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Remote conditioning as protective strategy influencing ubiquitin-mediated stress response in the rabbit spinal cord after ischemia/reperfusion

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Abstract. The aim of presented study was to investigate the model of non-invasive method of remote conditioning induced by compression of left forelimb with a tourniquet in three cycles of 2 min of ischemia each followed by 2 min of reperfusion and its influence on the rabbit spinal cord ischemia/reperfusion injury *via* ubiquitin-mediated stress response. Ubiquitin immunoreaction in spinal cord motor neurons as well as detection of neuronal survival in ventral horns of spinal cord were evaluated. Significantly increased ($p < 0.001$) number of ubiquitin positive neurons was registered in all remote conditioned groups *versus* both spinal cord ischemia (SC-ischemia) groups. Our results indicate that remote conditioning significantly attenuated degeneration of motor neurons in all conditioned groups *versus* SC-ischemia groups in each time point. According to our results, we concluded that the remote conditioning induced by transient limb ischemia is relevant stimulus that provides potent neuroprotection in a model of spinal cord ischemia/reperfusion injury.

Key words: Ischemia/reperfusion injury — Rabbit — Remote conditioning — Spinal cord — Ubiquitin

Introduction

Paraplegia is a serious and unpredictable complication of surgical procedures on the abdominal aorta or interventions in the thoracic region (Arai et al. 2002; Kim et al. 2019). Although a number of strategies have been used to reduce a risk of spinal cord injury associated with this surgery, including induced hypothermia and cerebrospinal fluid drainage, the therapeutic benefits of these interventions remain uncertain (Huang et al. 2007). Nerve cells have different functions and their response to stressful situations also varies. The motor neurons in the spinal cord may degenerate selectively after experimental transient ischemia, which was compatible with the delayed deterioration of neurological function after spinal cord ischemia (Moore and Hollier 1991; Sakurai et al. 1997). At the moment, when the ischemic stimulus triggers destructive metabolic process in the nerve cell, the stopping of it and resumption of normal operating conditions in cells is difficult. In recent years, increased attention to the phenomenon of ischemic tolerance was recorded (Pignataro et al. 2020). It was confirmed in various animal models of focal cerebral ischemia, global ischemia (Riksen et al. 2004) and spinal cord ischemia (Mukai et al. 2020; Yazar et al. 2021).

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Ischemic tolerance lies in the bear relation to ability of cell response to sublethal stimulus by building of extremely powerful endogenous protection allowing them to survive otherwise lethal ischemic injury. Tissues or cells can provide tolerance to each other – remote tolerance. If only one part of the body undergoes stress conditions with adequate stressors, the full organ respectively the whole body can acquire the tolerance completely (Riksen et al. 2004). Remote ischemic perconditioning or postconditioning applicated as sublethal ischemia on the organ (e.g. limb) far away from the target organ, such as the brain or spinal cord (Changhong et al. 2015), induces global endogenous tolerance and protects distant target organs or tissues against subsequent prolonged ischemia/reperfusion injury. Altogether, the protective effect of ischemic conditioning can be induced not only directly *in situ*, but also in distant organs (Hess et al. 2015). The positive results were obtained by direct ischemic preconditioning in experimental studies (Peralta et al. 1996; Fernandez et al. 2002), but there are limits in the application to clinical practice. In clinical practice, the perconditioning and postconditioning application has a greater perspective (Ren et al. 2015).

Wei et al. (2011) reported that the remote ischemic perconditioning (application during ischemia) in combination with remote ischemic postconditioning (application after ischemia) increased cell survival in a rat model of myocardial infarction. To this moment, information regarding the neuroprotection in spinal cord is insufficient.

