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EVALUATION OF NEUROPROTECTIVE EFFECTS OF LOCAL HYPOTHERMIA IN A PORCINE SPINAL CORD INJURY MODEL

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ABSTRACT

The goal of this study was to assess the therapeutic potential of a 5-hour local spinal cord (SC) hypothermia by 4 °C saline on preservation of SC tissue at the injury epicentre and 3 cranial and caudal 10 mm long SC segments in a porcine experimental model of spinal cord injury (SCI). The SCI was inflicted through L3 laminectomy by a metallic rod moved by a velocity of 30 mm.sec^{-1}, and operated by a computer-controlled apparatus. A group of 15 female minipigs 5–8-month-old weighing 28–35 kg was randomly divided into 5 subgroups (each composed of 3 animals): 1) sham controls; 2) SCI by force 8N; 3) SCI by force 8N, 5-hour hypothermia; 4) SCI by force 15N; 5) SCI by force 15N, 5-hour hypothermia. After a 9-week survival period, the minipigs were in deep general anaesthesia transcardially perfused by 5000 ml of saline and fixed by 5000 ml 4 % neutral paraformaldehyde. White and grey SC matter damage was evaluated in specimens cut from the epicentre of injury as well as 3 cranial and 3 caudal 10 mm long SC blocks dyed according to Luxol fast blue (LFB) with cresyl violet (CV) protocol for light microscopic observations. The percentage of preserved SC white and grey matter was assessed in microphotographs and compared with data from sham controls (considered 100 %). The data were statistically evaluated by ANOVA test, the difference $P < 0.05$ was considered significant. Results of the study suggest that 5-hour local cooling of the epicentre of SCI is well tolerated and facilitates the preservation of SC tissue integrity. Additional experimental and preclinical studies are necessary before introducing the method in practice.

Key words: minipig; neuroprotection; spinal cord trauma; therapeutic hypothermia

INTRODUCTION

Spinal cord injuries (SCIs) resulting from mechanical insults inflicted on the fragile spinal cord (SC) tissue cause partial or complete, temporal or permanent loss of motor,

sensory, and autonomic functions caudal from the site of the lesion [4, 15]. Since severe SCIs are accompanied by significant morbidity and mortality, and there are only limited therapeutic options available at present, they remain the most challenging problems in both human and veterinary medicine. The neurological deficit in SCI develops through primary and secondary phases [9]. The primary phase encompasses the destruction of SC tissue. It occurs at the time of mechanical insult and is irreversible. The secondary phase develops as a result of pathological processes triggered by the primary event, continues for weeks and months after trauma, and is amenable to therapy [21]. One promising therapeutic strategy is suppressing a secondary injury cascade and restricting the secondary SC tissue damage area [28].

Multiple clinical observations demonstrated the protective capacity of hypothermia in cases of extensive soft tissue contusion, inflammation, complex cardiovascular or neurosurgical interventions, ischemic stroke, neonatal ischemic or hepatic encephalopathy, and brain and spinal cord trauma [5, 6, 11, 12, 14, 17, 19, 23, 24]. This inspired the authors to study the neuroprotective capacity of a 5-hour topical application of therapeutic hypothermia in a porcine SCI model.

MATERIALS AND METHODS

Fifteen female minipigs, 5–8-months-old, Göttingen-Minnesota-Liběchov crossbreed strains weighing 25–35 kg were randomly divided into 5 subgroups each of them comprising of 3 animals: 1) Sham controls, i.e. intact minipigs; 2) L3 laminectomy, SCI by force 8N, the velocity of impactor movement 30 $mm.s^{-1}$, without local application of 4 °C saline at the epicentre of SCI – therapeutic hypothermia (TH); 3) L3 laminectomy, SCI by force 8N, the velocity of impactor movement 30 $mm.s^{-1}$, 5-hour local application of TH; 4) L3 laminectomy, SCI by force 15N, the velocity of impactor movement 30 $mm.s^{-1}$, without TH; 5) L3 laminectomy, SCI by force 15N, the velocity of impactor movement 30 $mm.s^{-1}$, 5-hour local application of TH.

Technical aspects of experimental procedures (i.e. the preparatory phase, anaesthesia, surgical approach to the spinal medulla (SM) via laminectomy of L3 vertebra, spinal cord (SC) contusion by the computer-controlled and stepping motor handled metallic impactor, epidural application of 4 °C saline at the epicentre of the SC trauma, and post-experimental care) were described in detail in previous publications, that is why we do not report upon these issues repeatedly [26, 27].

