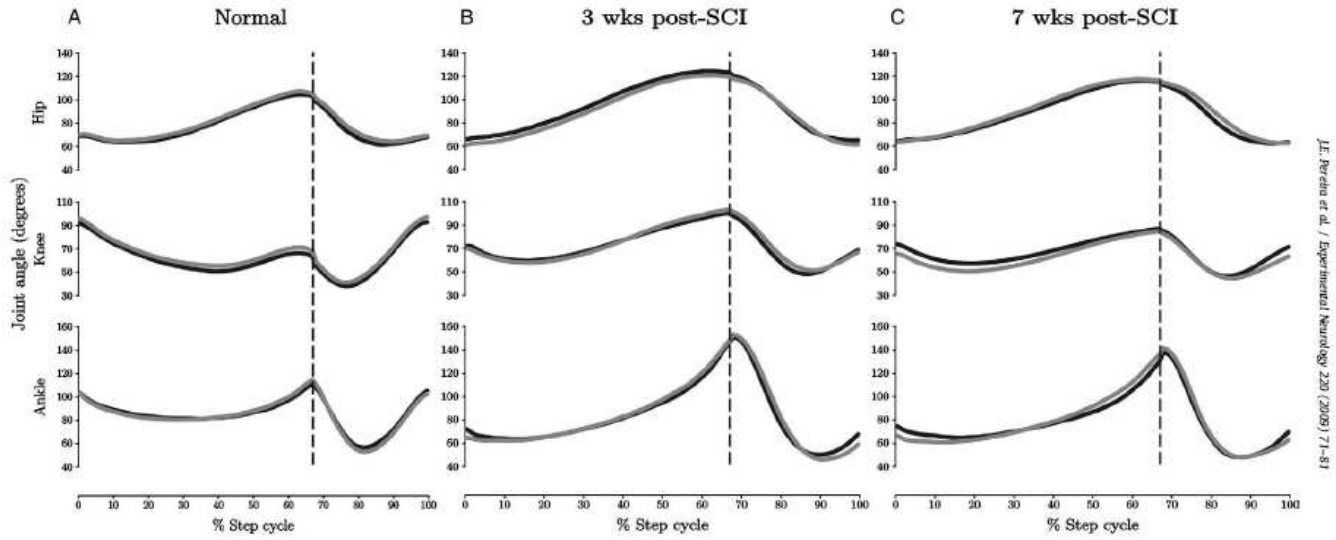

Fig. 6. Computerized analysis of 3D coordinates of the different joint markers was used to calculate the mean values for the joint angular positions of the hip, knee and ankle, for MPSS (black) vs. SS (gray) groups. Temporal analysis of the hindlimb movements in the form of angular excursions of the joints in normal (A), and injured rats at 3 weeks (B) and 7 weeks (C) post-contusion. The joint angular motion was very similar between MPSS- and SS-treated groups. Stance and swing phases were normalized. The stance duration was set at 67% of the step cycle duration. The vertical dashed line corresponds to the stance–swing transition.

13). There were no statistically significant differences between MPSS and SS groups in the weekly measurement of BBB scores.

We also tested locomotion on two more challenging terrains, a beam walk and a horizontal ladder. Before the injury, rats were able to cross the narrowest beam and the horizontal ladder with no errors in foot placement (Figs. 2 and 3). All animals showed severe deficits following injury. There were improvements over 7 weeks of testing and no differences between MPSS and SS groups, with the exception of a slight, yet significant (P =0.044) percentage of missteps in the SS group on day 28.

In the inclined plane test, severe motor disturbances were observed in rats with SCI during the first 2 weeks, with no significant differences between MPSS and SS groups (Fig. 4).

The base of support measured by footprint analysis also showed that there were no significant differences in the functional recovery of MPSS- vs. SS-treated rats (Fig. 5).