Ubiquitin is the highly conserved 76 amino acid protein, which is covalently attached *via* its C-terminal glycine to lysine residues or less frequently to cysteine, serine and threonine residues on substrate proteins. This process is called ubiquitination. Ubiquitination is an essential player in proteostasis regulation but also in orchestrating signalling pathways in response to various stress conditions (Weber et al. 2016). Ubiquitin conjugation to target proteins followed by their subsequent proteasomal degradation has become the hallmark of the mechanism by which cells specifically remove regulatory proteins, tune their activity, or destroy damaged proteins (Ziv and Ciechanover 2021). Ubiquitin-proteasome pathway is involved in the protection of nerve cells from ischemia/reperfusion injury. Zhang et al. (2010) confirmed that inhibition of ubiquitin-conjugated protein aggregation may have an essential role in inducing cerebral ischemic tolerance by isoflurane preconditioning in a transient global cerebral ischemia-reperfusion injury in mouse model. Preservation of ubiquitin activity for elimination of misfolded proteins is one of possible mechanisms in neuroprotection of rabbit's spinal cord after ischemia and bradykinin postconditioning (Fagová et al. 2019). Dysfunction in proteostasis regulation due to imbalances in protein synthesis, folding, and degradation challenges the integrity of the cellular proteome and favours the accumulation of aggregated proteins that can damage cells by a loss of their functions and/or a gain of adverse functions. The ubiquitin-proteasome system plays an important role in the numerous of these cellular processes, and its dysfunction is thought to contribute also to motor neurons disease (Bax et al. 2019).

This study is aimed to determine the relative potency of remote perconditioning or postconditioning for protection against ischemic spinal cord injury in rabbits. We focused on neuronal nuclei (NeuN) immunohistochemistry for detection of surviving nerve cells in anterior horns of the rabbit spinal cord and ubiquitin immunohistochemistry was applied to detect spatial location of ubiquitin in the nerve cells. Based on our investigation of the ubiquitin-mediated stress response, we expect that the present study may help to acquire new information regarding non-invasive method of remote conditioning as well as to support idea of its application to the clinical area in the future.

Material and Methods

The experiments were performed on 83 male, clinically healthy New Zealand white rabbits, weighing 2.5–3 kg. Rabbits were supplied by Velaz, s.r.o. (Prague, Czech Republic) and housed in a light, temperature (15–21°C) and humidity (55 ± 10%) controlled room in standard cages in user´s establishment SKP03014. Rabbits had free access to food and water. Handling and experimental protocols of Ro-1347/15-221, Ro–3524/17-221 were permitted by the Ethical committee of the Faculty of Medicine, Pavol Jozef Šafárik University in Košice in accordance with the European Community Council Directive (2010/609/EEC).

Rabbits were anesthetized with intramuscular administration of zolazepam (20 mg/kg; Virbac; Carros, France), tiletamine (20 mg/kg; Virbac) and xylazine (3 mg/kg; ECUPHAR N.V.; Oostkamp, Belgium) and operated under sterile conditions. Antibiotic cefotaxime (50 mg/kg, i.m.; Sandoz Pharmaceuticals d.d.; Ljubljana, Slovenia) was administered prior to 20 min of spinal cord ischemia. Spinal cord ischemia (SC-ischemia) was induced by occlusion of the aorta below the left renal artery for 20 min (Zivin and Girolami 1980). The time period 24 or 72 h after removal of aortic occlusion we referred as the reperfusion (R) period.

Remote ischemic conditioning of the spinal cord was induced by compression of left forelimb with a tourniquet in three cycles of 2 min of forelimb ischemia, each followed by 2 min of reperfusion. Remote conditioning procedures were performed at one of three time points: during the last 12 min of aortic occlusion (perconditioning; PerC), 1 h after occlusion (early postconditioning; early PostC), or 3 h after occlusion (late postconditioning; late PostC). Rabbits were divided into 9 groups.

The rabbits of sham control (Sham) were subjected to procedures related to the anesthesia, and surgical incisions, but not to the remote conditioning stimulus or spinal cord ischemia. The rabbits of sham group were, immediately after these procedures, transcardially perfused with 0.9% saline and 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4).

Rabbits of SC-ischemia groups (SC-ischemia/24h, SC-ischemia/72h) were subjected to spinal cord ischemia only, followed by no additional treatment.