Spinal cord tissue preparation

At the end of the survival period (9 weeks) the minipigs were premedicated by azaperon (2.0 $mg.kg^{-1}$) with atropin (0.5 $mg.kg^{-1}$) and in the thiopental general anaesthesia (10 $mg.kg^{-1}$, i. v.) transcardially perfused with 5 000 ml of heparinized saline and fixed with 5 000 ml of 4 % paraformaldehyde in 0.1 M phosphate buffered saline (PBS) at pH 7.4. Approximately 10 cm long segments of lumbar spinal cord with the epicentre of traumatic lesion in the middle were thoroughly dissected from the vertebral canal and the spinal dural sac, then post-fixed in the 4 % formaldehyde with PBS for subsequent ≈24 hours. The following day, the dissected and post-fixed pieces of the SC were cut into seven, ≈10 mm long blocks – one block involved the epicentre of injury, three the cranial (+3, +2, +1), and three the caudal (-1, -2, -3) segments of the SC. All seven blocks were subsequently cryopreserved in a solution of 30 % neutral sucrose (30 % saccharose in 0.1 M PBS) at 4 °C for two days, then cut into 30 µm thick sections on a cryostat (Leica Microsystems, Wetzlar, Germany) at -20 °C. The transverse serial sections were mounted on slides, which were processed in accordance with the combined histopathological staining method luxol fast blue (LFB) with cresyl violet (CV) [16]. The copper phthalocyanine dye in LFB helps to identify myelin (the basic component of the central nervous system) so the white matter myelin sheaths are stained blue to blue-green. The basic aniline dye CV selectively accentuates Nissle substance in neurons, their axons and dendrites so the basic components of the CNS grey matter appear rosy-violet to purple-blue in the light-microscopic images (Fig. 1). The combined LFB-CV histological staining also provides for the distinct contrast between intact and defective spinal cord tissue [7, 13, 20]. The percentage of preserved SC white and grey matter was assessed in microphotographs (taken by digital camera Olympus DP50, coupled with light microscope Olympus BX52, magnification 10x) of transverse serial sections of lesion epicentre and 3 rostral (+1, +2,+3) and 3 caudal (-1, -2, -3) blocks of the spinal cord of control and experimental minipigs by Java-based Image Processing Programme provided free by Laboratory for Optical

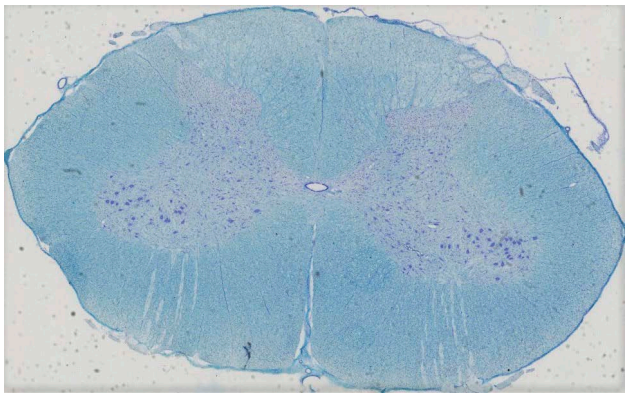

Fig. 1. Microphotograph of transverse spinal cord section of control minipig, LFB + CV histological staining. Magn. x 10
Personal observation

and Computational Instrument, USA. The proportion of damaged spinal cord white matter (WM) was assessed in 5 transverse sections prepared from every SC segment (3 cr, 2 cr, 1 cr, Epictr, 1 cd, 2 cd, 3 cd) bilaterally in the 800 x 600 µm rectangular areas of dorsal, lateral, and ventral funiculi, and the proportion of the SC grey matter (GM) in Rexed´s laminas IX was presented as percentage of similar intact WM/GM regions of spinal cords of control minipigs, considered 100 % [8, 32].

Statistical evaluation

The histological data were analysed in a blinded manner using one-way analysis of variance, i.e. ANOVA test with help of the GraphPad Prism 6 programme (GraphPad Software, Inc., La Jolla Ca, USA). $P < 0.05$ value was considered statistically significant difference. All results were arranged in tables and presented as mean values ± SD, i.e. standard deviation [2].

