Journal of Thermal Biology 83 (2019) 1–7

Contents lists available at ScienceDirect

Journal of Thermal Biology

journal homepage: www.elsevier.com/locate/jtherbio

Therapeutic hypothermia attenuates paraplegia and neuronal damage in the lumbar spinal cord in a rat model of asphyxial cardiac arrest

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ARTICLE INFO

Keywords:
Asphyxial cardiac arrest and cardiopulmonary resuscitation
Spinal cord
Motor neuron
Paraplegia
Therapeutic hypothermia

ABSTRACT

Spinal cord ischemia can result from cardiac arrest. It is an important cause of severe spinal cord injury that can lead to serious spinal cord disorders such as paraplegia. Hypothermia is widely acknowledged as an effective neuroprotective intervention following cardiac arrest injury. However, studies on effects of hypothermia on spinal cord injury following asphyxial cardiac arrest and cardiopulmonary resuscitation (CA/CPR) are insufficient. The objective of this study was to examine effects of hypothermia on motor deficit of hind limbs of rats and vulnerability of their spinal cords following asphyxial CA/CPR. Experimental groups included a sham group, a group subjected to CA/CPR, and a therapeutic hypothermia group. Severe motor deficit of hind limbs was observed in the control group at 1 day after asphyxial CA/CPR. In the hypothermia group, motor deficit of hind limbs was significantly attenuated compared to that in the control group. Damage/death of motor neurons in the lumbar spinal cord was detected in the ventral horn at 1 day after asphyxial CA/CPR. Neuronal damage was significantly attenuated in the hypothermia group compared to that in the control group. These results indicated that therapeutic hypothermia after asphyxial CA/CPR significantly reduced hind limb motor dysfunction and motoneuronal damage/death in the ventral horn of the lumbar spinal cord following asphyxial CA/CPR. Thus, hypothermia might be a therapeutic strategy to decrease motor dysfunction by attenuating damage/death of spinal motor neurons following asphyxial CA/CPR.

1. Introduction

Cardiac arrest (CA) abruptly halts blood flow and oxygen delivery in the whole-body. It induces whole-body ischemia and causes high morbidity and mortality (Chalkias and Xanthos, 2012; Schneider et al., 2009). Experimental animal models of transient whole-body ischemia resulting from CA/CPR have been established to investigate brain injury (Hickey et al., 2000; Katz et al., 1995; Paine et al., 2012). Specific regions in the central nervous system (CNS) are selectively damaged even after a brief ischemic episode. This phenomenon is called “selective vulnerability” (Pulsinelli et al., 1982).

Until now, most studies following whole-body ischemia have been carried out in the brain. Few studies have been demonstrated in the spinal cord. This is because it is generally believed that the spinal cord is less vulnerable to transient global ischemia than the brain (Duggal and Lach, 2002). Spinal cord ischemia or infarction is a major cause of serious spinal cord injury in clinical practice with very complicate mechanisms. It can lead to severe spinal cord disorders such as paraplegia that has a major invalidating impact on patient's life (Grassner et al., 2014; Nedeltchev et al., 2004; Watts, 1995). In the spinal cord, motor neurons are known to be particularly vulnerable during ischemia (Nohda et al., 2007). However, factors contributing to the vulnerability

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https://doi.org/10.1016/j.jtherbio.2019.04.018
Received 21 February 2019; Received in revised form 24 April 2019; Accepted 24 April 2019
Available online 30 April 2019
0306-4565/

of motor neurons in spinal cord ischemia have not been well documented yet.

It is well known that body temperature can influence the outcome of ischemia-reperfusion injury (Busto et al., 1989; Nurse and Corbett, 1996). It has been documented that hypothermia can improve survival and neurological outcomes after CA/CPR (Arrich et al., 2016; Bernard et al., 2002; Cronberg et al., 2015; Gebhardt et al., 2013). However, mechanisms underlying hypothermic protection against ischemia-reperfusion injury remain unclear. It is believed that hypothermia can trigger endogenous cellular adaptation. In addition, roles of hypothermia on paraplegia and spinal cord injury in rat models of asphyxial CA/CPR have not been studied, although there are various possible explanations for the protective effect of hypothermia against spinal cord injury (Dietrich, 2009; Martirosyan et al., 2017). In this regard, the objective of this study was to determine the effect of hypothermia on paraplegia and spinal cord injury in a rat model of CA/CPR. We examined effects of therapeutic hypothermia on paraplegia and neuronal damage in the lumbar spinal cord of rats following CA/CPR.

