

# Neuroprotective effect of local hypothermia in a computer-controlled compression model in minipig: Correlation of tissue sparing along the rostro-caudal axis with neurological outcome

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**Abstract.** This study investigated the neuroprotective efficacy of local hypothermia in a minipig model of spinal cord injury (SCI) induced by a computer-controlled impactor device. The tissue integrity observed at the injury epicenter, and up to 3 cm cranially and caudally from the lesion site correlated with motor function. A computer-controlled device produced contusion lesions at L3 level with two different degrees of tissue sparing, depending upon pre-set impact parameters (8N- and 15N-force impact). Hypothermia with cold (4°C) saline or Dulbecco's modified Eagle's medium (DMEM)/F12 culture medium was applied 30 min after SCI (for 5 h) via a perfusion chamber (flow 2 ml/min). After saline hypothermia, the 8N-SCI group achieved faster recovery of hind limb function and the ability to walk from one to three steps at nine weeks in comparison with non-treated animals. Such improvements were not observed in saline-treated animals subjected to more severe 15N-SCI or in the group treated with DMEM/F12 medium. It was demonstrated that the tissue preservation in the cranial and caudal segments immediately adjacent to the

lesion, and neurofilament protection in the lateral columns may be essential for modulation of the key spinal microcircuits leading to a functional outcome. Tissue sparing observed only in the caudal sections, even though significant, was not sufficient for functional improvement in the 15N-SCI model.

## Introduction

Currently there is robust experimental and some clinical evidence that hypothermia (local or systemic) may target multiple pathological mechanisms caused by SCI trauma. In principle, hypothermia reduces metabolic consumption and energy demands, having beneficial effects on cytoplasmic ATP stores and the maintenance of normal transmembrane ion and neurotransmitter gradients (1). Previous investigations have shown that the degree of ATP preservation depends on the hypothermic level as well as the severity of the insult. Erecinska *et al* (2) reported that hypothermia lowered the cerebral metabolic rate for oxygen and glucose two-fold to four-fold per 10°C reduction in temperature, in addition to reducing cerebral blood flow. Hypothermia can also provide benefits through its ability to preserve the blood-spinal cord barrier (BSCB), ameliorate local edema, suppress axonal swelling and development of gliosis in the peri-injury zones (3,4); also to reduce oxidative stress, apoptosis and inflammation (5-8). Spinal cord injury studies in animals treated with mild systemic hypothermia for 30 min to 4 h have provided positive results for locomotor function and neuronal tissue maintenance. The protective influence of hypothermia has also been reported following its local application to the CNS. The advantage of local cooling using low temperature applied directly to the epicenter of injury obviates many of the

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risks of systemic hypothermia, such as arterial hypertension, bradycardia or pneumonia (9,10). Better outcomes have been achieved when modest cooling was started as soon as possible after trauma (4,5,8,11,12).

The development of a treatment strategy for acute spinal cord injury requires reliable and easily reproducible animal models allowing further characterization and better understanding of secondary pathophysiological mechanisms which could represent targets for therapeutic interventions. Preclinical and clinical trials have demonstrated that no treatment successful in rodents has been effective in humans so far. With regard to the similarity of their morphology and anatomical structures with humans, minipigs have already been selected as a useful SCI animal model for preclinical therapeutic trials (13,14).

In the current study we used a computer-controlled chronic compression model in Göttingen-Minesota-Liběchov minipigs (15) to test local spinal cord hypothermia treatment with saline or oxygenated culture medium (4°C) administered to the epicenter of injury beginning 30 min after traumatic impact. Because previous experimental and human clinical studies have shown effective transdural diffusion of epidurally administered analgesics, anesthetics and antibiotics (16-18), we anticipated that transdural diffusion of the saline or oxygenated culture medium at the lesion site without opening the spinal dural sac could lead to a positive treatment effect by maintaining local cellular homeostasis. Local hypothermia carried out through a special spinal perfusion chamber, prepared using 3D scanner technology, provided outright 5 h-long perfusion of the lesion site (L3) with cold solutions (4°C) administered by means of a peristaltic pump (flow 2 ml/min), and allowed stable body temperature to be maintained during the whole perfusion period. The temperature of solutions in the perfusion chamber just above the lesion site was maintained at 19°C.

The study provides evidence of considerable loss of neurofilament immunoreactivity (NF-IR), and reduced tissue degradation seen at the lesion site and away from the lesion epicenter (3 cm caudally as well as cranially) nine weeks after SCI (8N and 15N force), and it supports the beneficial effect of hypothermia with saline to promote the number of neurofilaments and gray and white matter preservation, and although without statistical significance, also functional improvement.

## Materials and methods

**Animals.** Thirty adult female minipigs (Göttingen-Minnesota-Liběchov crossbred strains; 5-8 months of age, weighing 25-35 kg) were used in the experiment. The minipigs were delivered from Liběchov farm 3-4 months after their birth. They were group housed in connected pens and were maintained in standard conditions with *ad libitum* access to food and water. Female minipigs were used in order to avoid the development of penile prolapse that can be a serious complication after SCI performed at lumbar level. No estrus was observed in any experimental animal. The animals had only a basic feed ration compared with the ration used by breeders for pubertal gilts to stimulate sexual processes. This dose restriction therefore did not stimulate early sexual maturity. This fact, together with the stress (animal transport, daily manipulation) was probably the reason that no rut was reported during the period of housing and clinical experiments.

All procedures were carried out in accordance with the protocols approved by the State Veterinary and Food Administration in Bratislava (decision no. 1319/13-221) as well as by the Animal Use Committee at the Institute of Neurobiology, Slovak Academy of Sciences, and in accordance with the EC Council Directive (2010/63/EU) regarding the use of animals in research. All efforts were made to minimize the size of experimental groups and animal suffering.

**Experimental groups.** In a pilot study three minipigs were used for standardization of the compression technique by testing the U shape/circular aluminum bars, and two other minipigs were assigned for testing of spinal perfusion chambers. These animals were excluded from immunohistochemical, histochemical and behavioral analyses.

Twenty-five animals were randomly divided into six experimental groups: i) control group without surgical intervention (n=3); ii and iii) spinal cord compression at the L3 segment induced by 8N (n=4) or 15N (n=4) force; iv and v) groups that underwent spinal cord compression (8N force) and local hypothermia (5 h) with saline (n=4) or oxygenated culture medium (DMEM/F12) (n=3); vi) group that underwent spinal cord compression (15N force) and local hypothermia (5 h) with saline (n=7). All experimental animals survived for 9 weeks.

**Pre-surgical procedures.** The minipigs were premedicated with an intramuscular injection of Stresnil (2 mg/kg; Janssen Pharmaceutica, Beerse, Belgium) and Atropin (0.5 mg/kg; Biotika a.s., Slovenská Ľupča, Slovakia), and then anesthetized with Thiopental (10 mg/kg; Czech Pharma, Praha, Czech Republic). Prior to surgery, the precise location of the L3 vertebra was identified using plain X-rays in a lateral projection (Fig. 1) (19). Afterwards the animals were intubated with an endotracheal tube (diameter 5.0-6.0 mm) and the anesthesia was maintained with Sevoflurane (1.5%; Baxter Czech spol. s r.o., Praha, Czech Republic) mixed with oxygen. For both the surgical procedure and the duration of hypothermia, the analgesia was supported by the administration of Butomidor (0.4 mg/kg; i.v.; Richter Pharma AG, Wels, Austria). Catheters for the administration of infusions and medicaments were inserted bilaterally into the cephalic and/or auricular veins.

**Spinal cord compression.** The minipigs were placed in a special immobilization apparatus consisting of a basal oval stainless steel platform (50x90 cm) with four horizontal bars (2 cm in diameter) and four vertical bars (size 3x9 cm and 23 cm length) (Fig. 1A). The apparatus was placed on a standard operating table. The lumbar portion of the minipigs was fixed in a prone position with the horizontal bars slid bilaterally against the lateral portion of their paravertebral muscles. After disinfection of the lumbar area, a midline skin and subcutaneous fat incision was made from L2 to L4 vertebrae. Paraspinal muscles were retracted to access the L2-L4 laminae and a laminectomy was carried out. The SCI was induced at the L3 segment using a compression device consisting of a computer-controlled stepping motor (ViDiTo, Kosice, Slovakia) and a digital force gauge bridged to a 5-mm diameter circular aluminum bar (Fig. 1B). The computer software (FORCE; ViDiTo) controlling the compression arrangement permitted parameters such

as compression rate, pressure and force to be obtained (15). To control the uniformity of the impact force, the compression curve was recorded in each animal. After spinal cord compression (8N or 15N force; velocity: 30 mm/sec.), the paraspinal musculature and skin were sutured with several absorbable stitches and the animals were housed in separated cages to recover.

**Preparation of perfusion chamber.** Two types of chambers were used. In the initial experiments the closed chamber was fabricated to fit into the injury site. For that, in two animals the site of laminectomy was scanned with an eScan 3D laser scanner (3D Digital Corp., Sandy Hook, CT, USA). Data were then transferred to Rhinoceros 3D software (MCNeel & Associates, Seattle, WA, USA) where the scanned data were used to finalize a prearranged model chamber, to make the bottom surface of the chamber fit perfectly with the bones over the injury site. The spinal perfusion chamber therefore perfectly fitted the injury site (Fig. 2A). The finalized perfusion chamber was then printed in ABS plastic using a uPrint SE 3D printer (Stratasys, Eden Prairie, MN, USA). The spinal perfusion chamber was fitted with inflow and outflow tubing. However, the closed chamber did not allow cleaning of the spinal spinal dural sac, which was frequently covered with blood clots, preventing contact between the solution and the medullary tissue. For this reason these animals were excluded from the experiment. For all following experiments, an open design of the chamber was prepared. The chamber in the shape of a cone with both ends opened (Fig. 2B and C) was printed from ABS plastic using a uPrint SE 3D printer (Stratasys). The spinal perfusion chamber had ellipse-shaped openings, the lower opening measuring 20x14 mm and the upper one 40x30 mm, and the height of the chamber was 50 mm. The spinal perfusion chamber was fitted with two tubings (inflow and outflow). An individual chamber was fabricated for each animal.