Ethical considerations

The experimental protocols were prepared in compliance with the Animal Protection Act of the Slovak Republic No. 15/1995 and approved by the State Veterinary and Food Administration in Bratislava (Decision No. 1749/10-221) as well as by the Ethical Commision of the Institute of Neurobiology, Slovak Academy of Sciences in Košice, and in accordance with the EC Council Directive (2010/63/EU) regarding the utilization of animals in research. All efforts were made to restrict the number of animals in experimental groups and minimize their suffering.

RESULTS

Light microscopy image of the spinal cord white matter damage

Comparison of the extent of the SC white matter damage after SCI by the force of 8N and 15N.

The SC white matter (*substantia alba – SA*) of the minipigs from the subgroup 2 was significantly damaged in all spinal cord funiculi compared to the control animals (members of the subgroup 1). On average, 68.67 ± 0.61 % of SC white matter was damaged in the epicentre area in dorsal funiculi (*funiculi dorsales – FD*). On average, 63.47 ± 7.10 % of SC white matter was damaged in the epicentre in lateral funiculi (*funiculi laterales – FL*). The SC white matter loss was the smallest – 58.99 ± 2.93 % on average in ventral funiculi (*funiculi ventrales – FV*). Comparison with control animals showed significant differences between the preserved SC white matter of healthy minipigs (sham controls, subgroup No 1) and animals after the SCI by the force of 8N (subgroup No 2).

The contusion of the spinal cord by the force of 15 N caused histopathological changes in the SC white matter visible in its entire cross-sections. Maximum damage of the SC white matter epicentre was observed in dorsal funiculi (71.37 ± 1.22 %), a little less in the epicentre of lateral funiculi (70.03 ± 0.91 %) and the least in the epicentre of ventral funiculi (60.34 ± 1.45 %).

Compared to control animals (subgroup No. 1), the extent of damage to the SC white matter in minipigs from the subgroup No. 4 (contusion of SC by the force of 15 N without application of therapeutic hypothermia) was again significant. All details concerning the extent of damage of SC white matter after SCI by the force of 8 N and 15 N, respectively, and that in individual sections (3, 2, 1 cr, Epictr, and 1, 2, 3 cd) and in individual spinal cord funiculi (*FD*, *FL*, and *FV*) compared with each other and with values obtained from healthy (control) minipigs are presented in Tables 1-3.

Comparison of the SC white matter damage extent after SCI by the force of 8 N and 15 N in *FD* with the damage extent in *FV* showed the maximum difference in the section 1 cr of dorsal funiculi (significant at $P < 0.05$). Similarly, more SC white matter was damaged in *FL* than in *FV,* but the differences between the average values were insignificant. Details are more clearly presented in Tables

2 and 3. Comparison of the SC white matter damage extent in the epicentre of individual spinal cord funiculi in members of subgroup 2 and subgroup 4 showed that in the subgroup 2 a little more of SC white matter was preserved. However, the differences were not significant.

Effect of hypothermia on the spinal cord white matter preservation

Spinal cord contusion by the force of 8 N

The evaluation of a 5-hour local application of therapeutic hypothermia by 4 °C saline proved a protective effect of this treatment modality on SC white matter in all funiculi. As the tables 1–3 show, significantly more SC white matter ($P < 0.05$) was preserved in section 1 cd of *FD* compared with the untreated minipigs. In *FL,* favourable influence of a 5-hour hypothermia was also manifested in all animals in Epictr, and in sections 1 cd and 2 cd – the differences were statistically significant ($P < 0.05$). Favourable influence of a 5-hour therapeutic hypothermia application in *FV* was expressed by preservation of more SC white matter in all monitored sections of *FV* (compared with untreated animals). However, statistically significant difference in favour of treated animals was only in section 1 cd ($P < 0.05$).

Spinal cord contusion by the force of 15 N

Favourable influence of a 5-hour local application of 4 °C saline (therapeutic hypothermia) at the epicentre of SC lesion in minipigs from subgroup 5 was manifested by the reduction of the *SA* damage extent (in %) in all tracked *FD* sections, but the differences were statistically significant in Epictr, as well as in sections 1 cr, 1 cd and 2 cd. These facts are expressed more clearly in Table 1.

In the *FL* contusion epicentre the difference between the *SA* damage extent in untreated minipigs and in animals after a 5-hour local application of 4 °C saline was insignificant. The results only suggested a positive trend after the therapeutic hypothermia application. However, significant differences in favour of treated animals were recorded in sections 3 cr and 1 cr, 1 cd, 2 cd, and 3 cd (Table 2).