2. Materials and methods

2.1. Experimental animals and groups

We obtained male Sprague-Dawley rats (10 weeks of age; body weight, 310–320 g) from the Experimental Animal Center, Kangwon National University, Chunchon, South Korea. They had been maintained in pathogen-free conditions under temperature (23 °C) and humidity (60%). The experimental protocol of this study was approved (approval no. KW-151127-1) by the Institutional Animal Care and Use Committee (IACUC) at Kangwon University. The content of the protocol adhered to guidelines that are in compliance with the current international laws and policies (Guide for the Care and Use of Laboratory Animals, The National Academies Press, 8th Ed., 2011). Rats (total n = 35) were divided into three groups: (1) Sham group (n = 7), which was given identical anesthetic and surgical procedures without the induction of CA/CPR operation. (2) Control group (n = 17), which was given asphyxial CA/CPR operation, and body temperature was controlled (37 ± 0.5 °C) for 4 h after return of spontaneous circulation (ROSC). (3) Hypothermia group (n = 11), which was given asphyxial CA/CPR operation, and body temperature was controlled (33.0 ± 0.5 °C) for 4 h after ROSC.

2.2. Asphyxial CA/CPR operation and therapeutic hypothermia

Rat in each group were anesthetized with a mixture of 2.5% isoflurane in 33% oxygen and 67% nitrous oxide. They were endotracheally intubated with a 14-gauge cannula and mechanically ventilated with a mixture of 2% isoflurane in 33% oxygen and 67% nitrous oxide. The right femoral artery and vein were cannulated with PE-50 catheters to monitor arterial blood pressure and to administer drug, respectively. Electrocardiographic (ECG) probes were placed to provide three-lead in the limbs (GE healthcare, Milwaukee, WI). These data were continuously monitored. Body (rectal) temperature was maintained at 37 ± 0.5 °C with a heat blanket. At 5 min after stabilization, vecuronium bromide (2 mg/kg) (Reyon Pharmaceutical, Seoul, Korea) were intravenously administered, and anesthesia was stopped. The mechanical ventilation was discontinued, and the endotracheal tube was disconnected from the ventilator. Define CA was confirmed when mean arterial pressure (MAP) was below 25 mmHg and subsequent pulseless electric activity (PEA) was shown (Che et al., 2011; Han et al., 2010). Usually, CA was confirmed at about 3–4 min after the discontinuation of ventilation following vecuronium bromide injection in this study. At 5 min of CA, CPR was initiated by intravenously administering a bolus injection of epinephrine (0.005 mg/kg) (Dai Han Pharm, Seoul, Korea) and sodium bicarbonate (1 meq/kg) (Daewon Pham, Seoul, Korea) followed by mechanical ventilation with 100% oxygen. For ROSC, manual chest compression was done at a rate of 300/min until MAP reached 60 mmHg, and ECG activity was observed (Idris et al., 1996; Liachenko et al., 1998). If ROSC is not detected, half of the injected amount of epinephrine was administered with 1 min of CPR. However, the rats with the third time CPR were excluded from this experiment. After ROSC, body temperature in the control group was maintained at 37 ± 0.5 °C by using a heating pad and lamps, meanwhile body temperature in the hypothermia group was maintained at 33 ± 0.5 °C by using isopropyl alcohol wipes for 4 h after ROSC. Once each rat was hemodynamically stable and spontaneously breathing (usually 1 h after ROSC), the catheter was removed and the animal extubated. Thereafter, all animals were kept in a thermal incubator (Mirae Medical Industry, Seoul, Korea) to maintain to 37.0 ± 0.5 °C. Rats of the sham group underwent surgical procedure of CA except for the induction of CA, and they were kept under anesthesia and 37 °C for 4 h.

2.3. Evaluation of physiological variables and motor function

To examine physiological variables, we compared body weight and MAP between the groups at 1 days after ROSC. For motor function of hind limbs, we evaluated it at 1 day after ROSC by Tarlov Scale (Tarlov, 1972) as follows (motor deficit scoring): 0, complete paralysis with no lower extremity function; 1, slight movement in articulations; 2, unable to stand without support; 3, sit alone; 4, weak walking with poor jumping; 5, normal walking.