**Implantation of perfusion chamber.** The perfusion chamber was implanted over the exposed spinal dural sac containing the spinal cord (Fig. 2B and C). The smaller opening was about the size of the laminectomy, so it could be freely placed directly on the dura mater with no additional fixation to surrounding tissue. Only two stitches partially closed the wound, so the skin edges slightly embraced the chamber. No additional pressure was generated, since the weight of the chamber was minimal (4 g), and the paraspinal muscles and subcutaneous fat layer around conical chamber would lift it, rather than push it down. The contact of the chamber with dura was checked periodically during removal of blood clot from the surface of dura mater through perfusion chamber. No leakage was detected. Since the chamber was placed freely on the dural surface, without any fixture to the spine or paravertebral muscles, the edema which developed after SCI just lifted the chamber, without causing any additional harm.

**Hypothermia.** Hypothermia with saline or porcine medium (DMEM/F12) consisting of rh-Transferrin 100 mg/l, rh-Insulin 25 mg/l, Glucose 1.56 g/l, Progesterone 6.3 µg/l, Putrescine 16.1 mg/l, Selenium 5.2 µg/l (Cell Guidance Systems Ltd., Cambridge, UK) was carried out locally through each spinal

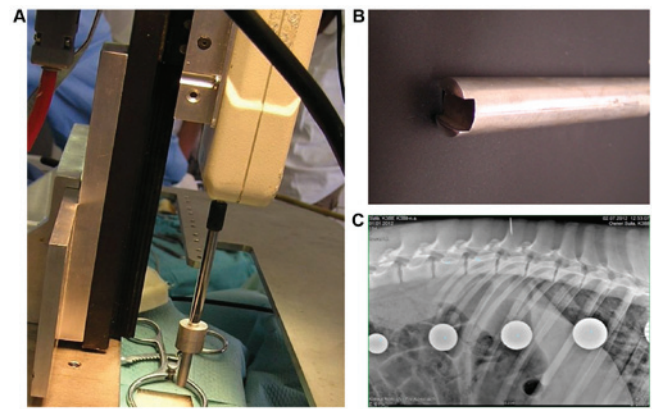


Figure 1. (A) Computer-operated compression apparatus consisting of a computer-controlled stepping motor and (B) digital force gauge bridged to a 5-mm diameter circular bar with U-shaped tip. (C) Correct localization of laminectomy was determined using plain X-rays of the lumbar vertebral column in a lateral projection.

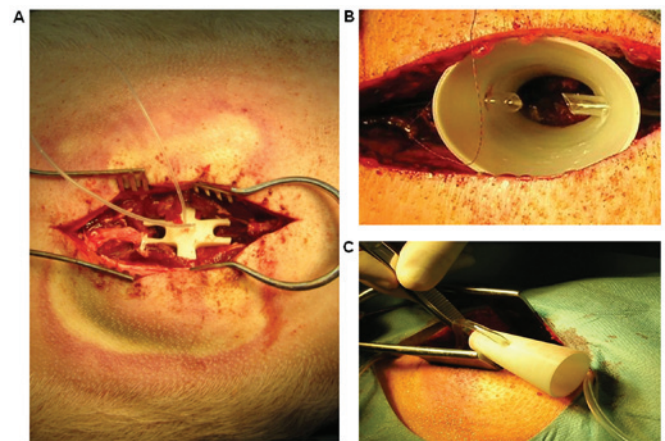


Figure 2. Special spinal perfusion chamber implanted over the exposed spinal dural sac to allow local perfusion of injured tissue with treating solutions. (A) Closed design of the chamber was not useful due to limited contact of solution with spinal tissue, so we used (B and C) an open chamber enabling cleaning and monitoring of the spinal dural sac surface.

perfusion chamber. The inflow tubing of the perfusion chamber was connected to the tank with sterile ice cold (4°C) perfusion solution. The cooling of the site of injury was initiated 30 min after SCI. At the beginning the solution drip rate was manually adapted to ensure that the temperature of fluid in the perfusion chamber (just above the SCI) was maintained at 19°C. Given that cooling was applied locally, the temperature of the solution in the chamber decreased to 19°C very rapidly, within 4-5 min. The temperature of solutions in the perfusion chamber was measured at 15 min intervals using a needle-tip thermometer (Omega Bio-Tek, Inc., Norcross, GA, USA) during the whole treatment procedure (5 h). Continuous inflow of the perfusion solution into the chamber was regulated by a peristaltic pump (Heidolph, BRD, the flow 2 ml/min). Outflow tubing was used to drain excess perfusion solution.

To minimize variability in body temperature during hypothermic treatment the minipigs were covered with a thermal blanket. The rectal temperature of each animal was measured at 15 min intervals using a digital predictive thermometer



Table I. 20-point neurological scale for minipigs.

Scale	Description
0	Complete paraplegia, no movement in joints of hindlimbs, spontaneous defecation and urination
1	Slight movement in one joint (hip joint) of hindlimbs, not capable of sitting and plantar placement of hindlimbs during sitting, spontaneous defecation and urination
2	Slight movement in two joints (hip and knee joint) of hindlimbs, not capable of sitting and plantar placement of hindlimbs during sitting, spontaneous defecation and urination
3	Slight movement in all three joints of hindlimbs, capable of sitting on the side without plantar placement of hindlimbs during sitting, spontaneous defecation and urination
4	Extensive movement in one joint (hip joint) and slight movement in two joints of hindlimbs, capable of sitting on the side without plantar placement of hindlimbs during sitting, spontaneous defecation and urination
5	Extensive movement in two joints of hindlimbs and slight movement in third joint of hindlimbs, capable of straight sitting and plantar placement of hindlimbs during sitting, spontaneous defecation and urination
6	Extensive movement in all three joints of hindlimbs, capable of straight sitting and plantar placement of hindlimbs during sitting, spontaneous defecation and urination
7	Extensive movement in all three joints of hindlimbs, not capable of standing up, sweeping with one hindlimb, spontaneous defecation and urination
8	Extensive movement in all three joints of hindlimbs, not capable of standing up, sweeping with both hindlimbs, spontaneous defecation and urination
9	Sweeping with both hindlimbs, not capable of plantar hoof stepping, attempt to hold stand with initial help to stand up
10	Sweeping with both hindlimbs, capable of weigh support with plantar surface of hoof with initial help to stand up only, not capable of walking
11	Capable to stand up itself, not capable to maintain balance, walking with support only (falling back on wall), position only on dorsal surface of hoof
12	Capable to stand up itself on hindlimbs and capable standing, able to walk 1-3 steps with occasional support without forelimb-hindlimb coordination, occasional plantar-hoof stepping
13	Capable of standing up spontaneously and able to take 1-3 steps without support before losing balance, no forelimb-hindlimb coordination, frequent plantar-hoof stepping and capable to keep balance between stepping episodes
14	Capable of standing up spontaneously and able to take 3-5 steps, capable to keep balance between stepping episodes with occasional support only, no forelimb-hindlimb coordination, occasional to frequent plantar-hoof stepping
15	Capable of standing up spontaneously and able to take 3-5 steps, capable to keep balance between stepping episodes, frequent plantar-hoof stepping, no forelimb-hindlimb coordination
16	Capable of standing up spontaneously and able to take 5-10 steps, capable to keep balance, frequent plantar-hoof stepping, no or inconsistent forelimb-hindlimb coordination
17	Capable of standing up spontaneously and able to take 5-10 steps, capable to keep balance, frequent plantar-hoof stepping, occasional forelimb-hindlimb coordination
18	Capable of standing up spontaneously and able to take 5-10 steps, capable to keep balance, frequent plantar-hoof stepping, frequent forelimb-hindlimb coordination
19	Capable of standing up spontaneously and able to take more than 10 steps, capable to keep balance, consistent plantar-hoof stepping, frequent forelimb-hindlimb coordination
20	Capable of standing up and walking spontaneously, consistent plantar-hoof stepping, consistent forelimb-hindlimb coordination

Scale 1-8, the movement of hindlimbs in each individual joint; scale 9-11, the ability to stand up; scale 12-20, the ability to walk.

with the use of lubricant. Each single rectal measurement took 10-20 sec. When the temperature dropped below 36°C the animals were warmed with a stream of warm air (a hair dryer blew warm air below the blanket covering the animal). This heating method was very efficient and maintained the minipigs at normothermia during the whole procedure. The rectal temperature varied during hypothermia from 35.4°C±0.4 to 36.6°C±0.4. After finishing the therapy the perfusion chamber was explanted and the incision was closed with absorbable sutures. The animals were housed in separated cages to recover.

*Post-operative animal care.* Post-operative care included bladder expression twice a day, antibiotic administration (Penstrepten (0.5 ml/30 kg/day; Biotika a.s.) for 10 days, cleaning the hind limbs and monitoring of skin irritation and development of decubitus ulcers. Abrasions of the tail and perianogenital region occurred in 18% of animals (3 out of 4) subjected to 15N SCI and 2 out of 7 treated with cold saline after 15N SCI. The affected areas (bruises around the tail) were treated twice a day with 0.1% solution of Rivanol and with Alamycín spray (Norbrook, Newry, Northern Ireland,

UK) for about one week. The animals survived for an additional 2 months without further complications. Pressure sores or other skin lesions were not observed in the 8N SCI group, 8N SCI animals treated with cold saline or medium, or in the rest of animals after 15N SCI or 15N SCI and treatment.

*The 20-point porcine neurological scale to determine hind-limb motor recovery after spinal cord compression.* Recently, the porcine neurological motor score was re-graded on a 10-point scale according to Lee *et al* (20) or a fourteen-point grading scale according to Navarro *et al* (15). Although both protocols are well validated and easily replicated, the assessment of functional recovery in this study took into principal consideration the hind-limb movement for each individual joint, the capability of each animal to get up by itself, its trunk stability and stepping coordination. The scoring points range from complete paraplegia (zero) to normal ambulation (20 points) and represent three sequential recovery stages which the minipigs attained after SCI and hypothermic treatment. The 20-point neurological scale for minipigs is described in detail in Table I. Porcine neurological motor scores (1-8) represent slight or extensive movement for each individual joint of the hind limbs, points (9-11) represent sweeping and the ability to get up by itself (with or without assistance; these animals were not able to maintain balance), and points (12-20) characterize the ability to walk from a few steps without fore-limb/hind-limb coordination to consistent plantar-hoof stepping and consistent fore-limb/hind-limb coordination (see representative pictures in Fig. 3).