Next rabbits were also subjected to SC-ischemia, followed by three alternating 2-min on/2-min off sessions of left forelimb tourniquet compression during the last 12 min of the SC-ischemia (PerC/24h, PerC/72h groups), or 1 h after SC-ischemia (early PostC/24h, early PostC/72h groups), or 3 h after SC-ischemia (late PostC/24h, late PostC/72h groups).

After reperfusion period, rabbits were anesthetized and transcardially perfused with 0.9% saline and 4% paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4). Spinal cords were dissected and postfixed in 4% paraformaldehyde solution. The spinal cord segments L_4–L_6 were processed for light microscopy.

Neurologic assessment

The hind limb motor function was assessed at 24 h and 72 h of reperfusion by trained, independent observer, who was unaware of the animal groups. The modified Tarlov scoring system was used for assessment. A score 0–5 was assigned to each animal: 0 – complete paraplegia, 1 – slight movement of hind limbs, 2 – active movement but unable to sit without assistance, 3 – able to sit but unable to hop, 4 – weak hop, 5 – complete normal hind limb motor function.

Nissl staining method

The histological method invented by Nissl is widely used by neuroanatomists and pathologists to study the morphology and pathology of nerve tissue (Da Mota 2019). Paraffin sections (5-μm thickness) mounted on silane-coated slides were deparaffinized and rehydrated with descending series of ethanol, treated with 5% formol for 5 min followed by treatment with 5% acetic acid for minutes. Then, the sections were treated with 0.1% cresyl violet (Sigma-Aldrich, Co; St. Louis, Missouri, USA) solution in thermostat at 37.7°C for 20 min and then the sections were moved from thermostat and allowed to cool at the room temperature. After washing in distilled water for 1 min, cooled sections were differentiated in 96% ethanol twice for 3 min. Subsequently, the sections were cleared with xylene and coverslipped using Pertex (Histolab Products AB; Göteborg, Sweden).

Ubiquitin immunohistochemistry

Primary antibody for ubiquitin was applied to detect spatial localization of ubiquitin in the nerve cells (Fagová et al. 2019). Paraffin sections of 5 μm thickness were mounted to silane-coated slides and dried. Deparaffinized and rehydrated sections were treated with 20% methanol with 3% H_2O_2 in phosphate buffer (PBS). After washing with 0.1 M PBS for 5 min slides were blocked and permeabilised in 0.1 M PBS with Triton X-100 (9002-93-1; SIGMA-ALDRICH, Co.) and 1% bovine serum albumin and 5% normal goat serum. For detection of ubiquitin, sections were treated with primary polyclonal rabbit anti-ubiquitin antibody (U-5379; 20K9001; 1:100; Sigma-Aldrich, Co.) overnight at 4°C. After washing in PBS, sections were incubated with secondary biotinylated anti-rabbit IgG (B6648-3ML; 077K4848; 1:20; Sigma-Aldrich, Co.) for 70 min and subsequently with ExtrAvidin-Peroxidase (E8386-3ML; 077K4849; 1:20; Sigma-Aldrich, Co.) for 70 min in humidity chamber at room temperature. Reaction was visualized by 3,3'-diaminobenzidine (32750; Sigma-Aldrich, Co.). Mayer's hematoxyline was used for counterstaining. Sections were cleared by immersion in xylene and coverslipped with Pertex (Histolab Products AB).

Neuronal nuclei (NeuN) immunohistochemistry

NeuN immunohistochemistry was used for detection of surviving nerve cells in anterior horns of the spinal cord (Fagová et al. 2019). Paraffin sections (8-μm thickness) were mounted to silane-coated slides and dried. Deparaffinized and rehydrated sections were treated with 20% methanol with 3% H_2O_2 in phosphate buffer (PBS). After washing with 0.1 M PBS for 5 min slides were blocked and permeabilised in 0.1 M PBS with Triton X-100 (9002-93-1; Sigma-Aldrich, Co.) and 2% horse serum. Sections were incubated with diluted primary mouse anti-neuronal nuclei (NeuN) monoclonal antibody (clone A60; MAB377; LV1457494; 1:500; Merck Millipore) overnight at 4°C. After washing with PBS, secondary biotinylated anti-mouse IgG (BA-2001; ZC1230; 1:400; Vector Laboratories, Inc., Burlingame, California, USA) was applied for 2 h at room temperature in humidity chamber. Subsequently, sections were rinsed in PBS and VECTASTAIN ABC KIT (Elite PK-6100 Standard; Vector Laboratories, Inc.) was applied for 1 h at room temperature in humidity chamber. Mayer's hematoxyline was used for counterstaining. Sections were cleared by immersion in xylene and coverslipped with Pertex (Histolab Products AB).