2.4. Tissue processing for histology

The animals were used for histological and immunohistochemical staining at 1 day after ROSC as follows. The animals were perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (in 0.1 M PB) (pH 7.4). We removed lumbar spinal cords and postfixed them in the same fixative for 6 h. The lumbar spinal cord tissues were cryoprotected by infiltration with 25% sucrose for 8 h. Thereafter, the cord tissues were frozen and serially sectioned into 25 μm coronal sections in a cryostat (Leica, Wetzlar, Germany).

2.5. Cresyl violet (CV) and Fluoro-Jade B (F-J B) histofluorescence staining

To elucidate neuronal damage or death after CA/CPR, CV staining and F-J B histofluorescence staining were performed as previously described (Lee et al., 2010). In brief, for CV (a marker for Nissl's body) staining, the sections were stained with solution of 1.0% (w/v) CV acetate (Sigma–Aldrich, St. Louis, MO, USA). For neuronal degeneration, F-J B (a high affinity fluorescent marker for the localization of neurodegeneration) histofluorescence staining, the sections were immersed in solution of 0.0004% F-J B (Histochem, Jefferson, AR, USA). The immersed sections were washed and placed on a slide warmer (50 ± 1 °C) to be reacted. For the count of F-J B-positive cells or neurons, the ventral gray matter (anterior to a line drawn through the central canal perpendicular to the vertical axis) was used from 7 sections of each rat. F-J B positive cells were captured by using an epifluorescent microscope (Carl Zeiss, Göttingen, Germany) with blue (450–490 nm) excitation light and a barrier filter, which was equipped with a digital camera (Axiocam, Carl Zeiss) connected to a PC monitor. The mean number of F-J B-positive cells were obtained by averaging the total cell numbers from each animal per group.

2.6. Immunohistochemistry for neuronal nuclei (NeuN)

To examine neuronal damage, immunohistochemistry for NeuN (a neuron-specific soluble nuclear antigen) was carried out according to our published procedure (Lee et al., 2014). In brief, the sections were

incubated with primary mouse anti-NeuN (diluted 1:1,000, Chemicon International, Temecula, CA), reacted with secondary antibody (Vector Laboratories Inc., Burlingame, CA) and developed by Vectastain ABC (Vector Laboratories Inc.). Finally, they were visualized with solution of 3,3′-diaminobenzidine. For the count of NeuN immunoreactive cells, the cells were captured by using an AxioM1 light microscope (Carl Zeiss) equipped with a digital camera (Axiocam, Carl Zeiss) connected to a PC monitor. The mean number of NeuN-immunoreactive cells were obtained by averaging the total cell numbers from each animal per group.

2.7. Statistical analysis

Sample size was at least seven rats per group with an alpha error of 0.05 and a power of > 80%, and the sample size was calculated with a power calculator (UCLA Department of Statistics, http://www.stat.ubc.ca/~rollin/stats/ssize). All statistical data were performed with SPSS software (version 15.0, SPSS Inc., Chicago, IL, USA) and expressed as mean ± SEM. The significance of differences between the groups was assessed using the Non-parametric analyses with Mann-Whitney *U* test, one-way analysis of variance followed by post hoc Tukey test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Physiological variables and body temperature

There were no significant differences in characteristics including body weight, anesthesia duration for preparation and MAP among the groups ($P > 0.05$). The induction of CA was about 3–4 min after vecuronium bromide injection (2 mg/kg, *i.v.*) and confirmed with isoelectric ECG and MAP. MAP was changed as expected according to the experimental protocol between onset and after ROSC. The values had no significant difference among the groups (Fig. 1A). Compared to the sham and control groups, rectal temperature in the hypothermia group was decreased to target temperature (33 ± 0.5 °C) for 4 h after ROSC and followed by gradually rewarming to 37 ± 0.5 °C (Fig. 1B). The recovery rate of ROSC after CPR was about 85% in this study and survival rate is 50% in control group and 80% in hypothermia group at 1 day after CA/CPR.