*Behavioral assessment.* Three weeks before the surgery the minipigs underwent half an hour of training for 5 days/week to walk upon command up and down a rubber mat (width 0.8 m, length 5.2 m). One week before SCI the minipigs were videotaped. Post-operative neurological impairment was tested under blinded outcome assessment two days after surgical procedures, and continued weekly for nine weeks of survival. Each minipig was allowed to walk freely on a rubber mat for 10 min without assistance. A video recording was made of each evaluation. In order to reduce bias, three independent observers were involved in the development of the neurological scale. To blind the outcome assessment, the observers graded the neurological functions independently without knowledge of the treatment. To minimize the variability, each camera record was re-scored by the same three observers. The inter-rater differences in the scores varied max by 1 point. The discrepancies obtained by the raters from two independent assessments were discussed and clarified. Based on direct behavioral observations of neurological recovery and re-scoring the videos we developed our own porcine neurological scale to determine hind-limb motor recovery after spinal cord compression (Table I).

*Spinal cord tissue preparation.* At the end of survival, the minipigs were deeply anesthetized with an intravenous overdose of thiopental and transcardially perfused with heparinized saline (5l), followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4, 5l). The lumbar spinal cord (approximately 10 cm) was carefully dissected out,

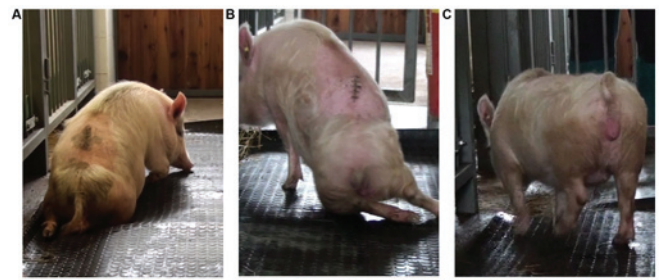


Figure 3. Representative pictures of the porcine behavioral scoring system (points 0-20). (A) Points 1-8 represent the movement of hind limbs in each individual joint. (B) Points 9-11 represent hind-limb sweeping and the animal's ability to stand up by itself. (C) Points 12-20 represent the ability to walk. The neurological scale for minipigs is described in detail in Table I.

cleaned of the dura mater and post-fixed in the same fixative overnight. Next day, the spinal cord was cut into one 1 cm block (lesion site) and three 1 cm blocks caudally (-1, -2, -3) as well as cranially (+1, +2, +3) from the epicenter of injury. In the next step, the spinal cord pieces were cryopreserved in a solution of 30% sucrose in PBS at 4°C for 48 h. Afterwards, each tissue block was cut into transverse serial sections (30  $\mu$ m thick) on a cryostat (Leica Microsystems, Wetzlar, Germany) and stored in PBS. Transverse serial sections were mounted on slides and were processed in accordance with immunofluorescence and histochemical procedures.

*Immunofluorescence staining.* For processing of neurofilament immunoreactivity, the slices taken from the rostral and caudal blocks were incubated in blocking solution consisting of 5% normal goat serum in 0.1 M phosphate-buffered saline (PBS) with 0.3% Triton-X 100 for 1 h at room temperature. After blocking the slices were incubated overnight at 4°C with primary monoclonal mouse antibody anti-neurofilaments (Pan-Axonal Neurofilament Marker SMI312, 1:1,000; cat. no. ab24574; Abcam, Cambridge, UK) diluted in 0.1 M PBS with 5% normal goat serum and 0.3% Triton-X 100. Next day, the sections were washed three times in 0.1 M PBS for 10 min and incubated with secondary anti-mouse antibody (Rhodamine Red™-X conjugated AffiniPure goat anti-mouse IgG, 1:200; cat. no. 115-295-003; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in Tris-buffered saline containing 0.1 M phosphate buffer (7.4 pH) with 5% normal goat serum and 0.3% Triton-X 100 for 90 min at room temperature. The sections were finally rinsed in 0.1 M PBS and cover-slipped with Fluoromount-G (cat. no. 21644; Serva Electrophoresis. GmbH, Heidelberg, Germany). Microphotographs were taken using a fluorescence microscope (Olympus BX51/BX52; Olympus, Hamburg, Germany) with an Olympus DP50 digital camera coupled with a computer equipped with Olympus DP Image software (version 3.1). Neurofilament immunoreactivity was evaluated using the Image J graphics software. SMI312 labeled axons were counted by a blinded observer using a 20x lens in a 800x600  $\mu$ m area in three specific regions of the white matter (dorsal, lateral and ventral columns) (Fig. 4). The number of neurofilaments (expressed as percentage of NF/mm<sup>2</sup>) was evaluated bilaterally from five randomly-selected transverse slices of each spinal cord block.

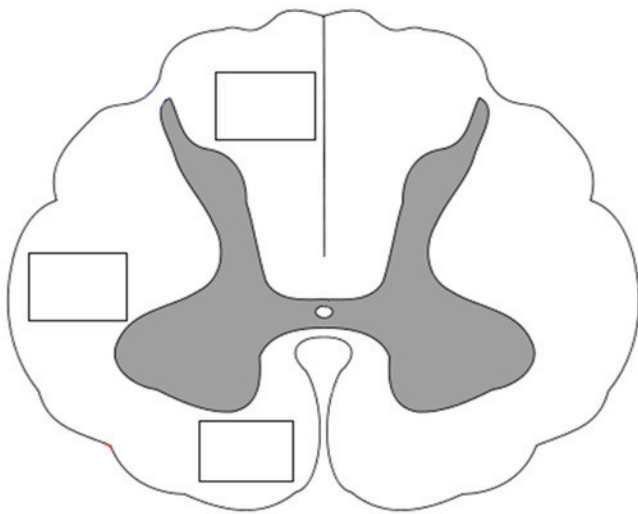


Figure 4. Representative picture of the spinal cord showing localization of three areas of interest taken for quantitative analysis of neurofilaments.

**Histological analyses.** The spinal cord slices (lesion site and 1 cm blocks in rostral and caudal directions) were stained according to the protocol for luxol fast blue and cresyl violet staining to recognize myelinated fibers in white matter and the residual preserved tissue. The percentage of total volume of preserved spinal tissue was evaluated using an Olympus (BX51/BX52) light microscope and ImageJ software.

**Data acquisition and statistical analysis.** The results from neurological analyses were assessed in a blinded manner and statistically evaluated using Kruskal-Wallis or Mann-Whitney tests. All immunohistochemical and histological data were analysed in a blinded manner using one-way ANOVA test.  $P < 0.05$  was considered to indicate a statistically significant difference. All outcomes were expressed as mean values with standard errors of the mean (SEM). The correlation between degree of neurological outcome, number of neurofilaments and gray and white matter preservation was assessed using the Pearson correlation test. The test was used to evaluate the coefficient of determination  $r^2$ .

## Results

**Behavioral outcomes.** Recovery of motor function was assessed using a 20-point scoring system representing the recovery stages which the minipigs attained after SCI and local hypothermia with saline or porcine medium (DMEM/F12). In the first two weeks after SCI (impact force 8N and 15N) the behavioral status of each animal was very low in both experimental groups (Fig. 5). These animals showed total loss of locomotor function in the hind-limbs or just slight movement in hip and knee joints (score 0-2). The outcome improved notably from the 2nd to 5th week in the group of minipigs which underwent impact with 8N force. Hind-limb movement in this group of animals recovered spontaneously from the 5th week and later on, during the last two weeks the outcome was stabilized. In the 9th week the motor score reached  $10.8 \pm 2.2$ . In the animals which underwent 15N impact, very slow spontaneous functional recovery was observed. At the end of the

survival time the mean score for motor outcome in this group was graded  $6.3 \pm 0.5$ .

Animals treated with hypothermic saline perfusion following 8N compression demonstrated continuous slight improvement during the whole survival period (Fig. 6A). Although the difference in outcome between the two groups (SCI vs. SCI and hypothermia with saline) was not significant at the end of the survival period, the improvement after treatment exceeded compression by 3 scoring points ( $10.8 \pm 2.2$  SCI;  $13.7 \pm 1.8$  SCI and hypothermia with saline). The saline-treated animals were able to take 1-3 steps with initial help to stand up and keep balance between stepping episodes, but the movement was without fore-limb/hind-limb coordination. Surprisingly, this group of animals had substantially better recovery rate than the minipigs treated with hypothermia using porcine medium (DMEM/F12) containing a supplement of nutrients. During the first post-injury week, no animal treated with  $4^\circ\text{C}$  medium recovered and complete absence of movement in the hind-limbs or at most slight movement in only one joint was observed. Up to the end of the survival period the medium-treated minipigs showed lower motor function than untreated animals (8N compression) and at the 9th week the motor score reached only  $9.0 \pm 2.3$  points. The behavioral study also showed that recovery in the 15N SCI group treated with cold saline for 5 h was only slightly better compared with the untreated group (Fig. 6B). From the 4th to 9th week the minipigs treated with  $4^\circ\text{C}$  saline improved their outcome over compression only by 1 scoring point. This group of animals was not capable of standing up, but we noted sweeping with one hind limb.

**Quantitative analysis of neurofilament immunoreactivity.** The immunoreactivity for neurofilaments was strong in the dorsal funiculi compared to other white matter columns, and was equally distributed throughout the lateral and ventral column axons of control animals (Fig. 7). Nine weeks after SCI axons in the white matter funiculi were weakly stained with various degrees of staining. Quantitative analysis showed significant decrease in NF intensity generally in all rostral and caudal segments, with the lowest number of NF-IR profiles near the epicenter of injury (+1 and -1 segments) (Figs. 8 and 9). In the group which underwent 15N SCI we found the most striking alterations at the -1 segment in dorsal and lateral columns (38, 58%) (Fig. 9). Hypothermia with saline significantly increased the number of neurofilaments in each white matter column, with the most extensive improvement seen in the lateral funiculi (Figs. 8 and 9). The return of NF staining appeared greater in segments (-1, -2, -3) below the SCI than in the rostral segments. Extensive improvement was also noticed in the dorsal column, displaying a similarly high proportion of NF staining in the caudal segments in saline-treated minipigs. In contrast, treatment of injured spinal cord with hypothermic porcine medium (DMEM/F12) solution demonstrated no significant increase in neurofilament intensity in any of white matter columns in comparison with the compression group (Fig. 8).