To characterize 3D hindlimb kinematics during treadmill locomo-tion, a total of 75 step cycles from each experimental group were obtained before, at 3 weeks and 7 weeks after spinal cord contusion injury. The average toe-off time was calculated to be 67% into the step cycle. Records of treadmill performance produced by all pre-surgical animals were similar to our previous study using the same 3D motion analysis methodology (Couto et al., 2008). In both groups, impair-ments were evident and very similar in the treadmill locomotion of the lesioned rats for all joint kinematic analysis (Fig. 6). Three weeks following injury the rats exhibited increased hip extension along the step cycle. The knee and ankle joints showed reduced extension at initial contact (IC) and all the joints exhibited a substantial increment in the extension at TO. In the knee angle trajectory, maximum extension occurred during the stance–swing transition. Unlike normal animals, the injured animals lost the double peak pattern of the ankle angle. In addition, the switch from extension to flexion of all joints showed a more gradual transition in the SCI animals. Seven weeks post-injury we found identical changes in the hindlimb kinematics. In both groups animals walked with a decreased extension at TO compared to the previous functional assessment.

Table 1
Kinematic data for the SS and MPSS groups at week 0.

Variables	SS	MPSS	P value
Stance			
Hip			
Initial contact	70.3±6.4	68.9±6.6	0.694
MAX	108.5±9.7	106.1±10.5	0.633
MIN	64.1±8.0	62.3±7.1	0.694
Knee			
Initial contact	95.8±4.2	91.6±5.9	0.04
MAX	96.0±4.2	92.0±5.9	0.049
MIN	54.4±5.6	49.5±6.8	0.059
Ankle			
Initial contact	103.1±8.4	103.6±8.8	0.95
MAX	115.7±9.8	112.7±9.0	0.31
MIN	78.3±8.9	78.4±8.7	0.633
Swing			
Hip			
Toe-off	105.7±10.4	103.6±10.3	0.633
MAX	105.8±10.5	103.6±10.3	0.633
MIN	62.9±8.9	59.5±8.4	0.351
Knee			
Toe-off	68.0±9.6	63.3±11.3	0.165
MAX	97.3±5.0	93.1±6.8	0.062
MIN	40.1±5.3	37.4±6.0	0.13
Ankle			
Toe-off	114.5±11.2	110.4±11.2	0.29
MAX	117.9±10.0	115.1±9.3	0.468
MIN	51.3±10.0	55.4±8.6	0.206
Extension before toe-off	43.3±3.5	43.2±4.3	0.836

Note. Values are presented as mean±SD. Abbreviations: MAX, angular maximal; MIN, angular minimal.
Significant at P b 0.05.

Table 2
Kinematic data for the SS and MPSS groups at week 3.

Variables	SS	MPSS	P value
Stance			
Hip			
Initial contact	61.5±10.0	66.1±10.3	0.299
MAX	122.0±12.7	125.9±9.7	0.248
MIN	60.2±10.6	64.8±10.7	0.356
Knee			
Initial contact	70.3±11.2	72.5±11.7	0.773
MAX	103.5±13.0	100.6±15.7	0.564
MIN	56.4±8.4	57.8±8.5	0.862
Ankle			
Initial contact	64.7±13.8	71.8±17.8	0.419
MAX	143.7±12.2	141.8±13.0	0.908
MIN	58.2±12.6	60.0±14.2	0.908
Swing			
Hip			
Toe-off	119.8±12.9	123.2±9.9	0.225
MAX	120.0±12.7	123.3±10.0	0.248
MIN	60.4±10.5	63.2±11.7	0.326
Knee			
Toe-off	103.4±13.8	100.3±15.9	0.644
MAX	104.0±13.8	100.7±15.9	0.624
MIN	47.7±7.6	45.5±8.0	0.525
Ankle			
Toe-off	147.6±11.6	146.2±12.3	0.954
MAX	154.2±11.4	152.2±10.8	0.954
MIN	42.9±9.1	47.2±11.9	0.564
Extension before toe-off	61.2±5.2	58.8±4.2	0.166

Note. Values are presented as mean±SD. Abbreviations: MAX, angular maximal; MIN, angular minimal.

The statistical analysis of differences in the joint angular positions between the MPSS and SS groups revealed almost no significant differences with respect to the magnitude of the angles (Tables 1–3). At the end of the experiment knee kinematics for the MPSS group showed a sagittal plane extension shift

through the stance phase. This increased extension was significantly different during the IC (P =0.013) and minimal (MIN) values (P =0.019).

Table 3
Kinematic data for the SS and MPSS groups at week 7.

