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MOTOR RECOVERY AFTER SPINAL CORD TRAUMA AND EFFECT OF LOCAL HYPOTHERMIA IN A PORCINE EXPERIMENTAL MODEL

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ABSTRACT

This study was aimed to assess the therapeutic potential (expressed by improvement of pelvic extremities motor functions) of a 5-hour local application of hypothermia with cold saline (4 °C), or saline at room temperature (≈ 24 °C) conveyed via perfusion chamber placed epidurally over the epicenter of spinal cord lesion in minipigs paraplegic due to acute spinal cord injuries (SCIs) inflicted through L3 laminectomy with the force of 8N, 15N, or 18N by a computer operated contusion apparatus. Eighteen 5–8-month-old minipigs (Göttingen-Minnesota-Liběchov crossbreed strains weighing 28–35 kg) were randomly divided into 6 subgroups (each containing three animals) another 3 minipigs were added as sham controls. To evaluate the pelvic extremities motor recovery was used the porcine 20-point neurological scale. Regular evaluations of motor scores showed gradual spontaneous recovery of this parameter in all experimental animals, however, the best results achieved minipigs after SCI inflicted by 8N impacts. The data achieved in the study suggest that local application of therapeutic hypothermia (TH) is well tolerated and may improve functional outcomes after SCI. Further experimental and preclinical studies in different SCI animal models are required before the introduction of the method in healthcare practice.

Key words: hypothermia; minipig; motor functions; spinal cord injury

INTRODUCTION

Spinal cord injuries (SCIs) occur in humans as well as in other animals. The incidence of severe SCIs is not high, but they are devastating neurological disorders characterized by partial or complete derangement of motor, sensory, and autonomic functions caudal from the site of the spinal cord (SC) lesion, and prominent causes of morbidity and mortality [1, 3, 18, 19]. The neurological deficit in SCIs develops through two phases. The primary phase (injury) represents the immediate destruction of the SC tissue that arises at the time of insult. Hence, it is irreversible and unpreventable [26]. Within minutes, the initial impact is followed by a cascade of destructive processes (secondary injury, phase) which persist for months and considerably increase the area of the original SC lesion, but they are amenable to therapy [26, 27, 35]. To better understand the pathological processes and assess the efficacy of therapeu-

tic modalities utilization of animal experimental models is necessary. The most frequently used are rodent SCI models [1, 11, 35]. However, the positive results acquired in mice or rats are untranslatable neither to veterinary nor human clinical practice [1, 38]. That is why, the preclinical SCI trials are accomplished in animals with higher similarity to primate central nervous system (CNS) anatomy and function, currently [1, 7, 10, 22, 24, 34]. Multiple clinical observations demonstrated the protective capacity of therapeutic hypothermia (TH) in cases of extensive soft tissue contusion or inflammation, complex cardiovascular or neurosurgical interventions, ischemic stroke, neonatal ischemic or hepatic encephalopathy, brain and spinal injuries [4, 9, 13, 17, 21, 31, 32, 36, 37]. These facts inspired the authors to assess the effect of the local application otherapeutic hypothermia (TH) on the motor functions of minipigs paraplegic due to experimental SCIs.

MATERIALS AND METHODS

A total of 21 female minipigs 5–8-months-old, Göttingen-Minnesota-Liběchov crossbreed strains weighing 25–35 kg, were randomly divided into 7 subgroups: 1) Sham controls (n = 3); 2) Laminectomy of L3 vertebra, SCI by force 8N, the velocity of impactor movement 30 mm.s^{-1}, without application of TH (n = 3); 3) L3 laminectomy, SCI by force 8N, the velocity of impactor movement 30 mm.s^{-1}, 5-hour local application of 4 °C saline at the epicentre of SCI (n = 3); 4) L3 laminectomy, SCI by force 15N, the velocity of impactor movement 30 mm.s^{-1}, without TH (n = 3); 5) L3 laminectomy, SCI by force 15N, the velocity of impactor movement 30 mm.s^{-1}, 5-hour local application of 4 °C saline (n = 3); 6) L3 laminectomy, SCI by force 18N, the velocity of impactor movement 10 mm.s^{-1} without application of TH (n = 3); 7) L3 laminectomy, SCI by force 18N, the velocity of impactor movement 10 mm.s^{-1}, 5-hour local application of saline at the room temperature (≈24 °C) saline (n = 3).