**Histological assessment.** Luxol fast blue combined with cresyl violet staining of white and gray matter produced a marked contrast between intact and damaged spinal cord tissue.

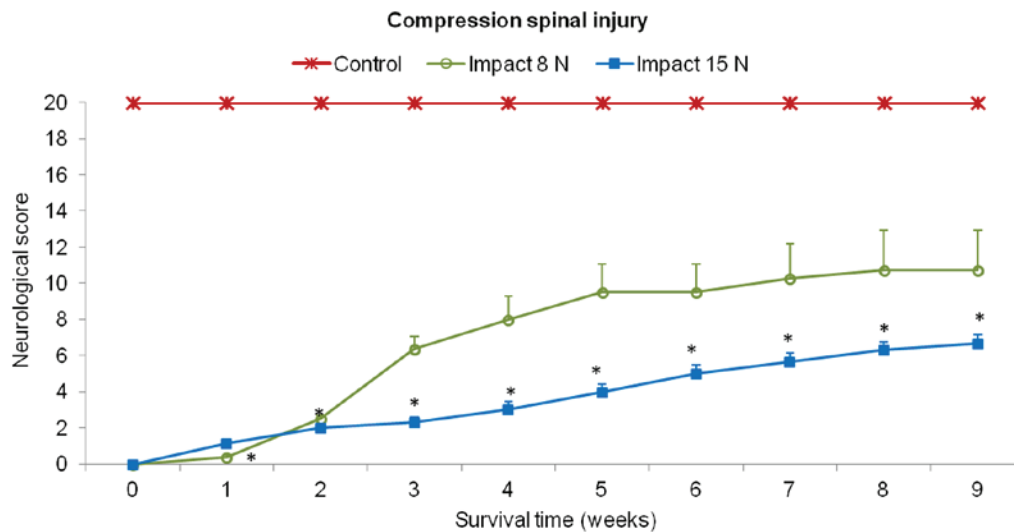


Figure 5. Graph representing motor function recovery analyzed in control animals and those after L3 spinal cord compression (8N force impact; 15N force impact) using the porcine behavioral scale (0, complete paraplegia with loss of hind-limb movements; 20, normal ambulation and balance) during nine weeks of survival. Kruskal-Wallis testing revealed statistically significant differences between control and spinal cord compression groups (\* $P < 0.05$ ). Data are given as means  $\pm$  SEM (n=3 in control group, n=4 in SCI groups). SCI, spinal cord injury.

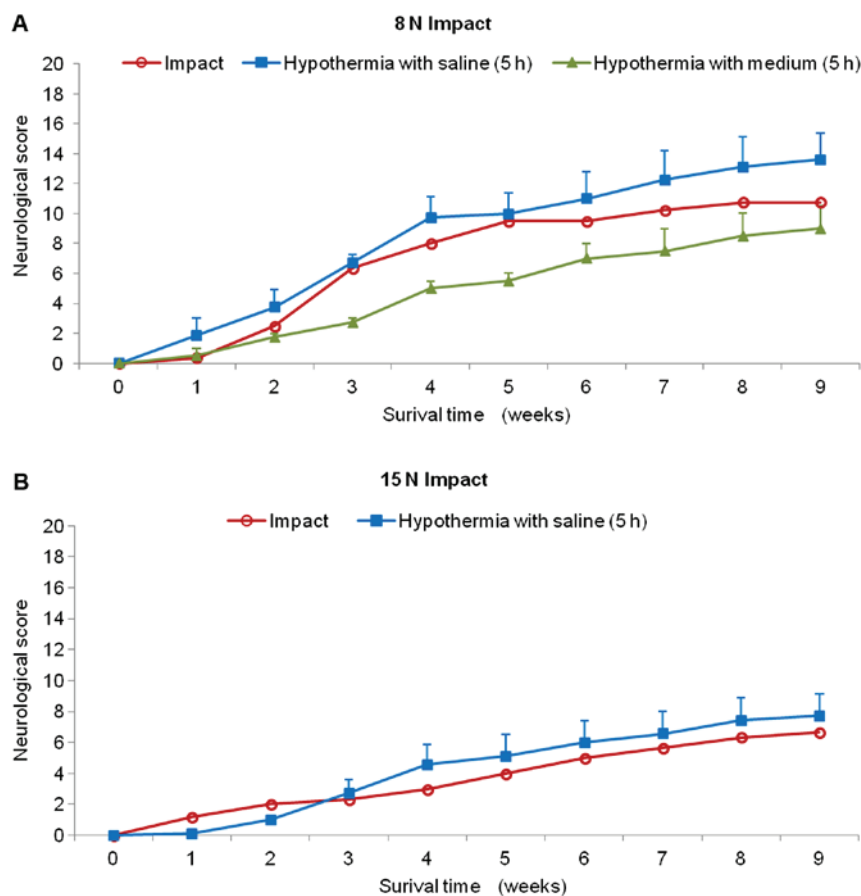


Figure 6. Locomotor function recovery assessed using a 20-point porcine scale in minipigs after undergoing L3 spinal cord compression (force 8N in A; force 15N in B) and treatment with local hypothermia (saline or oxygenated culture medium DMEM/F12 in A; saline in B). All experimental animals survived for nine weeks. Data are given as means  $\pm$  SEM (n=3-7).

Histopathological assessment of transverse serial sections showed congruous loss of myelin sheaths after trauma inflicted with 8N as well as 15N force and nine weeks of survival. Tissue degradation was visible in white as well as gray matter in both

compression groups. Major spinal tissue destruction occurred at the lesion epicenter and in segments near the site of injury (Fig. 10). The lesion area extended in both directions cranially and caudally from the epicenter. With increased compression



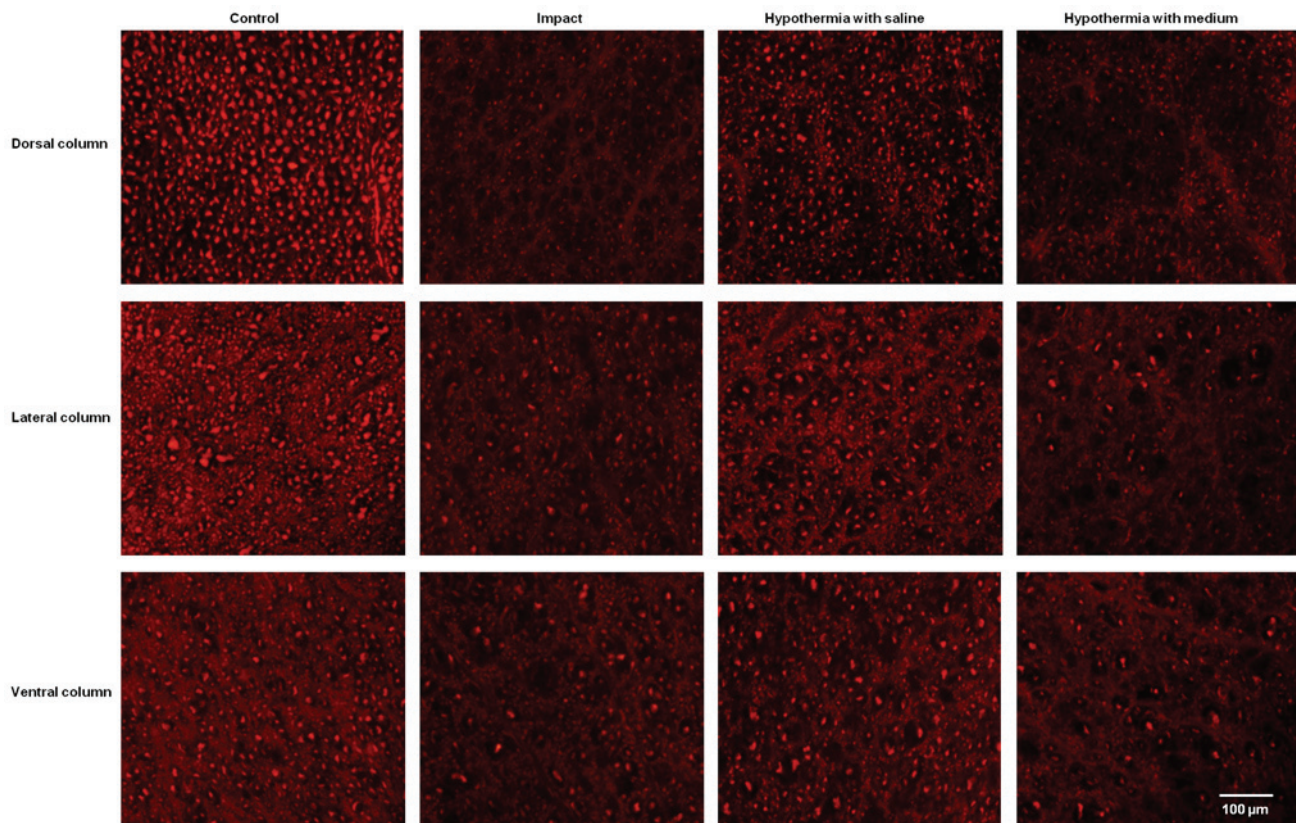


Figure 7. Microphotographs showing neurofilament immunoreactivity in the dorsal, lateral and ventral columns of the lumbar spinal cord (segment -1). Spinal sections were taken from the control group, the group with 8N- force impact injury and the group treated with local hypothermia with saline or porcine medium DMEM/F12 for 5 h after spinal cord injury. Scale bar=100  $\mu$ m.

of the spinal cord the structural loss of spinal tissue became greater.

In both compression groups, the gray matter preservation at the lesion site was quite low (53 and 33%). The preservation was significantly higher in saline-treated animals in +3, +1, -1 and -2 segments, compared with untreated 8N SCI minipigs, and almost no gray matter loss was seen in +3 and -2 segments. In the animals which underwent 15N force impact and were treated with saline hypothermia, the improvement in gray matter preservation was significant in the caudal (-1, -2) segments (Fig. 11).