The positive influence of hypothermia on SC white matter damage also appeared n *FV*. Although only 3.50 % decrease was detected in white matter in the epicentre of SC contusion in untreated animals in comparison with animals after the therapeutic hypothermia local application, a favourable influence of this treatment method was manifested also in other monitored sections of *MS*, and in the section 1 cd the difference was statistically significant ($P < 0.05$). All details are presented in Table 3.

Light microscopy image of the spinal cord grey matter damage

Comparison of the size of the SC grey matter damage after SCI by the force of 8N and 15N

In the minipigs after the SCI inflicted by the force of 8N without hypothermia application (subgroup No. 2), the extent of SC grey matter (*SG*) damage was maximal in the epicentre of contusion (47 ± 1.98 %), and was decreased in the cranial and caudal directions. Differences between Epictr and the other monitored SC sections were significant in this subgroup of animals ($P < 0.05$).

In the minipigs after the SCI inflicted by the force of 15N without hypothermia application (subgroup No. 4), the extent of SC grey matter damage was maximal in the epicentre of contusion (67 ± 2.54 %), and decreased with the distance. The differences between Epictr and the monitored spinal cord sections were significant ($P < 0.05$). The difference between grey matter lesions in individual monitored SC sections was bigger in animals after the SCIs inflicted by a force of 15N. The average values were insignificant, with the exception of the epicentre (Table 4).

Effect of hypothermia on the spinal cord grey matter preservation

Spinal cord contusion by the force of 8 N

The statistical analysis showed a favourable influence of the therapeutic hypothermia application on the preservation of SC grey matter in the treated minipigs. The differences between the *substantia grisea* (*SG*) percentage damage in animals from subgroup 2 (SCI by the force of 8 N without subsequent local SC cooling), and animals from subgroup 3 (the SCI followed by 5-hour local application of 4 °C saline) were significant in sections 3 cr and Epictr, as well as in sections 1 cd and 2 cd ($P < 0.05$).

Spinal cord contusion by the force of 15 N

The extent of damage to SC grey matter was, as expected, more prominent in subgroup 4 (SCI by the force of 15 N without the hypothermia application) than in subgroup

Table 1. The proportion of dorsal funiculi (*FD*) white matter damage (in %) after SCI inflicted by force 8N versus 15N, and neuroprotective effect of 5 h local application of hypothermia with 4 °C saline

SC section	SCI 8N		SCI 8N+5h HT		SCI 15N		SCI 15N+5h HT	
	MV	SD	MV	SD	MV	SD	MV	SD
3 cr	26.95	2.79	22.04	2.19	37.08	1.89	29.65	1.56
2 cr	28.15	3.15	27.40	2.76	36.71	2.76	32.41	1.85
1 cr	29.51	3.25	26.65	1.67	49.03	2.33	29.80	2.04*
Epictr	68.67	0.61	63.82	0.93	71.37	1.22	56.54	1.59*
1 cd	61.25	3.00	40.03	2.79*	42.99	3.55	28.43	4.76*
2 cd	48.63	4.88	41.56	2.67	40.37	2.58	29.43	1.78*
3 cd	35.68	3.16	33.92	3.05	35.97	2.39	31.87	3.77

SC – spinal cord; 5-h – 5 hours; HT – hypothermia; SCI – spinal cord injury; N – Newton; MV – mean value; SD – standard deviation; cr – cranial; Epictr – epicentre; cd – caudal; * – significant ($P < 0.05$) difference between percentage of white matter lesions

Table 2. The proportion of lateral funiculi (*FL*) white matter damage (in %) after SCI inflicted by force 8N versus 15N, and neuroprotective effect of 5h local application of hypothermia with 4 °C saline

SC section	SCI 8N		SCI 8N+5h HT		SCI 15N		SCI 15N+5h HT	
	MV	SD	MV	SD	MV	SD	MV	SD
3 cr	38.62	1.90	36.54	2.71	36.21	3.31	18.42	1.95*
2 cr	39.89	3.82	34.51	2.05	35.07	2.51	21.48	2.09
1 cr	51.40	3.72	43.67	2.36	42.98	2.78	27.80	1.98*
Epictr	63.47	7.10	50.14	2.20*	70.03	0.91	63.34	0.85
1 cd	55.39	3.46	34.95	4.02*	49.53	2.85	30.33	3.39*
2 cd	46.60	1.75	26.40	2.93*	40.98	3.86	24.53	2.92*
3 cd	30.90	1.11	21.31	3.42	37.32	1.85	24.18	5.19*