The technical aspects of the experimental procedures (i. e. the preparatory phase, anaesthesia, surgical approach to the vertebral canal through L3 laminectomy, SC trauma by the computer-controlled impactor, topical application of 4 °C saline, and post-experimental care) have been described in detail in previous papers, so we do not deal with these issues repeatedly [7, 34]. Three weeks before the execution of experimental procedures, the minipigs completed 30 min walking practice along a rubber path for five days a week. Following SCIs, all minipigs were left to survive for 9 weeks. During this time period, the motor functions of pelvic extremities, as well as walking ability in experimental animals (members of subgroups No. 2–7) were assessed utilizing a 21-point porcine locomotor scale elaborated by Gedrova and co-workers [7]. The zero point in the scale stands for paralysis of pelvic extremities (paraplegia). Points 1–8 indicate a minimum to modest improvement of pelvic extremities motor functions, but minipigs are not able to get up. Points 9–11 indicate the ability of minipigs to arise by themselves, but they are not able to walk without support and maintain balance. Points 12–19 indicate the ability of minipigs to stand up, to take several coordinated steps, and keep balace. The 20-point value indicates the ability of minipigs to walk and run with plantar hoof stepping and coordinate the movement of thoracic and pelvic extremities [7]. The first examination was accomplished 2 days after SCI, then they were carried on in 7-day intervals for 9 weeks [7, 39]. During the locomotor testing the experimental animals were allowed to move freely up and down the rubber path for 10 min. With an aim to consolidate judgment, three unattached observers were involved in the determination of motor scores, and a video recording was executed of every walk-test procedure. The eventual differences between reviewers were discussed and settled following re-scoring the videos [7, 39].

Statistical evaluation

The results of locomotor tests presented as mean values ± standard deviations (SD) were arranged into tables and statistically evaluated using the Kruskal-Wallis non-parametric test. $P < 0.05$ was taken for a significant difference.

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 Commission 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 subgroups and minimize their suffering.

RESULTS

The SCI caused paraplegia in all experimental animals unrelated to the force of impact. Two days after SCIs minipigs were unable to induce contraction of muscles innervated from spinal cord segments caudally from the crushed SC segment; they also lost voluntary control of defecation and urination. To consider the difference between the quality of motor functions in sham controls (minipigs from subgroup No. 1) and experimental animals (minipigs from subgroups No. 2, 4, and 6) we also introduced mean scores (20.00 points) of intact animals (Table 1). The first minimal to modest motion in hip and knee joints in untreated minipigs (spontaneous recovery) was registered during the examination on the 7th post-SCI day (week 1). From the 2nd post-SCI week, the values of motor scores increased gradually in all experimental animals (members of subgroups No. 2, 4, and 6). As expected, the spontaneous improvement of pelvic extremities motor functions in minipigs from subgroup No. 4 (SCI with force 15N) and subgroup No. 6 (SCI with force 18N) was slower and these animals achieved fewer points at weekly examinations than minipigs from subgroup No. 2 (SCI with force 8N), but at week 1. As demonstrated Table 1, the difference in mean values between subgroups No. 2 and No. 4 was statistically significant ($P < 0.05$) in weeks 3, 4, 5, and 6. Later on, the difference between these two subgroups became less marked and insignificant. There also was an insignificant difference in mean values between members of subgroups No. 2 versus No. 6, as well as between subgroups No. 4 versus No. 6. The minipigs from subgroup No. 6 (SCI with force 18N) achieved higher scores than minipigs after SCI with force 15N. At the end of the survival period (9 weeks after the SCI), the minipigs from subgroup No. 4 were able to sit straight and move all three joints of their pelvic extremities, but they neither were able to stand up spontaneously nor by help. At the same time, the minipigs from subgroup 2 were able to stand up with help, but they were not able to walk. The spontaneous recovery of motor functions of minipigs after SCI with force 8N (members of subgroup No. 2), exceeded the mean motor score of minipigs after SCI with force 15N (members of subgroup No. 4) by 4.08 points, and also exceeded the mean motor score of minipigs after SCI with force 18N (members of the subgroup No 6), specifically by 1.95 points. The difference between mean motor scores of minipigs from subgroup No.4 and No. 6 were less than 2 points in favour of minipigs from group No. 6, i. e. minimal, and not significant (Table 1).