Confirmation of antibody specificity

In order to establish the specificity of the immunohistochemistry, a negative control test was carried out. Primary

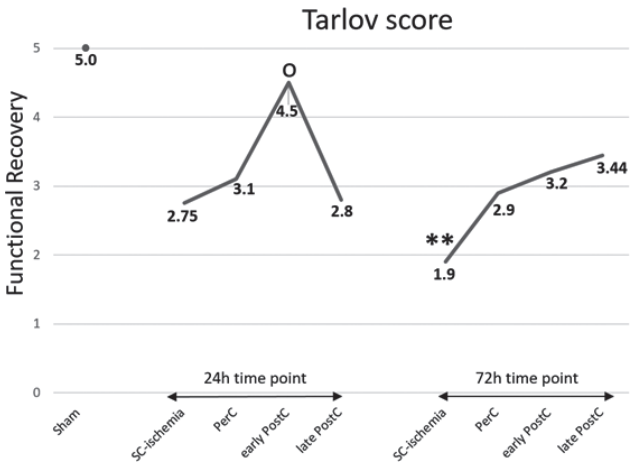

Figure 1. Neurologic function assessment by modified Tarlov score. Graph illustrating the effects of remote conditioning on hind limb functional recovery at 24 and 72 h time point. Note the statistically significant decrease in Tarlov score in the SC-ischemia/72h group and significant increase in Tarlov score in early PostC/24h group. Data are expressed as mean ± SEM; ANOVA and Tukey-Kramer test were used. ** $p < 0.01$ *vs.* Sham group, ^{o} $p < 0.05$ *vs.* SC-ischemia/72h group. SC, spinal cord; PerC, perconditioning; PostC, postconditioning.

antibodies were omitted in both immunohistochemical methods for negative control test. The negative control test was conducted in all groups.

Quantification of data and statistical analysis

All measurements were performed in order to ensure objectivity in blind conditions, by two observers (unaware of the experimental groups) for all experimental groups and methods, carrying out the measures of control and experimental samples of each spinal cord segment (L_4, L_5 and L_6) under the same conditions. For the qualitative and quantitative analyses of ubiquitin and NeuN immunohistochemistry, the motor neurons were evaluated at both grey matter sides of the anterior horns. Area for anterior horns was determined ventrally to a line drawn through the central canal perpendicular to the anterior median fissure (Mechírová et al. 2014). All measurements were done using magnification 200×. For evaluation of all processed methods, we used five sections from all spinal cord segments in all animals' groups. For quantitative and qualitative analyses of immunohistochemical methods for detection of ubiquitin and NeuN positivity, light microscope Olympus BX50 with a digital camera Olympus SP350 (Olympus; Tokyo, Japan) and QuickPHOTO Industrial 2.3 image analyzer software (Promicra, Prague, Czech Republic) were used. The statistical analysis was performed in GraphPad InStat ver. 3.10 for Windows (GraphPad Software Inc., San Diego, CA, USA). Quantitative evaluation of studied markers is expressed as mean ± SEM (standard error of the mean). The significance of the differences between experimental groups was analyzed using one-way analysis of variance ANOVA test followed by a Tukey-Kramer multiple comparison test. The value of $p < 0.05$ was considered to be statistically significant.