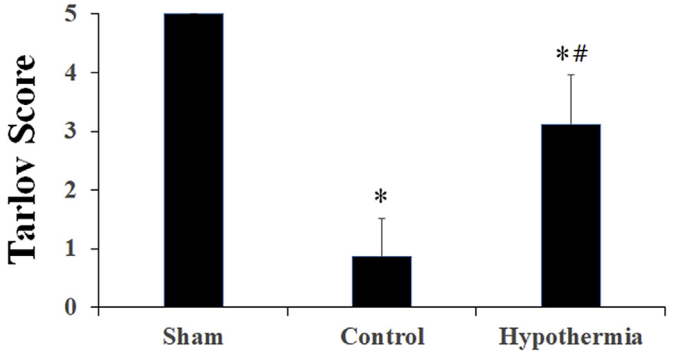

Fig. 2. Motor function of the hind limb in the sham, control and hypothermia group according to the Tarlov Scoring System. Motor deficit score was measured at 1 day after CA/CPR (n = 7). High score represents good motor function. The bars indicate the means ± SEM ($^{*}p < 0.05$ vs. sham group; $^{\#}p < 0.05$ vs control group).

3.2. Motor deficit score

Motor deficit of hind limbs was evaluated with modified Tarlov score at 1 day after ROSC (Fig. 2). Rats of the sham group showed normal function in the hind limb. In the control group, motor function score in the hind limb was significantly lower (average 1.3 point) than that in the sham group (average 5 point) ($P < 0.01$). However, in the hypothermia group, motor function of the hind limb was significantly

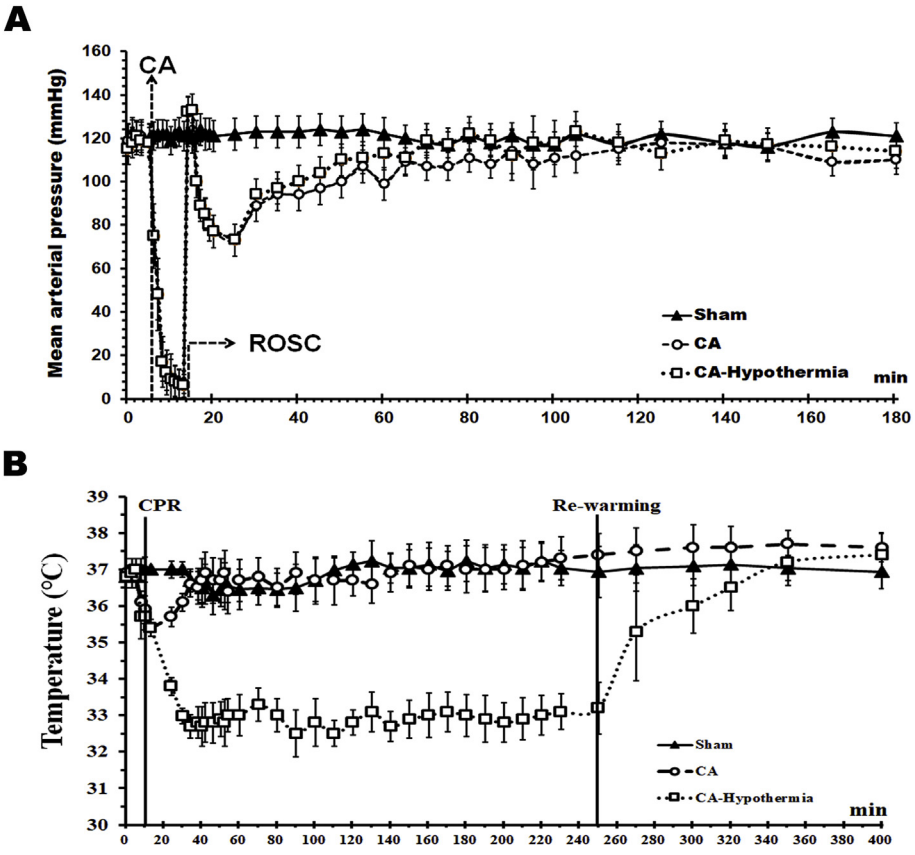

Fig. 1. Physiological variables after asphyxial CA/CPR. (A) MAP during CA, CPR and ROSC in the sham, control and hypothermia group. The bars indicate the means ± SEM. (B) Time course of changes in rectal temperature in the sham, control and hypothermia group. Body temperature in the hypothermia group was controlled at 33 ± 0.5 °C for 4 h after ROSC. The bars indicate the means ± SEM.