The pelvic extremities motor score values of minipigs from subgroup No. 3 (SCIs with force 8N treated for 5 hours by locally applied 4 °C saline) were higher than motor score values of minipigs from subgroup No. 5 (SCI with force 15N treated for 5 hours by locally applied 4 °C saline), and higher than motor score values of minipigs from subgroup No. 7 (SCI with force 18N treated for 5 hours by locally applied saline at room temperature, i.e. TH by ≈24 °C saline) at each examination. However, the differences were not statistically significant. At the end of the survival period (week 9), the minipigs from subgroup No. 3 were able to stand up without help, make 3–5 steps, and keep the balance between the stepping periods. Although at the last examination (after 9 weeks from SCIs) the minipigs from subgroup No. 5 moved all joints of their paretic pelvic extremities, they were unable to stand up or walk. The maximum difference in motor scores between members of subgroup No. 3 versus members of subgroup No. 5 (specifically 5.92 points) was in favour of minipigs after SCIs with lesser force (8N). The difference between motor score values in members of these two subgroups was insignificant. At the end of the survival period, the minipigs from subgroup No. 7 were able to move all joints of pelvic extremities, but they were not able to stand up without help. Their mean motor score was 7.67 points. The difference between mean motor scores of minipigs from subgroups No. 3 and No. 7 was 5.96 points in favour of animals after SCIs with 8N force (Table 2). The difference was not significant.

The maximum pelvic extremities mean motor score difference between untreated animals and minipigs treated after SCIs with force 8N five hours by TH (members of subgroup No. 2 versus members of subgroup No. 3) was 2.88 points in favour of treated animals. The maximum motor score difference between untreated animals and minipigs treated by TH after SCIs with force 15N (members of subgroup No. 4 versus members of subgroup No. 5) was 1.57 points in favour of animals treated 5 hours by TH. The values were not statistically significant.

Surprisingly, from week 4 the untreated minipigs (members of subgroup No. 6) attained better pelvic extremities mean motor scores than minipigs treated 5 hours by ≈24 °C saline (members of subgroup No. 7). The differ-

Table 1. The spontaneous recovery of pelvic extremities motor functions after SCI imposed by the force of 8N, 15N, and 18N.

Post SCI Interval	Sham ctrs Score	SCI 8N		SCI 15N		SCI 18 N	
		Score	SD	Score	SD	Score	SD
Week 0	20.00	0.00	0.00	0.00	0.00	0.00	0.00
Week 1	20.00	0.38	0.13.	1.17	0.12	0.80	0.37
Week 2	20.00	2.50	0.20	2.00	0.25	2.60	0.51
Week 3	20.00	6.38*	0.67	2.33	0.24	3.80	0.37
Week 4	20.00	8.00*	1.29	3.00	0.48	5.20	1.16
Week 5	20.00	9.50*	1.55	4.00	0.41	6.00	1.23
Week 6	20.00	9.50*	1.55	5.00	0.48	6.80	1.39
Week 7	20.00	10.25	1.93	5.67	0.48	7.60	1.50
Week 8	20.00	10.75	2.18	6.33	0.41	7.90	1.49
Week 9	20.00	10.75	2.18	6.67	0.48	8.70	1.50

Post-SCI interval – time after SCI; Sham ctrls – control animals; N – Newton; Week 0 ≈ 48hours after SCI; SD – standard deviation;
* – significant ($P < 0.05$) difference between spontaneous of motor functions recovery score after SCI by force of 8N and 15N.

Table 2. Pelvic extremities motor functions recovery after SCI imposed by the force of 8N, 15N, and 18N in untreated animals and in mini-pigs treated for 5-h with TH applied at the epicentre of SC trauma.

Post SCI Interval	SCI 8N +TH 4 °C		SCI 15N + TH 4 °C		SCI 18N + TH ≈24 °C	
	Score	SD	Score	SD	Score	SD
Week 0	0.00	0.00	0.00	0.00	0.00	0.00
Week 1	1.88	1.13	0.14	0.14	1.33	0.67
Week 2	3.75	1.18	1.00	0.22	2.33	0.88
Week 3	6.75	0.51	2.71	0.87	4.00	1.00
Week 4	9.75	1.38	4.57	1.31	4.67	1.67
Week 5	10.00	1.38	5.14	1.37	5.67	2.67
Week 6	11.00	1.78	6.00	1.38	6.67	2.19
Week 7	12.25	1.97	6.57	1.43	7.00	2.59
Week 8	13.13	2.03	7.43	1.46	7.00	2.52
Week 9	13.63	1.77	7.71	1.44	7.67	2.73