Results

Neurologic assessment

The assessment of neurological function of hind limbs was evaluated at two different time points: 24 and 72 h after SC-ischemia. Locomotor function, measured by the modified Tarlov score, showed trend toward decreasing in all ischemic treatment groups, compared to the Sham group. A statistically significant decrease of values was recorded 72 h after aortic occlusion in the SC-ischemia/72h group *versus* Sham group. Results of Tarlov score in all remote conditioned groups showed trend towards improvement in comparison to both SC-ischemia groups. The highest functional score from all of groups exposed to spinal cord ischemia was observed in early PostC/24h group. Values scored for animals in this group were also significantly increased compared to the SC-ischemia/72h group (Fig. 1).

Nissl staining method

In the cytoplasm of neurons in the Sham group, the angular or spindle shaped Nissl bodies were well showed (Fig. 2A). In the SC-ischemia/24h group, vacuolized neuropil areas surrounded by glial cells were observed in the center of anterior horns grey matter. Inside of this damaged grey matter areas, the injured motor neurons with excentrically located nuclei and chromatolysis in their cytoplasm were found (Fig. 2B). The PerC/24h, early PostC/24h and late PostC/24h groups did not show dramatic morphological changes in motor neurons. In the majority of motor neurons, Nissl bodies were evenly distributed in their soma and proximal dendritic region, only in a few motor neurons they were concentrated near the plasmalemma (Fig. 2D, F, H). In the anterior horn grey matter of SC-ischemia/72h group, the neuropil vacuolization, ghost cells and gliosis were observed (Fig. 2C). On the other side, in the PerC/72h, early PostC/72h and late PostC/72h group, surviving motor neurons with normal distribution of Nissl bodies in their cytoplasm were found. Their pale nuclei were present in the center of their soma. In close vicinity of surviving neurons, the nuclei of glial cells were visible. Some damaged motor neurons with chromatolysis were

observed only in the center of anterior horn grey matter together with gliosis (Fig. 2E, G, I).

Qualitative and quantitative analyses of ubiquitin immunohistochemistry

The ubiquitin-proteasome system is a crucial protein degradation system in eukaryotic cells. Damage of ubiquitin-proteasome system may be related to deficits in the clearance of misfolded proteins leading to intracellular protein aggregation, cytotoxicity and cell death (Pasquini et al. 2000). In the present study, ubiquitin distribution in the rabbit's nerve cells of spinal cord grey matter was evaluated. In the cytoplasm of nerve cells in spinal cord anterior horns in the Sham group, the normal-weak ubiquitin positivity was observed (Fig. 3A). In the cytoplasm of nerve cells in the SC-ischemia/24h group, dark brown aggregates of ubiquitin were visible as well as dark ubiquitin positive nuclei (Fig. 3B). The central area of grey matter in ventral horns was rich in injured nerve cells and vacuolization of neuropil was visible (Fig. 3B). In the SC-ischemia/72h group, the damage of majority of nerve cells in anterior horns grey matter was present. The cytoplasm of injured nerve cells was pale, without ubiquitin immunoreaction and in some of them irregular shaped dark brown nuclei with strong ubiquitin positivity were visible (Fig. 3C). The PerC/24h, early PostC/24h and late PostC/24h groups showed weak ubiquitin immunoreaction of motor neuron´s cytoplasm. The nuclei of motor neurons were without ubiquitin immunoreaction (Fig. 3D, F, H). Cytoplasmic ubiquitin positivity was present by forming of fine ubiquitin aggregates in the cytoplasm of motor neurons in PerC/72h, early PostC/72h and late PostC/72h groups (Fig. 3E, G, I). Some of motor neurons presented strong ubiquitin immunoreaction in irregular shaped nuclei (Fig. 3E, G), the other nuclei of motor neurons were without ubiquitin immunoreaction (Fig. 3E, I).