SC – spinal cord; 5-h – 5 hours; HT – hypothermia; SCI – spinal cord injury; N – Newton; MV – mean value; SD – standard deviation; cr – cranial; Epictr – epicenter; cd – caudal; * – significant ($P < 0.05$) difference between percentage of white matter lesions

5 (SCI by the same force followed by a 5-hour therapeutic hypothermia) in all sections of the spinal cord except for the epicentre where the extent of damage was greater in treated animals by 9 %. The result could be an accidental finding caused by a small number of minipigs in individual monitored subgroups. A significant ($P < 0.05$) favourable effect of therapeutic hypothermia was observed in sections 1 cd and 2 cd (Table 4).

DISCUSSION

The experimental studies, as well as clinical observations, showed that hypothermia can improve the neurological outcome in patients following traumatic or ischemic brain and spinal cord lesions [3, 6, 10, 11, 14, 24, 25, 29–31]. It acts through several mechanisms and can be applied shortly after the injury [8, 10, 24, 32]. Depending on the degree of the reached core temperature (or the temperature of surrounding tissues during local application) hypothermia is referred to as: deep (< 30 °C), medium (30–32 °C), mild (32–34 °C), and light (35 °C) [11, 29]. It was found that hypothermia reduces cellular metabolism by 5–8 % per each 1 °C, slows down enzymatic activity, reduces oxygen, and energy consumption [1, 3, 8]. That helps to save ATP reserves, stabilizes transmembrane ion, and transmitter gradients, supports the preservation of the BSCB ("blood-spinal cord barrier"), reduces oedema of the spinal cord and individual MS cells, suppresses the formation of gliosis, reduces oxidative stress, inflammatory reactions, and stabilizes mitochondrial membranes, the increased permeability of which means the key impulse (the so-called "point-of-no-return") for starting programmed cell death, i.e. apoptosis [1, 3, 8, 11]. However, it is known that even a slight drop in body temperature can act negatively. Hypothermia slows down metabolism, reduces effectiveness of medications, increases blood viscosity, can cause tremors (when due to increased activity of skeletal muscles, the consumption of O_2 rises sharply), can cause cardiac arrhythmia, induce in-

Table 3. The proportion of ventral funiculi (*FV*) white matter damage (in %) after SCI inflicted by force 8N versus 15N, and neuroprotective effect of 5 h local application of hypothermia with 4 °C saline

SC section	SCI 8N		SCI 8N+5h HT		SCI 15N		SCI 15N+5h HT	
	MV	SD	MV	SD	MV	SD	MV	SD
3 cr	26.95	2.79	22.04	2.19	32.86	2.68	29.22	3.79
2 cr	28.15	3.15	27.40	2.76	33.69	1.59	22.62	2.10
1 cr	29.51	3.25	25.65	1.67	35.98	1.42	26.00	2.09
Epictr	68.67	0.61	63.82	0.93	60.34	1.45	56.84	1.74
1 cd	61.25	3.00	40.03	2.79*	47.05	1.87	35.15	2.81*
2 cd	48.63	4.88	41.56	2.67	33.88	1.13	29.92	5.72
3 cd	35.68	3.16	33.92	3.05	31.67	1.52	24.51	5.19

SC – spinal cord; 5-h – 5 hours; HT – hypothermia; SCI – spinal cord injury; N – Newton; MV – mean value; SD – standard deviation; cr – cranial; Epictr – epicentre; cd – caudal; * – significant (P < 0.05) difference between percentage of white matter lesions

Table 4. The proportion of Rexed´s lamina IX grey matter damage (in %) after SCI inflicted by force 8N versus 15N, and neuroprotective effect of 5h local application of hypothermia with 4 °C saline

SC section	SCI 8N		SCI 8N+5h HT		SCI 15N		SCI 15N+5h HT	
	MV	SD	MV	SD	MV	SD	MV	SD
3 cr	15.00	1.94	5.00	2.65	23.00	0.85	17.00	2.05
2 cr	23.00	2.27	16.00	2.39	26.00	1.51	21.00	2.26
1 cr	29.00	2.16	19.00	2.06	38.00	2.67	31.00	1.82
Epictr	67.00	1.98	36.00	2.82	47.00	2.54	56.00	1.69
1 cd	35.00	1.78	9.00	3.84*	31.00	3.44	15.00	2.46*
2 cd	20.00	2.78	2.00	1.44*	36.00	1.89	16.00	2.11*
3 cd	21.00	2.26	21.00	2.22	30.00	2.13	22.00	3.41