Post SCI interval – time after SCI; N – Newton; TH 4 °C – therapeutic hypothermia by 4 °C saline;
TH ≈24 °C – therapeutic hypothermia by saline at room temperature; SD – standard deviation.

ence between scores in these two subgroups slightly vacillated, until the maximum mean difference (1.03 points in favour of untreated animals, members of subgroup No. 6) was detected at the end of the survival period (Table 1 and 2). The differences at individual week points were minimal, virtually negligible, and not significant.

DISCUSSION

The morphological and functional changes accompanying traumatic SC lesions have been studied in a multitude of experimental models. The most commonly used animal species in SCI research are small rodents [2, 6, 11, 35, 40]. However, rats and mice have different metabolisms, the structure and function of their CNS, and the consequences of severe SCIs differ from histopathologic changes observed in cats, dogs, sheep, pigs, apes, and humans [1, 10, 11, 12, 16, 24, 38, 40]. For example, the supraspinal control of motor functions in rodents is provided by the rubrospinal tract, and in bigger mammals, apes, and humans by the corticospinal tract [3, 19, 40]. The consequence of severe SCI in rats, bigger mammals, and primates is the development of a cavity (syringomyelic cyst) inside the spinal medulla which is bordered with a layer of preserved white matter. The destroyed spinal cord tissue within the SC of mice is replaced with connective tissue producing fibronectin and collagen [2].

The highest similarity to humans, especially regarding the corticospinal tract anatomy, thoracic extremities sensorimotor control, and bowel and urinary bladder function is in non-human primates [1, 12, 16, 23, 25, 40]. On the other hand, apes are expensive, and their prolonged care (e.g. urine and fecal discharge management, decubital ulcer prevention, and treatment) is difficult to maintain; also, emotional barriers may obstruct their utilization in SCI experiments [12, 25]. During the past two decades, the porcine SCI models have acquired increasing popularity [1, 24]. The manipulation of rapidly growing and massive domestic farm pigs is demanding, so the use of minipigs is preferred nowadays [6, 7, 23, 24, 39]. We decided to use 5–8-month-old Göttingen-Minnesota-Liběchov crossbreed strain minipigs in the study [34]. The female minipigs were employed with an aim to exclude the possibility of the development of penile prolapse followed by the potential occurrence of decubital necrosis of phallus (a life-threatening complication in paraplegic hogs), as well as to provide for the approximately equal hormonal status in all experimental animals [20, 28].

The majority of traumatic SCIs in humans are caused by motor vehicle accidents and falls [18]. The damage to the spinal medulla in these events is caused by fractured or luxated vertebrae, which act as impactors contusing the fragile medullary tissue [1]. To replicate this type of injury mechanism in the preclinical experimental study the authors used the circular metallic rod (5 mm in diameter) moved in the vertical direction by a computer-controlled stepping motor with prearranged velocity (30 $mm.sec^{-1}$, or 10 $mm.sec^{-1}$) and force (8N, 15N or 18N), crushing the spinal medulla located inside the spinal dural sac exposed by L3 laminectomy [7, 34, 39].

Remarkable attention of neuroscientists, neurologists, and neurotraumatologists aroused reports describing considerable improvement of neurological deficit in patients with complete tetra- or paraplegia caused by severe SCIs after application of TH or TH in combination with further therapeutic measures [5, 9]. The reduction of the whole body temperature or temperature of the medullary tissue in the epic enter of SCI (systemic/local hypothermia) inhibits cellular metabolism, retards enzymatic activity, reduces O_2 consumption, and energy demands along with increase of ATP stores, preservation of the blood-spinal cord barrier (BSCB), amelioration of development of local oedema, suppression of axonal swelling, inflammation, and mitochondrial membrane permeabilization which is a point-of-no-return in apoptosis [17, 26, 38]. The systemic application of TH is a complex procedure, often accompanied with serious complications, and the rewarming process requires 24–48 hours [5, 13, 36]. By contrast, the local cooling of the damaged spinal cord segment at the epicentres of injury is a technically simpler intervention, than systemic TH, the target temperature may be lower without serious jeopardy to the patient´s core (rectal) temperature, and development of the systemic hypothermia [36]. The positive outcomes were also reported after shorter application of hypothermia [9]. Hence, the authors decided to test the topical application of 4 °C saline and saline at room temperature (≈24 °C) via a perfusion chamber placed in the L3 laminectomy (the epic enter of SC trauma) with early onset (starting 30 min after experimental SCI) and lasting 5 hours [7, 34, 39]. The 30 min time interval between spinal trauma and the starting of TH application was necessary for removal of the impactor device from the L3 laminectomy, evacuation of potential epidural blood clot, coagulation of bleeding dural capillaries, and implantation of the perfusion chamber.