At both time points, significantly decreased number of ubiquitin positive neurons in both SC-ischemia groups ($p < 0.001$), both groups with PerC ($p < 0.001$), both groups with early PostC (at 24 h time point at $p < 0.01$ and at 72 h time point at $p < 0.001$), and both groups with late PostC (at 24 h time point at $p < 0.5$ and at 72 h time point at $p < 0.001$) was detected in comparison to Sham group. Ubiquitin positive neurons were significantly decreased in the SC-ischemia/24h group compared to the PerC/24h ($p < 0.001$), early PostC/24h and early PostC/72h ($p < 0.001$), late PostC/24h ($p < 0.001$) and late PostC/72h ($p < 0.5$) groups.

At the 72 h time point, groups with remote conditioning showed significant increase ($p < 0.001$) of ubiquitin positive

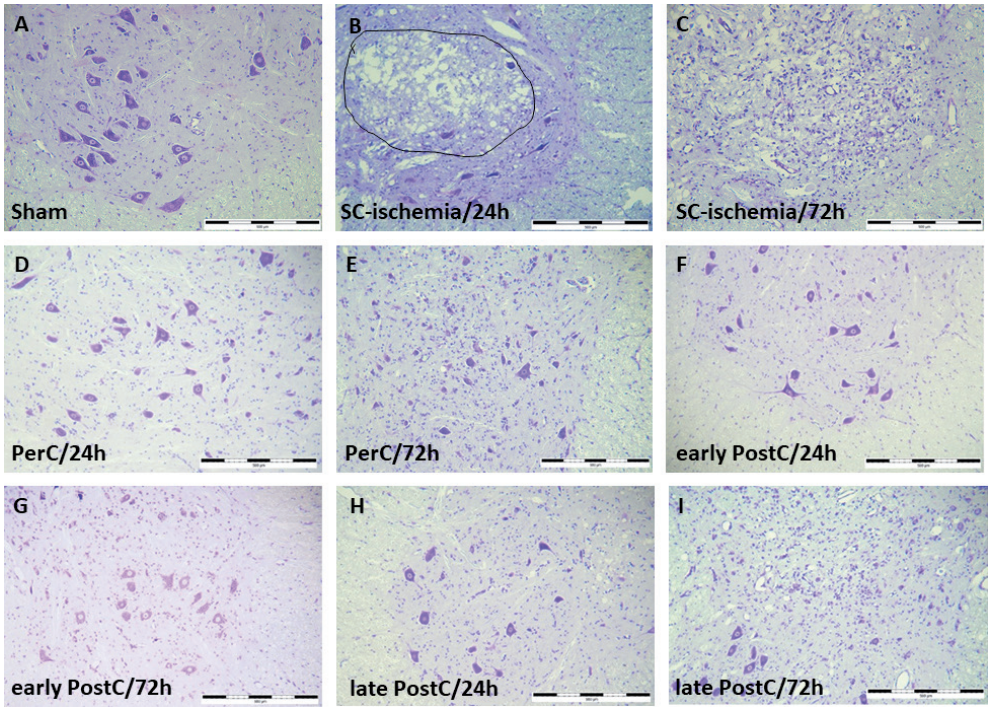

Figure 2. Nissl staining method. Micrographs of representative regions in the grey matter of the anterior horns in spinal cords in the Sham (**A**), 24 h after ischemia in the SC-ischemia (**B**), PerC (**D**), early PostC (**F**), and late PostC (**H**) conditions, respectively, and 72 h after ischemia in the SC-ischemia (**C**), PerC (**E**), early PostC (**G**) and late PostC (**I**), respectively. Scale bar = 500 μm. Nissl staining showed that in the Sham group (A) neurons exhibited a large amount of Nissl bodies in the cytoplasm. In the SC-ischemia/24h, the Nissl bodies were found as clusters in the cytoplasm of nerve cells and some damaged areas of grey matter occupied by neurons with chromatolysis were visible (B, area determined by line). At 24 h time point in conditioned groups, the neurons with normal distribution of Nissl bodies were observed (D, F, H). The neurons of SC-ischemia/72h showed dramatic morphological changes. Nissl bodies were even disappeared in neurons and gliosis was present (C). For abbreviations, see Fig. 1.