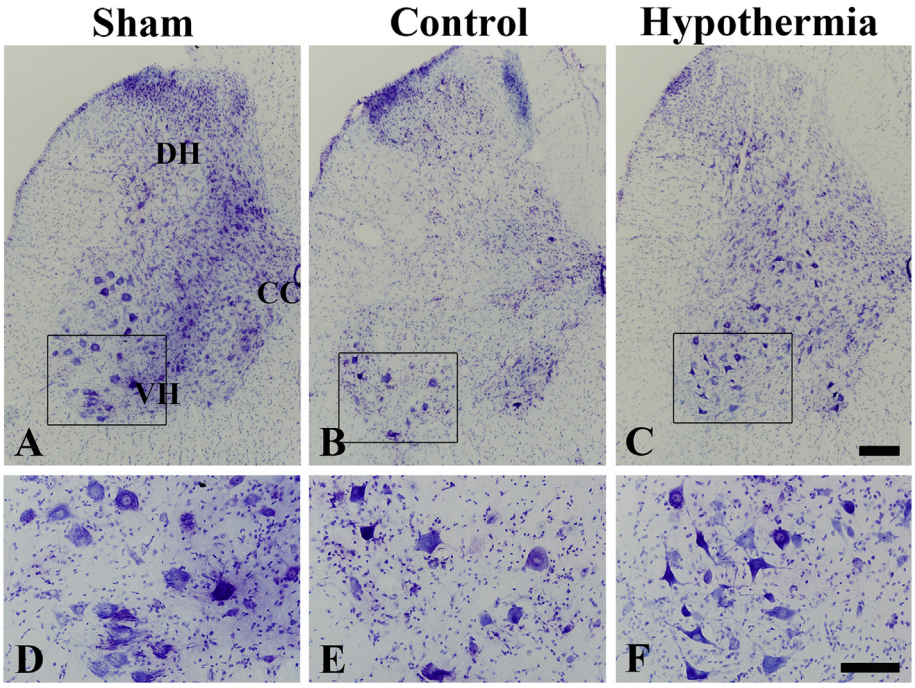

Fig. 3. CV staining in the lumbar spinal cord of the sham (A, D), control (B, E) and hypothermia (C, F) group at 1 day after CA/CPR. CV^+ cells are significantly lost in the ventral horn (VH) after CA/CPR, whereas, many CV^+ cells survive in the VH of the hypothermia group. DH, dorsal horn. CC; central cannel, Scale bar = 200 (A–C) and 100 (D–F) μm.

improved (average 3.7 point) compared to that in the control group ($P < 0.05$).

3.3. Histopathological findings

3.3.1. CV staining

We examined cellular distribution and change in the ventral horn of the lumbar spinal cord after CA/CPR by using CV staining, which is a very useful method to detect Nissl's body in cells (Fig. 3). In the sham group, CV-positive (CV^+) cells were easily detected in all subregions of the gray matter. CV^+ cells in the lateral part of the ventral horn showed typical motor neurons in shape, and they were large and pyramid-like or round (Fig. 3A, D). In the control group, CV^+ cells in the ventral horn were significantly changed in their morphology at 1 day after CA/CPR compared to that of the sham group (Fig. 3B), namely, the CV^+ cells were shrunken in size of somata, showed the rupture of cell membrane and contained dark and polygonal nuclei (Fig. 3E). In the hypothermia group, many CV^+ cells in the ventral horn were much less damaged at 1 day after CA/CPR than those in the control group (Fig. 3C, F).

3.3.2. NeuN immunohistochemistry

Neuronal damage in the ventral horn of the lumbar spinal cord was assessed by NeuN immunohistochemistry, which is useful to detect damage of neuronal nuclei (Fig. 4). In the sham group, motor neurons in the ventral horn were well stained with NeuN in their nuclei (Fig. 4A and D). In the control group, only a few NeuN-immunoreactive ($NeuN^+$) neurons were observed in the ventral horn at 1 day after CA/CPR (Fig. 4B and E). In this group, the mean percentage of $NeuN^+$ neurons in the ventral horn was 17.4% of that in the sham group (see Fig. 4G). In the hypothermia group, many $NeuN^+$ cells were found in the ventral horn at 1 day after CA/CPR (Fig. 4C and F), showing that the mean percentage of neurons was 56.5% of the sham group (Fig. 4G).