SC – spinal cord; 5-h – 5 hours; HT – hypothermia; SCI – spinal cord injury; N – Newton; MV – mean value; SD – standard deviation; cr – cranial; Epictr – epicentre; cd – caudal; * – significant (P < 0.05) difference between percentage of grey matter lesions

sulin resistance, slow down chemotaxis of leukocytes and phagocytes. Suppression of immune reactions promotes the emergence of infections, which (as it turned out) is not prevented even by prophylactic administration of antibiotics [1, 11]. Long term application of systemic therapeutic hypothermia in experimental conditions and clinical practice makes high demands on technical equipment of the workplace and on experience of the staff. In our experiments we therefore decided to investigate the possibilities to complete the local cooling of the injured *medulla spinalis* and the entire experimental procedure within approximately 12 hours – including the awakening of the minipigs from the total anaesthesia and stabilization of their condition [8, 27, 32].

We evaluated the degree of damage to *substantia alba* and *substantia grisea* in experimental animals after a 9-week survival period by comparing the extent of damage of these tissues with the situation in control minipigs (subgroup 1, i.e. intact animals). Monitoring the degree of damage to the white matter of the spinal cord confirmed the expectation that after SCI by a force of 8 N it was maximally damaged (≈69 %) in the epicentre of the *funiculi dorsales*, slightly less (≈63 %) in Epictr of *funiculi laterales* and least (≈59 %) in Epictr of *funiculi ventrales*. We observed a similar trend after SCI by a force of 15N in which case the maximum proportion of SC white matter damage (≈71 %) was observed in Epictr of *FD*, smaller (≈70 %) in Epictr of *FL*, while again the smallest (≈60 %) in Epictr of *FV*.

A mutual comparison of the extent of *SA* damage in the epicentre of the contusion in *FV* after the impact by a force of 8N and 15N and statistical processing of the results showed that after contusion of the SC by a greater force more white matter was destroyed, but the differences were not significant. Conversely, in *FD*, and similarly in *FL*, more *SA* was missing after contusion of the SC by a force of 8N than 15N. We also found a similar phenomenon in the ventral funiculi (*FV*). We assume that these were ran-

dom findings (the differences were not statistically significant) caused by the small number of experimental animals in the subgroups. Monitoring the effect of a 5-hour local application of 4 °C saline showed that this procedure had a beneficial effect on the preservation of *SA* in spinal cords of all experimental animals. However, the statistical significance fluctuated without a clearly expressed relationship between the distance from the epicentre of the SCI, individual spinal cords and the monitored SC sections. Similar observations were reported also by other authors [18, 29].

After contusion of the spinal medulla by the force of 8 N, and 15 N, the SC grey matter (*SG*) was less damaged than the SC white matter (*SA*). The number of nerve cells in Rexed's lamina IX decreased with the distance from the epicentre of the SCI and was proportional to the force by which the metal rod damaged the *medulla spinalis* (*MS*). This tendency was manifested in all monitored sections of the spinal cord, while in the epicentre the differences were significant ($P < 0.05$). The results showed that a 5-hour local application of therapeutic hypothermia had beneficial effects on the preservation of both the *substantia alba* and the *substantia grisea,* as observed for example by N a v a r r o and coworkers [18].

A similar therapeutic strategy was also proven by H a n s e b o u t and H a n s e b o u t who achieved a remarkable improvement in the neurological condition of 20 patients with clinical symptoms of a complete interruption (transverse lesion) of the spinal cord [10]. We therefore consider the result as promising and the method of topically applied therapeutic hypothermia suitable for inclusion in preclinical testing, especially in patients with spinal traumatic lesions accompanied with fractures or dislocations of vertebrae demanding surgical intervention [22]. Since local application of therapeutic hypothermia appears to be safe, the method has a potential to improve outcomes in patients with spinal cord traumatic lesions caused by dislocations of vertebrae or their fragments demanding surgical decompression and stabilisation [22, 24, 25, 29].

CONCLUSIONS

The maximum spinal cord tissue damage was observed in the epicentre of the injury. The extent of white and grey matter SC damage declined with increasing cranial and caudal distance from the impact site. The 5-hour local cooling (therapeutic hypothermia by 4 °C saline) of the epicentre of the spinal cord traumatic lesion is well tolerated and facilitates the preservation of SC tissue integrity. Additional experimental and preclinical studies are necessary before introducing the method into practice.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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