Variables	SS	MPSS	P value
Stance			
Hip			
Initial contact	63.3±11.5	64.5±9.8	0.696
MAX	118.3±13.1	118.4±7.4	0.884
MIN	62.4±11.4	63.0±10.3	0.807
Knee			
Initial contact	65.4±10.2	75.5±9.1	0.013
MAX	84.7±9.7	89.1±6.6	0.283
MIN	49.8±7.8	57.3±7.5	0.019
Ankle			
Initial contact	66.4±10.3	76.5±17.8	0.306
MAX	132.7±15.1	126.6±11.7	0.188
MIN	57.5±10.1	63.8±15.3	0.283
Swing			
Hip			
Toe-off	116.5±12.9	116.3±7.0	0.961
MAX	116.5±12.9	116.4±7.0	0.922
MIN	61.2±12.6	60.7±10.0	0.961
Knee			
Toe-off	84.6±9.5	88.5±7.1	0.329
MAX	85.0±9.7	89.1±7.2	0.306
MIN	42.0±6.0	45.1±6.2	0.306
Ankle			
Toe-off	136.0±14.8	130.5±12.5	0.188
MAX	142.8±14.5	140.0±13.3	0.558
MIN	45.2±7.6	46.2±10.9	0.77
Extension before toe-off	58.5±5.2	56.2±3.8	0.174

Note. Values are presented as mean±SD. Abbreviations: MAX, angular maximal; MIN, angular minimal.
Significant at P b 0.05.

Additionally, changes in the transverse plane kinematics were assessed throughout the analysis of the foot progression. The 3D motion analysis for the foot was calculated in normal and injured animals (Fig. 7). During normal gait, the foot is rotated slightly external to the direction of progression. In contrast, lesioned animals showed increased external rotation during the entire stance and the second period of the swing phase (E1). While the analysis of the mean waveform of the MPSS group shows less external foot rotation, the individual trials reveal that some of the animals walked with an abnormal internal foot rotation.

Histology and stereology

Histological analysis showed that acute spinal cord contusion caused a large lesion characterized by spread tissue disruption. Tissue damage involved both white and gray mater and extended for about 10 mm upstream and downstream to the lesion epicentre where spinal cord organization was completely disorganized (Fig. 8).

Results of the comparison of stereological estimates of lesion extent are shown in Fig. 9. Statistical analysis by one-way ANOVA showed that none of the numerical differences observed between the two experimental groups was significant.