In the white matter columns, the volume of preserved tissue after SCI (8N and 15N) at the lesion site was less (28-41%) (Figs. 12 and 13) than in the gray matter. Low percentages of preserved white matter was also detected in the section (-1) of the lateral (39%) and dorsal column (45%) after 15N SCI, while after 8N SCI the preservation in the same segments was better and in the 9th week of survival it reached 50 and 57% respectively. Hypothermia with porcine medium solution produced only minimal difference between untreated and treated animals (Fig. 12). Quite different results were noted after perfusion of the injured spinal cord with 4°C saline. We noted white matter preservation in the rostral and caudal sections of each white matter column, with substantial protection seen around the lesion site in the dorsal column. The most significant attenuation of white matter damage as a whole was seen after SCI (8N) treatment in the lateral column. In the 15N compression group the most effective percentage of preserved white matter was noted in the dorsal column at the site of injury and caudally (-1, -2) from the

lesion site. The differences in preserved white matter between groups are shown in Figs. 12 and 13.

*Correlative analyses between behavioral outcome, neurofilament immunoreactivity and histological assessment.* To determine the relationship between the histological damage to the spinal cord and behavioral outcome in animals treated with saline for 5 h, we utilized both SCI groups (8N, 15N force). As shown in Fig. 14, the number of neurofilaments counted in the white matter positively correlated with behavioral recovery. Similarly, positive correlation was observed between behavioral score and cumulative white and grey matter preservation.

Correlative analysis between the extent of axonal loss quantified in the dorsal, lateral or ventral funiculi and the degree of neurological dysfunction demonstrated positive correlation in the lateral funiculi with values  $r^2=0.4691$  in the rostral section,  $r^2=0.4992$  at the epicenter of injury, and a relatively high degree of correlation  $r^2=0.8164$  ( $P<0.05$ ) in the caudal section. Significant correlation was also found in the dorsal column, but only in the rostral section ( $r^2=0.9511$ ;  $P<0.05$ ) (Fig. 15). Measurements of gray matter preservation (lesion site, and caudally as well as cranially from the epicenter of injury) significantly correlated with the neurological score (Fig. 16).

## Discussion

*Is the preservation of the lesion site in the minipig SCI model a predictor of functional outcome?* Hypothermic treatments have been reported to be beneficial for some



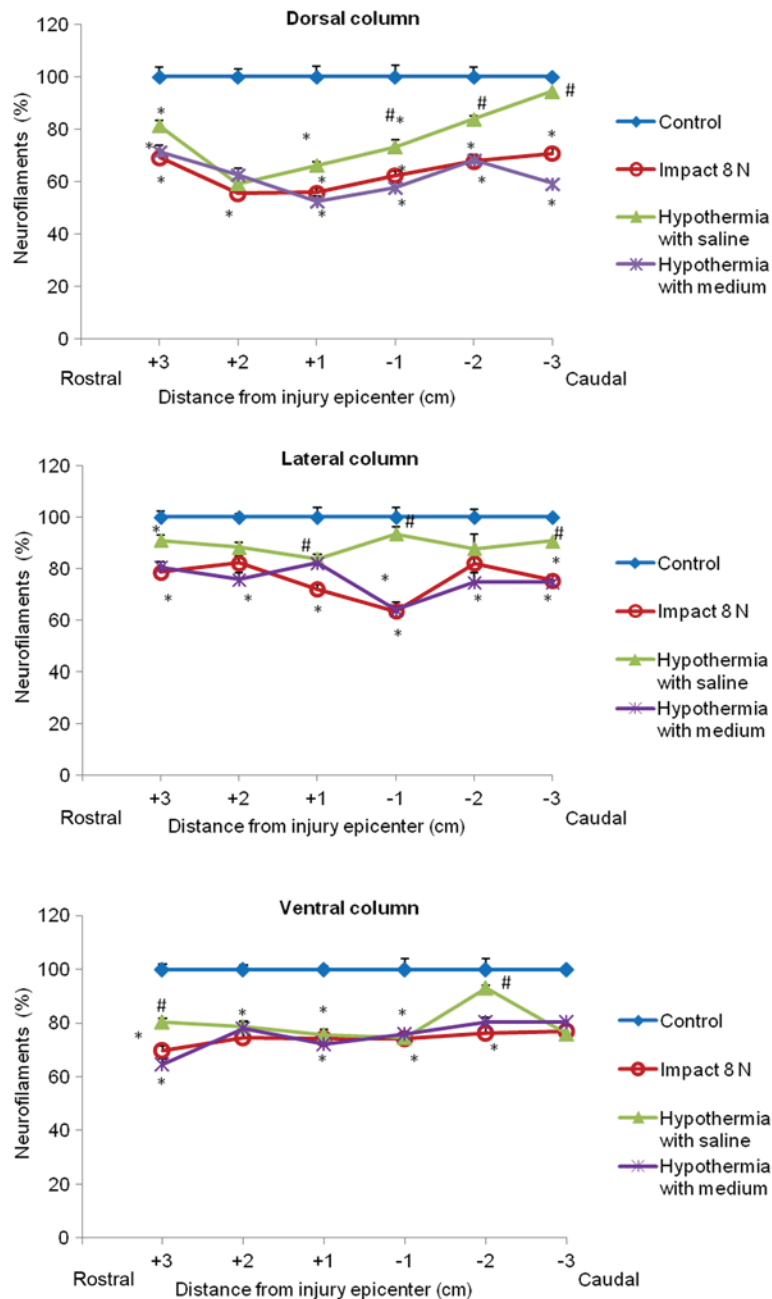


Figure 8. Graph representing the number of neurofilaments (expressed as percentage of NF/mm<sup>2</sup>) in three areas of interest (dorsal, lateral and ventral columns) in control animals, those after spinal cord compression (8N force impact) and treatment with cold saline or cold porcine medium DMEM/F12. All experimental animals survived for nine weeks. Asterisks indicate significant differences between control and spinal cord compression groups without treatment (\*P<0.05). The sharp symbols indicate significant differences between treated and untreated animals (\*P<0.05). Data are given as means  $\pm$  SEM (n=3-4).

recovery of sensory or motor function in a variety of animal SCI models (21-25). Although animal studies of SCI have revealed consistent neuroprotective effects of hypothermia applied either locally or systemically (26), further experimental/clinical studies are required to determine the optimal cooling parameters (therapeutic window, optimal temperature and duration) and to test the effectiveness of hypothermic therapy in individuals afflicted with this devastating neurological deficit. In the present study, the computer-controlled compression device produced graded contusion lesions at L3 level with two different degrees of tissue preservation and functional recovery, depending upon pre-set impact parameters. Nine weeks after SCI there was a large contusion

lesion at the epicenter, with 47% reduction of the gray matter after 8N force impact and 67% reduction in the 15N-force SCI group. Early and intense local hypothermia applied 30 min after SCI in the epidural space increased the gray matter preservation at the lesion site in both SCI groups by ~11%. It seems that neurons whose axons have been damaged within a few cell diameters of the cell body upregulate the regeneration-associated genes (27) and may lead to partially enhanced plasticity and reorganization of preserved neuronal circuits. Yoshitake *et al* (28) demonstrated the neuroprotective effects of local epidural cooling in pigs which underwent ischemic injury. The hypothermic group showed better recovery of somatosensory evoked potentials,

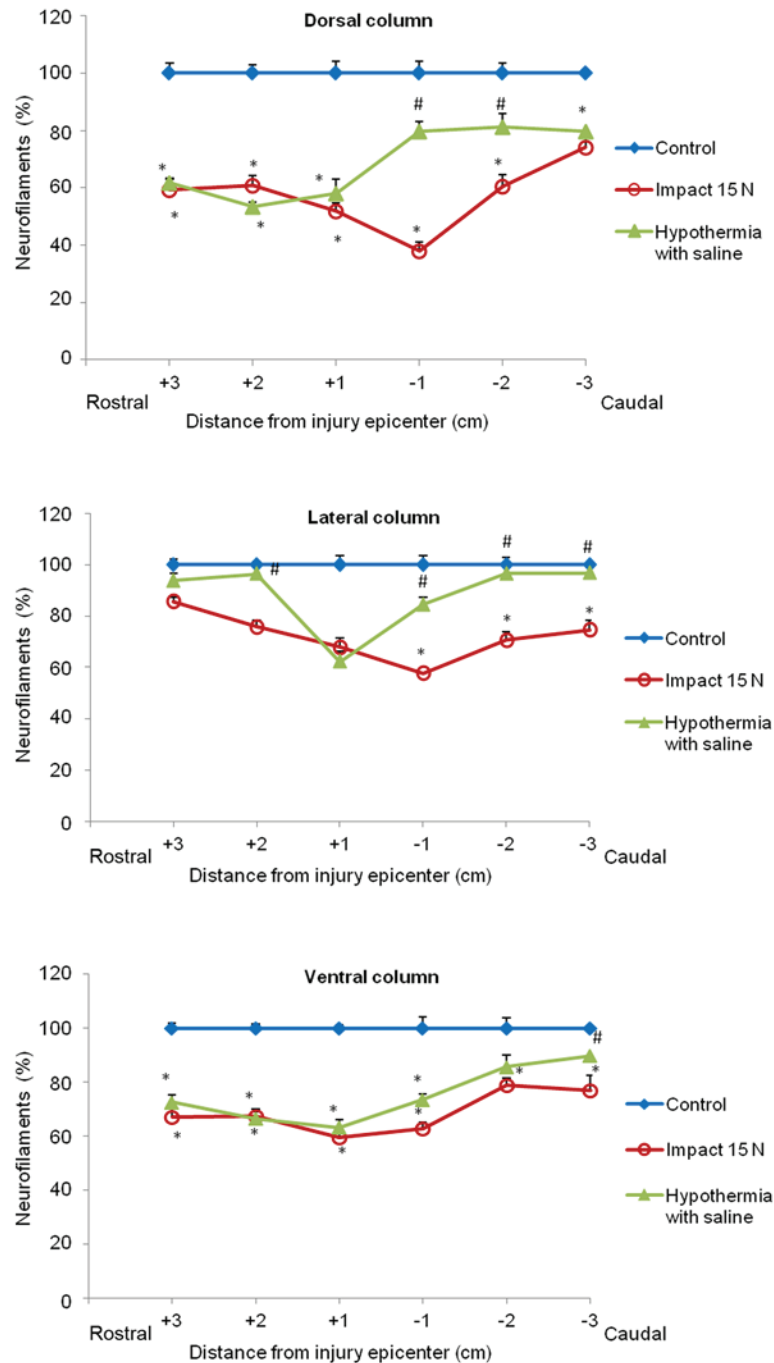


Figure 9. Number of neurofilaments (expressed as percentage of NF/mm<sup>2</sup>) in the dorsal, lateral and ventral columns in control animals, those after spinal cord compression (15N force impact) and treatment with cold saline. All experimental animals survived for nine weeks. Asterisks indicate significant differences between control and spinal cord compression groups without treatment (\**P*<0.05). The sharp symbols indicate significant differences between treated and untreated animals (#*P*<0.05). Data are given as means  $\pm$  SEM (*n*=3-7).

behavioral ability and increasing numbers of motor neurons in the anterior horn.