Spinal cord traumatic lesions are classified as complete and incomplete. A true anatomical transection/rupture of the spinal medulla occurs exceptionally, so there is a far lower incidence of complete than incomplete SCIs in both human as well as veterinary healthcare practice [14, 33]. The majority of conversions from complete to incomplete SCIs occur within the first 3 to 12 months after spinal trauma and during one year following SCI, up to 70 % of initially paraplegic subjects spontaneously recover one, sometimes two levels according to ASIA/ISNCSCI classification scale [8, 30]. It means the prognosis for ASIA C recovery to ASIA D is more propitious for patients with initial motor incomplete than for initial motor complete lesions, i.e. ASIA A or ASIA B [14, 30, 33]. The mechanisms concerned with spontaneous functional recovery after spinal traumatic lesions are poorly understood, so far. Usually, it is assumed that crucial for this phenomenon are spared axons of corticospinal tracts, and to a lesser degree the structural adaptation of spared neurons [33].

Immediately after the traumatic SCI develops in mammal spinal shock. It is characterized by transient flaccid paralysis caudal to the level of damaged SC segment without motor responses to external stimuli caused by axonal depolarization. The phenomenon lasts minutes to hours in

animals, but days to weeks in humans [15, 29]. To prevent the possible negative impact of this clinical entity on pelvic extremities motor scores, the initial performance tests in minipigs from subgroups No. 2–7 were postponed ≈48 hours after SCI (recorded „week 0“ in Table 1 and 2).

The phenomenon of spontaneous functional recovery also manifested itself in the presented study. The maximum spontaneous improvement of pelvic extremities motor functions was observed in minipigs after SC contusion inflicted by 8N force. On weeks 3, 4, 5, and 6, the differences were significant. Later on, they became less expressive, nevertheless, they exceeded 4 points in favour of subgroup No. 2 members at each examination. There also were differences in motor score rates between minipigs from subgroup No. 2 versus minipigs from subgroup No. 6. The results fluctuating from 2.05 to 3.50 points in favour of minipigs from subgroup No. 2 were statistically insignificant, nevertheless functionally important.

The correlation of mean motor scores of minipigs from subgroup No. 4 versus minipigs from subgroup No. 6 showed that at weeks 2–9, minipigs after SCIs inflicted by 18N force achieved higher mean motor scores than minipigs after SCIs inflicted by 15N force. The results could be incidental or caused by an error of small numbers as values were insignificant.

The maximum positive effect of therapeutic hypothermia (5-hour local application of 4 °C saline) on pelvic extremities motor functions recovery, became evident in minipigs after SCI inflicted by 8N force (experimental animals from subgroup No. 3). The results were not significant (mean motor scores vacillated until reached 2.88 points in favour of treated minipigs on week 9). However, the improvement of motor scores by almost 3 points (from not being able to stand up versus being able to stand up on its own and take 1–3 steps without support) could be considered an important positive change. The positive effect of TH application in minipigs after SCI inflicted by 15N force (experimental animals from subgroup No. 5) was lesser and insignificant. Surprisingly, minipigs from subgroup No. 6 (without application of TH) achieved higher motor scores than experimental animals from subgroup No. 7. The substantial difference in temperature of cooling solutions (saline 4°C, versus saline ≈24 °C) disqualifies a liable comparison between results obtained in these subgroups.

CONCLUSIONS

The data achieved in the minipig experimental study showed that a 5-hour application of 4 °C saline at the epic enter of SCI inflicted by force of 8N, 15N, or 18N via L3 laminectomy is well tolerated and may improve pelvic extremities motor score. However, further experimental and preclinical trials are requested to determine the optimal cooling parameters (therapeutic window, optimal temperature, and duration of hypothermia application), to test the effectiveness of the method in the various large animal as well as non-human primate SCI models.

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