3.3.3. F-J B histofluorescence staining

Neuronal death in the ventral horn of the lumbar spinal cord was assessed by F-J B histofluorescence staining, which is a very useful method to detect dead neurons (Fig. 5). No F-J B-positive (F-J B^+) neurons were found in the ventral horn of the sham group (Fig. 5A and D). In the control group, many F-J B^+ cells were shown in the ventral horn at 1 day after CA/CPR (Fig. 5B and E). In the hypothermia group, numbers of F-J B^+ cells in the ventral horn were significantly decreased at 1 day after CA/CPR (Fig. 5C and F), and the mean percentage of the F-J B^+ cells was 31.3% of the control group (Fig. 5G).

4. Discussion

In recent years, advances in medical technology have increased the number of patients surviving from CA/CPR (Bunch et al., 2003; Ewy and Bobrow, 2016; Ewy and Sanders, 2013). However, few studies have examined the impact of CA/CPR on spinal motor neurons (Duggal and Lach, 2002; Imaizumi et al., 1994). Clinically, the spinal cord is extremely vulnerable to ischemic insults due to a variety of circulatory abnormalities, including embolic disease, vasculitis, cardiac arrest, thoracic aortic aneurysm, and complications after aortic surgery or spinal angiography (Cheng et al., 2009; Dublin et al., 2010; Turkoz et al., 2007). It has been reported that about 2–18% patients display neurological deficits due to ischemic spinal cord injury (Patel et al., 2011). These patients undergo delayed neurological deficits and develop paraplegia in several hours or a few days after surgery (Safi et al., 2005). In this study, we found that CA could lead to a loss of motor function in hind limbs at 1 day after CPR. However, motor deficit of hind limbs was significantly attenuated after animals received therapeutic hypothermia compared to control animals. Despite continuous efforts, CPR is still associated with high mortality. In addition, many survivors suffer from neurological deficits (Chen et al., 2013; Ehlenbach et al., 2009; Nolan et al., 2014). This problem is associated with the degree of interruption of spinal cord blood flow as severe spinal cord ischemia following CA is obviously due to neglect after CPR (Cheshire et al., 1996; Turkoz et al., 2007).

Previous studies have suggested large motor neurons in the brain are more sensitive to ischemia than small sensory neurons (Harukuni and Bhardwaj, 2006; Lee et al., 2000). Motor neurons in the spinal cord are also known to be vulnerable during ischemia (Jacobs et al., 1992; Nohda et al., 2007). Furthermore, it has been reported that paraplegia happens when motor neurons in the lumbar spinal cord are damaged or dead. Experimentally, many studies on ischemic injury of the spinal cord have focused on spinal cord ischemia models associated with local