Earlier studies documented strong correlation between neurological outcome and the extent of axonal dysfunction at the lesion epicenter (15,26,29-31). Dimar *et al* (32) reported that local hypothermic treatment at 19°C for 2 h improved neurological function in the rat spinal cord compression model, and indicated that directly-applied hypothermia may be beneficial in preventing injury secondary to ischemic cellular damage. However, the authors failed to find any improvement in the more severe contusion model. In this study, local

perfusion of the lesion epicenter with cold saline (19°C just above the lesion) produced evident protection of white matter integrity in the dorsal columns (improvement by 13-15%) with minimal 4-7% preservation of tissue in the lateral and ventral columns. We speculate that, as in other studies, a small increase in axonal survival at the lesion epicenter may be one of the parameters which enable improvement in neurological function. Nout *et al* (33) reported that the lesion size per se is not a strict predictor of functional outcome. Our results fully support this suggestion, showing that early local saline hypothermia after 8N SCI partially improved tissue integrity

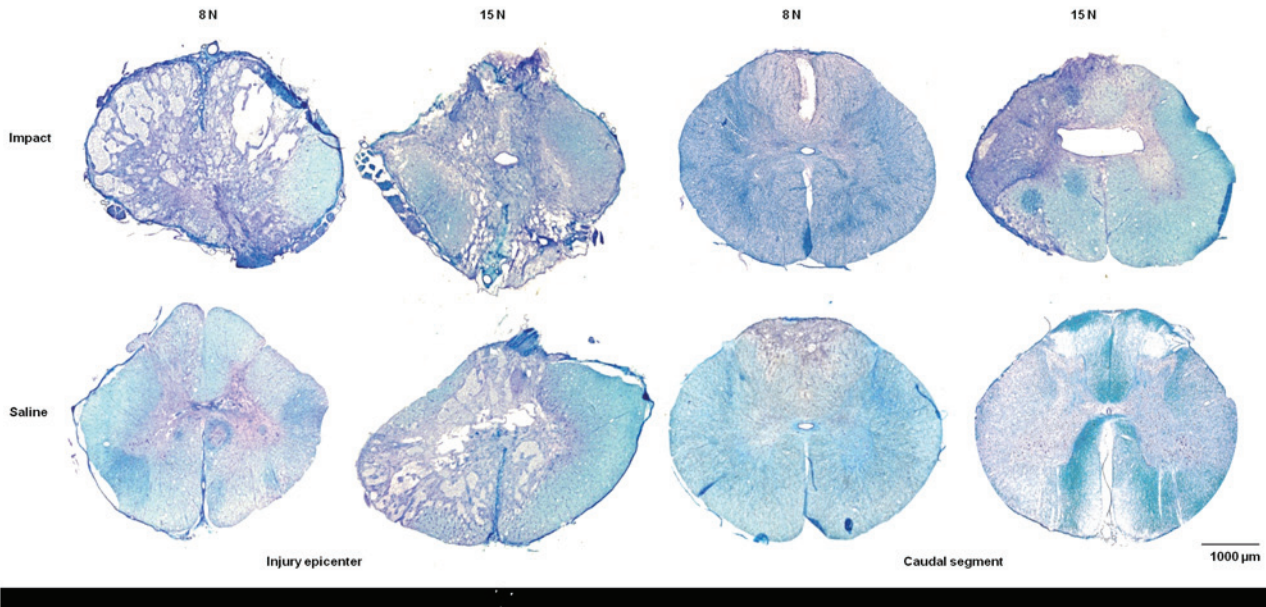


Figure 10. Transverse spinal cord sections taken from the epicenter of injury and caudal segments (-1) after L3 compression (8N and 15N force impact) and treatment with cold saline (5 h). Animals after SCI and hypothermia survived for nine weeks. Histological sections processed with Luxol fast blue and cresyl violet showed substantial reduction of cavity size at the lesion site and caudally to the epicenter of injury after SCI (8N impact force)+local hypothermia. SCI (15N impact force) and local hypothermia with saline show only partial white and gray matter preservation. Scale bar, 1,000  $\mu$ m.

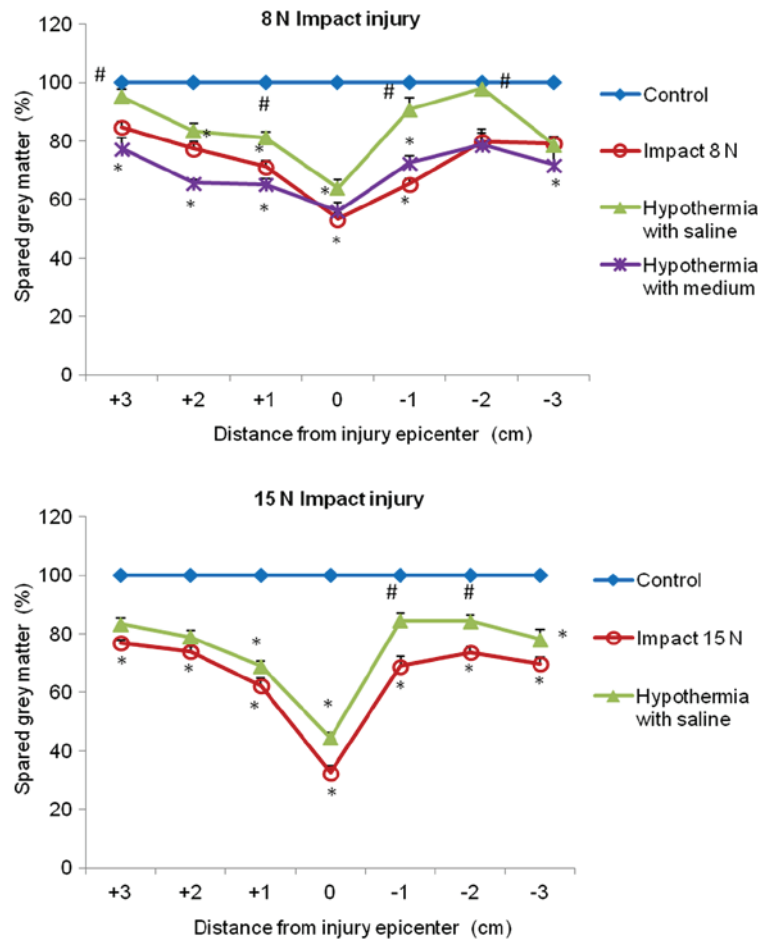


Figure 11. Quantitative analysis of gray matter preservation performed on transverse sections dyed with Luxol fast blue and cresyl violet staining. The percentage of preserved gray matter was calculated in the control group, in animals with 8N and 15N force spinal cord injury and after treatment with cold saline or oxygenated culture medium DMEM/F12. Experimental animals survived for nine weeks. Asterisks indicate significant differences between control and spinal cord compression groups without treatment ( $P < 0.05$ ). The sharp symbols indicate significant differences between treated and untreated animals ( $P < 0.05$ ). Data are given as mean  $\pm$  SEM (n=3-7).



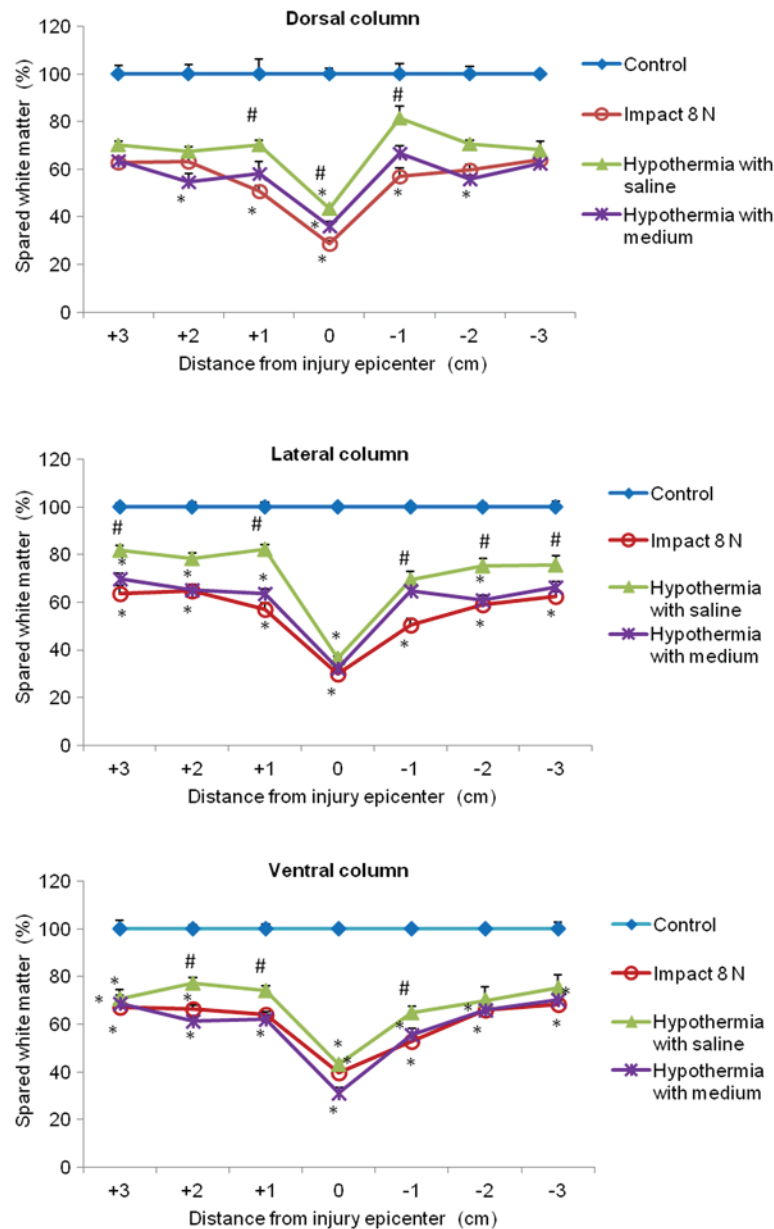


Figure 12. Analysis of preserved white matter in three areas of interest (dorsal, lateral and ventral columns). The percentage of preserved white matter was calculated in control animals, in the group after spinal cord injury (8N force impact) and in the group after injury and treatment with cold saline or oxygenated culture medium DMEM/F12. Experimental animals survived for nine weeks. Asterisks indicate significant differences between control and spinal cord compression groups without treatment ( $P < 0.05$ ). The sharp symbols indicate significant differences between treated and untreated animals ( $P < 0.05$ ). Data are given as means  $\pm$  SEM ( $n = 3-7$ ).