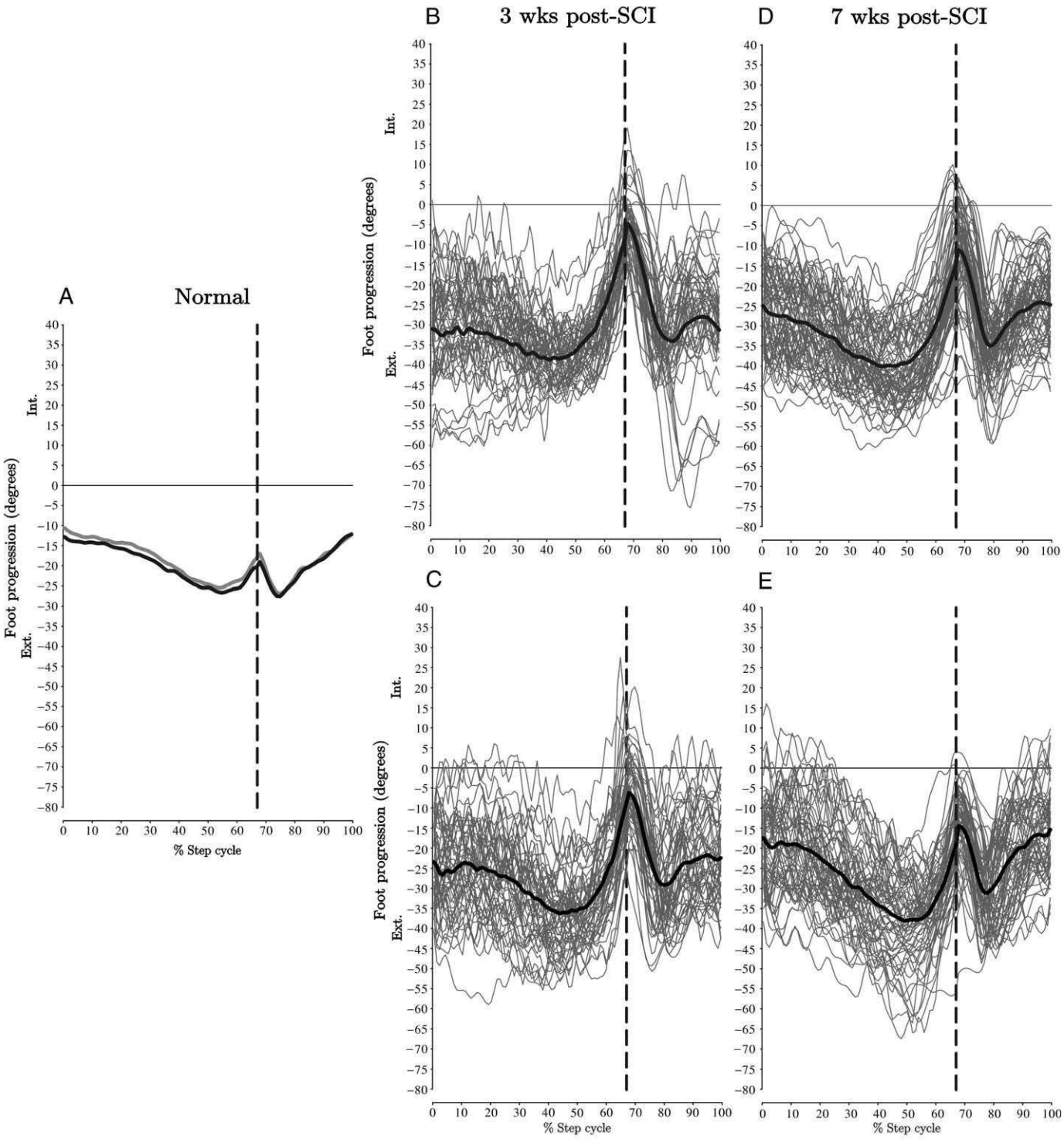

Fig. 7. The foot progression is the angle between the long axis of the foot and the line of progression. Temporal analysis of the foot progression in normal (A), and injured rats at 3 weeks for SS-treated (B) and for MPSS-treated (C), and at 7 weeks for SS-treated (D) and for MPSS-treated (E) post-contusion. The thin curves within each plot (B–E) represent seventy five individual trials. In pathological gait, the foot showed an increased external rotation; however there was a large intra-animal variability. Stance and swing phases were normalized. The stance duration was set at 67% of the step cycle duration. The vertical dashed line corresponds to the stance–swing transition.

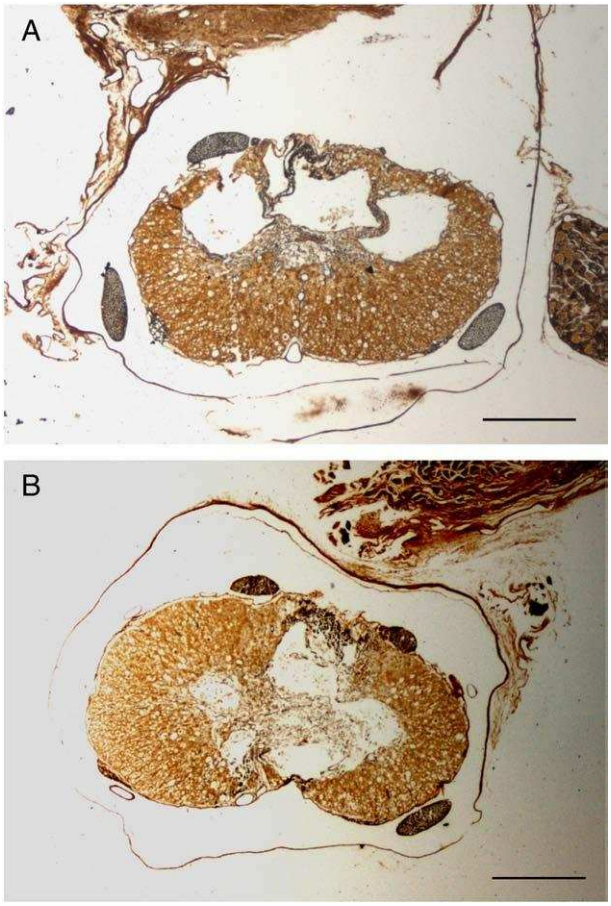

Fig. 8. Silver staining of spinal cord transverse sections in a SS (A) and a MPSS (B) treated animal taken at the lesion epicentre. Scale bar=500 μm.