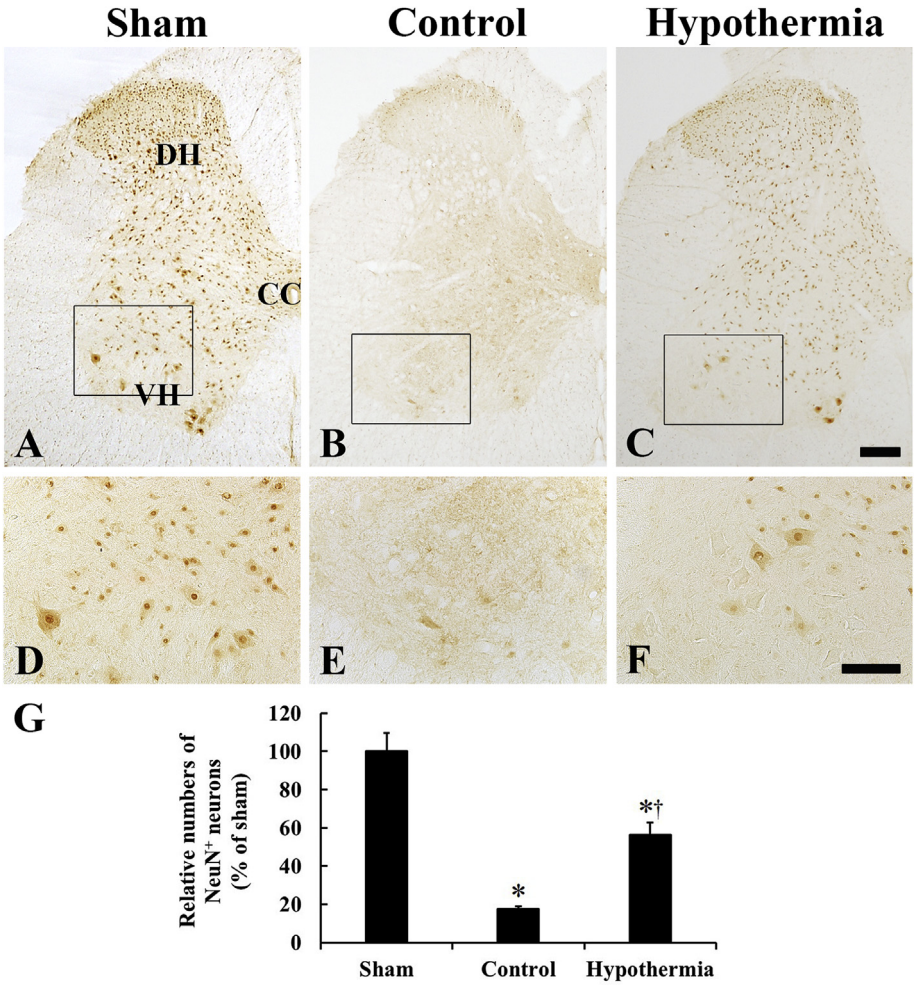

Fig. 4. Immunohistochemistry for NeuN in the lumbar spinal cord of the sham (A, D), control (B, E) and hypothermia (C, F) group at 1 day after CA/CPR. In the control group, $NeuN^+$ neurons are hardly shown in the ventral horn (VH) after CA/CPR, whereas, many $NeuN^+$ cells are shown in the VH of the hypothermia group. DH, dorsal horn. CC; central cannel, Scale bar = 200 (A–C) and 100 (D–F) μm. G: Quantitative analysis of $NeuN^+$ cells in the VH. The bars indicate the means ± SEM ($^*p < 0.05$ vs. sham group; $^\#p < 0.05$ vs control group).

vascular changes or aortic disease (Lang-Lazdunski et al., 2000; Lu et al., 2004; Marsala and Yaksh, 1994). Among simple models of spinal cord ischemia, selective motor neuronal death happens in the lumbar level within 1 day after transient ischemia in a rabbit model of spinal cord ischemia, a simple model by ligation of the spinal arteries which do not have collateral circulation (Lee et al., 2005; Mechirova et al., 2014; Yoo et al., 2017). However, the characteristic of paraplegia after CA/CPR is very complicated with few reports (Katz et al., 1995; Kofler et al., 2004; Krep et al., 2000). Kudo et al. (2006) have reported that neuronal death in the spinal cord of a rat model of hemorrhagic shock model followed by CA occurs in the intermediate gray matter of the lumbar spinal cord whereas most motor neurons in the ventral horn are intact. In this study, we found that severe cell damage or death occurred in the ventral horn of the lumbar spinal cord after CA/CPR in rats. From an anatomical view, spinal motor neurons are located within the ventral horn which is named lamina IX in the Rexed lamina system. Motor neurons provide motor component (general somatic efferent) of spinal nerves that innervate muscles of the body. Taken together, neuronal vulnerability in the spinal cord might be different according to the impact of ischemia due to proper neuronal function such as pumps and channels in the neuronal membrane (Sengupta et al., 2013). Our finding shows that motor neurons are most vulnerable to ischemia induced by asphyxial CA, suggesting that the extent and severity of spinal cord injury are related to the impact of CA.

For the past 20 years, many researchers have shown that hypothermia can lead to neuroprotection and improve functional outcomes in various animal models of brain and spinal cord injury (Dietrich et al., 2009). Although few clinical evidence concerning the efficacy of hypothermia in the treatment of spinal cord injury after CA/CPR has been demonstrated, many animal studies have shown that hypothermia has potent efficacy in spinal cord injury caused by ischemia (Allen et al., 1994; Horiuchi et al., 2009; Kwun and Vacanti, 1995; Saito et al., 2013; Wang et al., 2005). In this study, we investigated the efficacy of therapeutic hypothermia in rats with spinal cord injury after CA/CPR. As expected, we found that hypothermia (32 °C) improved paraplegia and significantly attenuated the damage/death of motor neurons in the ventral horn at 1 day after CA/CPR. Therapeutic hypothermia might exert neuroprotection by blunting pathologic mechanisms of ischemia such as decrease of cerebral metabolism, reduction of free radicals, and anti-inflammatory effect (Froehler and Geocadin, 2007; Janata and Holzer, 2009; Vaagenes et al., 1996).