at the epicenter of SCI (by 11% in gray matter and 15, 7 and 4% in the dorsal, lateral and ventral columns respectively), and that this improvement was essential for modulation of the key spinal microcircuits leading to better functional outcome. However, no functional improvement was seen in the more severe 15N SCI group treated with saline, although the preservation of both gray and white matter at the epicenter was very similar in both 8N- and 15N-force experimental groups. This finding raises the question to what extent the spinal cord must be preserved.

Fawcett (34) described in his review that in order to produce a two-spinal level improvement in motor function, corticospinal and other descending axonal pathways must regenerate over two or more spinal segments, or for around

2-3 cm. Although extensive spinal relay circuits exist in rodents, the extent to which this circuitry exists and is active in large animal models and primates is unknown and is still controversial. We document that rostro-caudal tissue integrity causing interconnections throughout the lesion site is needed for better neurological outcome in the minipig SCI model.

*Integrity of the tissue surrounding the lesion is essential for functional recovery.* We attempted to find out to what extent the spinal cord might be saved/repared after hypothermia, which could be beneficial to minipigs with SCI. For a patient with cervical SCI a return of function over even one segment would improve their quality of life, while return of function over three or four spinal segments would transform it (34,35). To address

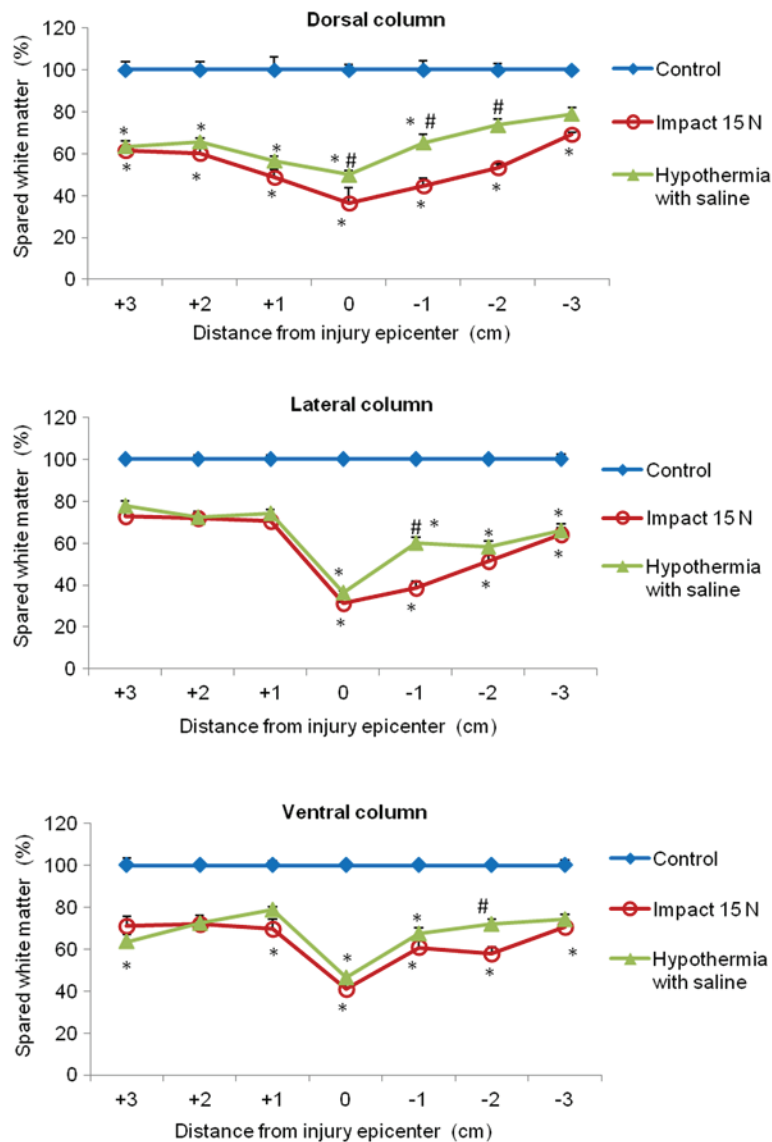


Figure 13. Analysis of preserved white matter in three areas of interest (dorsal, lateral and ventral columns). The percentage of preserved white matter was calculated in control animals, those after spinal cord injury (15N force impact) and after treatment with cold saline. Experimental animals survived for nine weeks. Asterisks indicate significant differences between control and spinal cord compression groups without treatment (\* $P < 0.05$ ). The sharp symbols indicate significant differences between treated and untreated animals (# $P < 0.05$ ). Data are given as means  $\pm$  SEM (n=3-7).

the potential effect of hypothermic treatment we systematically analyzed and quantified the histopathological changes in the spinal sections (rostral +3, +2, +1 and caudal -1, -2, -3) which are likely to be affected by secondary injury. Although the promoting of morphological preservation/recovery was detected up to a 3 cm extent rostral-caudally in both SCI models, we consider that saline hypothermia (which might be beneficial, partially returning motor function in minipigs) was strictly connected with significant tissue preservation, at least within the immediate rostral-caudal (+1 and -1) segments. Local hypothermia applied following 8N SCI for 5 h significantly enhanced the gray and white matter (dorsal, lateral and ventral columns) preservation near the lesion site (+1 and -1), and improved the preservation of neurofilaments in the lateral columns of the same segments. This experimental group achieved faster recovery of hind-limb function and the ability to walk from one to three steps with consistent plantar-hoof stepping at nine weeks in comparison with non-treated

animals. It seems that the preservation of axons in the lateral funiculi in the +1 and -1 segments (improvement by 25 and 19% respectively) and more rostrally or caudally, might be essential for favorable neurological outcome.

Axonal injury dispersion in the spinal cord includes mainly demyelination, loss of neurofilaments and failure of axolemma permeability. Tomko *et al* (36) reported that the lesion size after dorsal column lesion in rats expanded dramatically within the first two days, when its size reached the maximum ( $40.2 \pm 12.1$  mm). Decline in neurofilament numbers was detected in the dorsal and ventrolateral white matter funiculi at the lesion epicenter and 5 mm in rostral and caudal directions of injury diffusivity after spinal compression injury in rats (37). As shown previously, the fundamental role of neurofilaments is to preserve axonal caliber, so its breakdown represents deficiency of axonal conduction velocity (38,39). Schumacher *et al* (40) reported an association between neurofilament loss and hind-limb motor function deficiency after

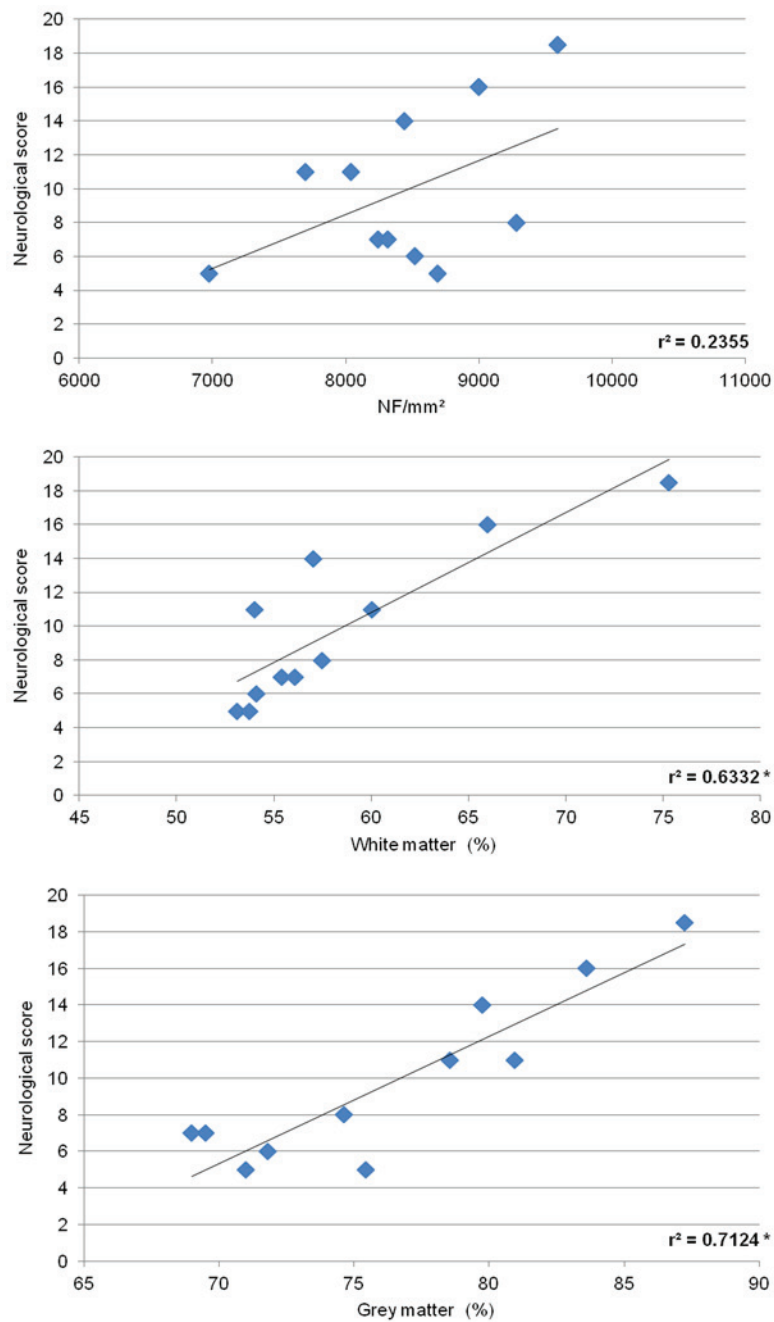


Figure 14. Correlation coefficients ( $r^2$ ) between the degree of neurological recovery and neurofilament immunoreactivity, and white and gray matter preservation measured nine weeks after spinal cord injury (8N and 15N impact) and hypothermia with saline. There was a high degree of correlation with significance in gray and white matter preservation. Data are given as means  $\pm$  SEM (n=11); \*P<0.05 with respect to neurological outcome.