Discussion

Despite major progress in pharmacological, surgical and rehabil-itative treatment approaches, SCI still remains a very complex medical and psychological challenge, with no curative therapy available (Rossignol et al., 2007). The animal model of contusion injury that we employed represents the typical injury mechanism in humans (Norenberg et al., 2004). The Infinite Horizon spinal cord injury impactor uses force as a user-defined variable rather than tissue displacement. This minimizes potential errors which may arise from any tissue movement during the surgical procedure (Scheff et al., 2003). In our study the injury was delivered with a force of 200-kdyn using the Infinite Horizon spinal cord injury device. This moderate injury was chosen in order to allow, during the recovery process, repetitive plantar stepping in the treadmill (Nessler et al., 2007).

A great variety of pharmacological agents have been studied extensively in acute SCI (Baptiste and Fehlings, 2008; Knafo and Choi, 2008). Although MPSS is not a Food and Drug Administration approved drug for use in acute SCI, it is still being used by many clinics as sole treatment for human SCI (Lee et al., 2008). The only pharmacological compound that has demonstrated neuroprotective ability in Phase III clinical trials of SCI has been MPSS. The results of the NASCIS II and NASCIS III trials recommended that MPSS should be given as a bolus dose of 30 mg/kg over 15 min followed by a continuous rate infusion of 5.4 mg/kg/h. If treatment is initiated within 3 h following SCI, the infusion would last for 23 h (total treatment time of 24 h). However, if treatment is initiated within 3–8 h following SCI, the infusion should be continued for 47 h (total treatment time of 48 h). MPSS should not be given if the patient arrives after 8 h or more following SCI (Bracken et al., 1990, 1997, 1998).

The precise mechanisms of action by which MPSS affect neuro-protection are not completely understood. In the mid-1960s the use of glucocorticoid steroids was based on the empirical notion that they would attenuate post-traumatic spinal cord oedema. More recently, proposed mechanisms include the

inhibition of lipid peroxidation and inflammatory cytokines, preservation of calcium homeostasis, preservation of spinal cord blood flow and modulation of the inflamma-tory/immune cells (Young, 2000). The central mechanism of the neuroprotective action of MPSS is closely linked to the inhibition of lipid peroxidation (Hall, 2003). A recent study provided evidence that MPSS selectively attenuates oligodendrocyte apoptosis after injury without affecting neuronal survival, via a glucocorticoid receptor-mediated mechanism (Lee et al., 2008).

An important goal of this investigation was to examine in detail the effects of MPSS on behavioral recovery following SCI in rats. While there are several ways to quantify recovery following SCI, the true measure of successful outcome is functional recovery (Metz et al., 2000). Several reviews have outlined the procedures for assessing functional recovery after spinal injury in experimental rodents (Goldberger et al., 1990; Wrathall, 1992; Kunkel-Bagden et al., 1993; Muir and Webb, 2000; Basso, 2004). These authors have recognized the need for a variety of tests to assess behavioral recovery in order to provide a means to monitor progressive, post-injury changes in function. Of all the detrimental effects of SCI, one of the most devastating is the inability to perform functional movement. For this reason, our emphasis was on quantifying functional motor behaviors. To accomplish this goal we included traditional methods such as the BBB score, the inclined plane, the beam walk, footprint analysis and the horizontal ladder.

In order to refine motor assessment we collected 3D continuous kinematic gait measures. The kinematic gait data produced by these measures are referred to as continuous because an infinite number of values are possible within a certain range, unlike data produced by ordinal scales (Muir and Webb, 2000). Recent technological advances have made the collection of 3D movement data both possible and desirable. Previously we showed that maximal precision and accuracy of the hindlimb kinematic parameters are achieved when the experimental protocol includes 3D calculation (Couto et al., 2008).

The functional results of this study suggest that using the NASCIS II dosage of MPSS following acute spinal cord contusion has no effect on neurological recovery after 7 weeks compared with SS. The results of the traditional methods showed that there were no significant differences in the functional recovery of MPSS- vs. SS-treated animals. Open-field locomotor testing with the BBB showed that both post-injury groups improved locomotor behavior. At the time a plateau in open-field recovery was reached, 4 weeks post SCI, the animals had a score of 12–13. At the end of the experiment the base of the support was still increased, reflecting disturbance in body balance and walking stability. Both experimental groups revealed similar deficits during beam walk and horizontal ladder testing. These tasks were used to assess deficits in descending fine motor control after spinal contusion. Following the currently used spinal cord contusion protocol, the most dramatic changes occur in the white matter of dorsal corticospinal and rubrospinal tracts (Cao et al., 2005). The dorsal corticospinal and rubrospinal tracts have been shown previously to be involved in precise hindlimb move-ments (Hendriks et al., 2006).