5. Conclusion

Collectively, our findings showed that paraplegia occurred at 1 day after CA/CPR by death of lumbar spinal motor neurons after asphyxial CA/CPR. In addition, we provided evidence that therapeutic hypothermia could improve spinal motor dysfunction by attenuating neuronal damage/death in the ventral horn of the lumbar spinal cord.

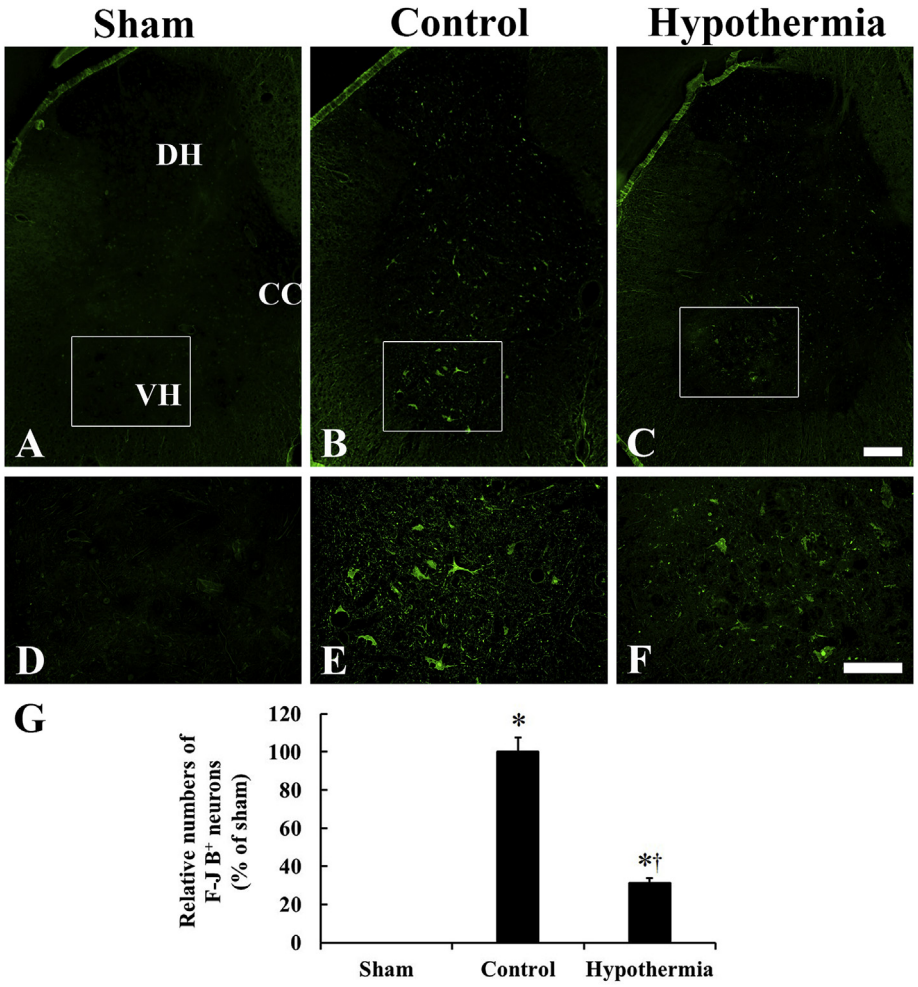

Fig. 5. Histofluorescence staining for F-J B in the lumbar spinal cord of the sham (A, D), control (B, E) and hypothermia (C, F) group at 1 day after CA/CPR. In the control group, many F-J B^+ cells are shown in the ventral horn (VH) after CA/CPR, whereas, numbers of F-J B^+ cells are decreased in the VH of the hypothermia group. DH, dorsal horn. CC; central cannel, Scale bar = 200 (A–C) and 100 (D–F) μm. G: Quantitative analysis of F-J B^+ cells in the ventral horn. The bars indicate the means ± SEM ($^*p < 0.05$ vs. sham group; $^\#p < 0.05$ vs control group).

Acknowledgements

This work was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2018R1D1A1B07049453, NRF-2018R1D1A1B07049594) and by the Ministry of Science, ICT & Future Planning (NRF-2017R1A2B4009079).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.04.018.

Declaration of interests

The authors declared that there are no conflicts of interest to this work.

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