SCI. Recently, Navarro *et al* (15) detected a loss of NF-positive axons four and nine months after Th12 contusion in minipigs in areas heavily infiltrated with IBA-1 positive cells. Nine weeks after SCI we observed significant reduction in the number of neurofilaments. Major increase in NF-IR profiles was detected after saline hypothermia in LC in the +1 and -1 segments, a finding which strongly correlates with significant white matter preservation. Our findings confirm the strong correlation between neurological outcome, the extent of tissue preservation within the immediate rostro-caudal (+1 and -1) segments, and preservation of neurofilaments which are almost exclusively found in myelinated axons. Such improvements were not observed in the saline-treated animals subjected to more

severe (15N) SCI or in the group treated with DMEM/F12 medium. DMEM/F12 perfusion medium was designed to support the growth and maintenance of a variety of cells. The lack of its positive effect may have been due to the use of this medium at low temperature, which reduces its efficiency.

Saline solution administered over the lesion site in the 15N SCI group significantly preserved the gray and white matter and protected neurofilaments in the dorsal and lateral columns, however the enhancement of tissue integrity was seen only in caudal segments. Devaux *et al* (41) reported that segments caudal to the lesion site hosted a robust inflammatory process, accompanied at least temporarily by local synthesis of neuro-protective and regenerative molecules. Most experimental



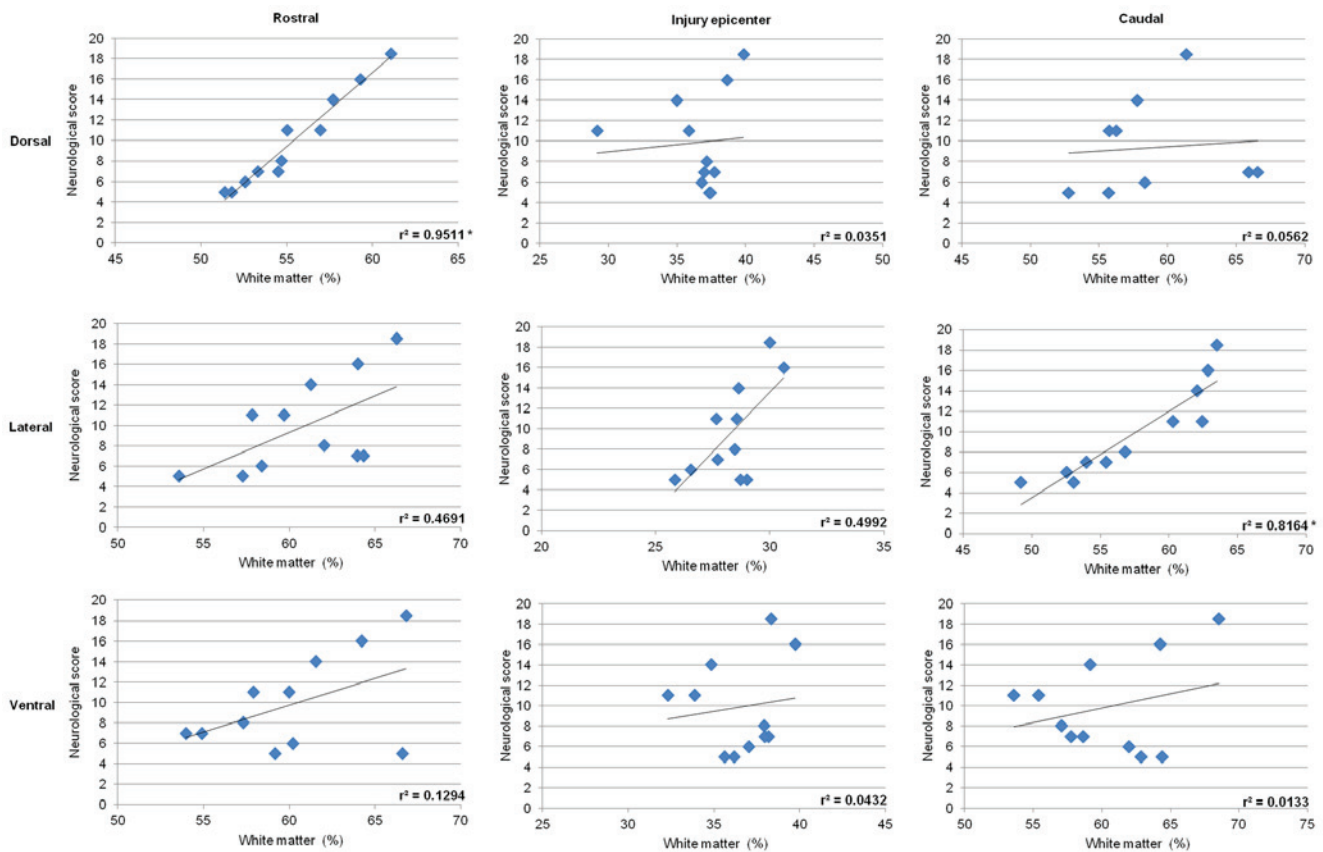


Figure 15. Plotted values show correlation coefficients ( $r^2$ ) between neurological score and white matter preservation analyzed in the dorsal, lateral and ventral columns nine weeks after spinal cord injury (8N and 15N force impact) and hypothermia with saline. Spinal cord was taken from the lesion site, and rostral and caudal sections. Data are given as means  $\pm$  SEM (n=11); \*P<0.05 neurological outcome with respect to white matter preservation.

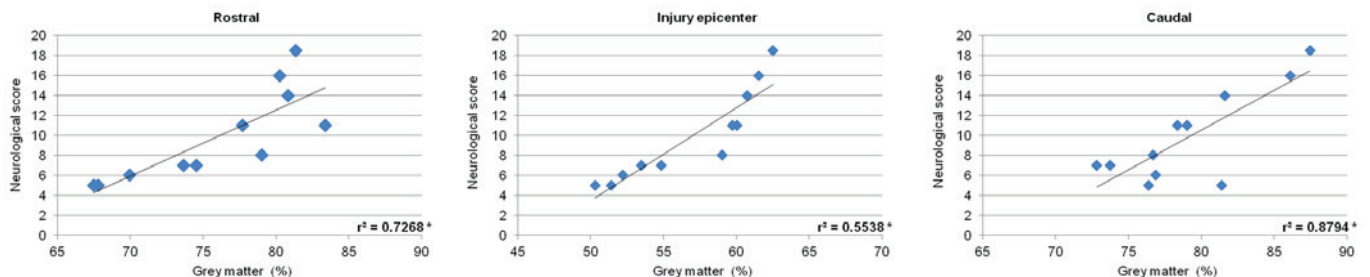


Figure 16. Correlation between locomotor function recovery and gray matter preservation nine weeks after spinal cord injury (8N and 15N impact force) and hypothermia with saline. There were high correlation coefficients ( $r^2$ ) with significance between behavioral score and preserved gray matter in each spinal cord section. Data are given as means  $\pm$  SEM (n=11); \*P<0.05 neurological outcome with respect to gray matter preservation.

SCI studies show proliferation and activation of microglia in the injured spinal cord (42,43). This proliferation accelerates neuronal damage by releasing toxic substances, including nitric oxide, super oxide and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (44-46). Our previous study revealed strong increase in NOS activity in the caudal segments immediately adjacent to the lesion site one day after mid-thoracic spinal cord constriction in rabbits (47). Morino *et al* (48) reported that peak proliferation of microglia in rats was around 48 h, and the subsequent production of cytokine and nitric oxide were key events in accelerating neuronal damage after spinal cord injury. Mild hypothermia for 48 h inhibited microglia proliferation and TNF- $\alpha$  production after traumatic SCI and improved hind-limb motor function (49). In another computer-controlled

minipig SCI model, the presence of high-density activated microglia and macrophages was identified up to nine months after SCI (15). This indicates that if the target of hypothermia treatment is inflammatory response, the hypothermia treatment in more severe SCI models should be continued for a longer time after the SCI.

In the present study, we validated the effectiveness of an implantable spinal perfusion chamber (using 3D scanner technology) in a computer-controlled spinal cord trauma minipig model. The perfusion chamber could be successfully used for application of selective spinal cord hypothermia in large pre-clinical animal SCI models, aimed at maximum functional benefit as defined by recovery of motor function. Although the superfusion of cold saline was restricted to a

relatively small spinal cord region (just above the lesion site), the effectiveness of treatment spread up to 3 cm both cranially and caudally. The treatment showed that tissue integrity in both cranial and caudal segments immediately adjacent to the lesion (+1 and -1) and substantial preservation of neurofilaments in the lateral funiculi of the same segments is needed for a better neurological outcome. No improvement of functional recovery was seen when treatment affected the tissue integrity only in the caudal segments. These results suggest that local saline hypothermia (4°C) applied through a spinal perfusion chamber for 5 h could be a promising therapeutic method when combined with clinically-proven surgical (decompression) and medical treatments for SCI. However, further experimental studies are required to evaluate hypothermia treatment for its possible utility in human patients with SCI.

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