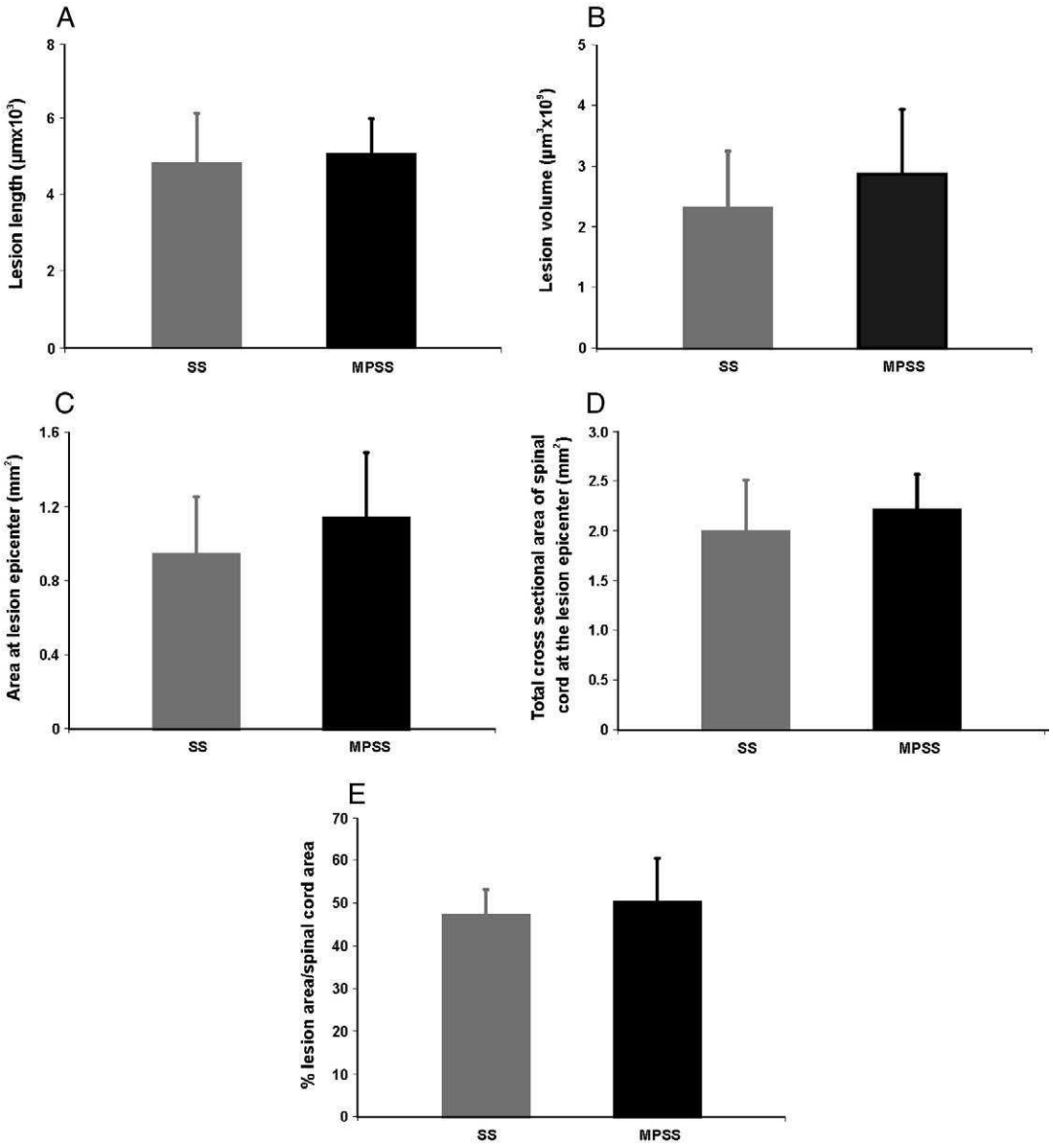

Fig. 9. Comparison of stereological estimates between SS (gray) vs. MPSS (black) regarding the lesion length (A), the lesion volume (B), the area of damaged tissue at the lesion epicentre (C), the total cross-sectional area of the spinal cord at the lesion epicentre (D) and the rate between the area of damage and total spinal cord area at the lesion epicentre (E) comparing SS (gray) vs. MPSS (black) groups.

Although 3D gait analysis is still an expensive proposition and time-consuming, it is a desirable method to more accurately describe the locomotor performance. In accordance with the results obtained from traditional tests we found that MPSS administration did not affect the flexion/extension of the hip, knee and ankle joints during the step cycle. The results of the 3D kinematic data before and after SCI showed that the general shapes of the joint angular motion were very similar for the MPSS and SS groups. Additionally, we included the transverse plane in order to describe the external/internal rotation of the foot during the stance and swing phases. Over the step cycle, the foot progression underwent large excursion about the transverse plane following SCI for both groups compared to normal walking. This may reflect their need to achieve additional lateral stability and body balance due to the spinal lesion (Metz et al., 2000).

Along with a comprehensive battery of behavioral tests for the assessment of functional recovery, we decided to include a morpho-logical evaluation of the extent of the lesion using stereology. Results of the stereological assessment were in line with the functional data and showed no significant differences in any of the parameters investigated in the present study.

The dose of methylprednisolone used in the most significant clinical trials, NASCIS II and NASCIS III, was the result of animal research at various MPSS dosing regimens after SCI (Braughler and Hall, 1982; McGinley et al., 1982; Braughler and Hall, 1983, 1984; Hall et al., 1984; Braughler et al., 1987). The initial

bolus and the continuous dosage of MPSS was based on NASCIS studies (Bracken et al., 1997, 1998) and on significant tissue sparing and behavioral recovery previously reported in spinal cord injured rats (Nash et al., 2002; Lee et al., 2008; Kim et al., 2009).

In agreement with our results, a number of experimental studies have shown that MPSS treatment does not have a positive effect on acute SCI. Despite promoting mild neuroprotection several authors found that MPSS did not improve functional recovery (Haghighi et al., 2000; Mu et al., 2000; Takami et al., 2000; Carlson et al., 2003). After a moderate contusion SCI, the administration of MPSS failed to increase the amount of spared gray and white matter and did not improve recovery of hindlimb function (Rabchevsky et al., 2002). Following a

photochemical lesion, administration of MPSS did not cause signifi-cant neuroprotection and recovery (Lopez-Vales et al., 2005). No locomotor improvement was recorded following moderate or severe clip-compression SCI after treatment with MPSS (Weaver et al., 2005). It has recently been shown that MPSS reduced cytokine production but failed to affect the rate of hindlimb recovery in rats following a contusion injury (Gorio et al., 2007). In a study of acute SCI, dogs treated with surgical decompression 6 h following injury did not show a significant difference in neurologic recovery with those treated with decompression and MPSS (Rabinowitz et al., 2008). A study designed to determine the levels and the duration of lipid peroxidation in a rat model of SCI suggested that MPSS had no effect at 24 h or later following the initial injury (Christie et al., 2008).

In addition, an increasing number of authors have criticized several aspects of NASCIS studies (Coleman et al., 2000; Hurlbert, 2006; Miller, 2008). Other clinical studies of MPSS treatment in acute SCI could not reproduce results obtained in the NASCIS II and III (Sayer et al., 2006). The most definitive finding of the NASCIS studies was that the use of a megadose of steroids was associated with detrimental effects such as wound infection, gastrointestinal hemorrhage, pulmonary embolism, severe pneumonia and sepsis and even death secondary to respiratory complications (Bracken et al., 1990, 1997).

In conclusion, the results of the current study add to the growing evidence that treatment of acute SCI with MPSS as a standard therapy may not be justified. Importantly, we have addressed the critical issue of a rigorous approach to the analysis of behavior following SCI. In addition to traditional functional and morphological assessment, we included a 3D hindlimb kinematic analysis during treadmill locomo-tion which gave more strength to our results. This analysis represents the most important novelty of our study.

Acknowledgments

This research was supported by the Luso-American Foundation (grant # L-V-315/2006), by the Operational Programme for Science and Innovation 2010 (Portuguese Ministry of Science, Technology and Higher Education) and by the Regione Piemonte (Progetto Ricerca Sanitaria Finalizzata).

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