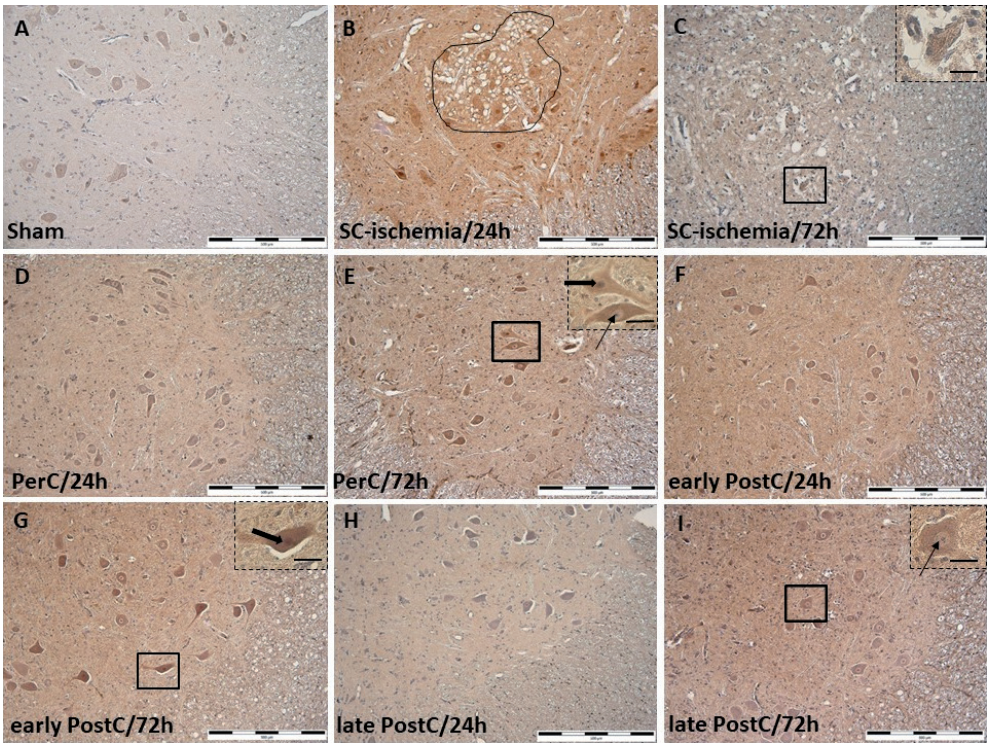

Figure 3. Ubiquitin immunoreaction in the neurons of spinal cords. Micrographs of representative regions of the grey matter of the anterior horns in spinal cords in the Sham (**A**), 24 h after ischemia in the SC-ischemia (**B**), PerC (**D**), early PostC (**F**) and late PostC (**H**) conditions and 72 h after ischemia in the SC-ischemia (**C**), PerC (**E**), early PostC (**G**), and late PostC (**I**) conditions. Scale bar = 500 μm (in insets, scale bar = 100 μm). Sham group with normal distribution of ubiquitin in the cytoplasm and nuclei of nerve cell (A); the damaged central area of grey matter in spinal cord ventral horns in the SC-ischemia/24h (B, area determined by line) and SC-ischemia/72h (C); moderate ubiquitin immunoreaction in the cytoplasm of nerve cells in the PerC/24h, early PostC/24h and late PostC/24h (D, F, H); the strong ubiquitin immunoreaction in irregular shaped nuclei of nerve cells in PerC/72h and early PostC/72h (E and G, thick arrows), nuclei of motor neurons without ubiquitin immunoreaction in the PerC/72h and late PostC/72h (E and I, thin arrows). For abbreviations, see Fig. 1.

neurons in comparison with SC-ischemia/72h group. We compared also all groups with different treatments in each group with each other. Ubiquitin positive neurons were decreased in PerC/72h, early PostC/72h, and late PostC/72h groups compared to PerC/24h ($p < 0.5$ and $p < 0.001$), early PostC/24h ($p < 0.01$), and late PostC/24h ($p < 0.001$) groups (Fig. 4).

Quantitative analysis of NeuN immunohistochemistry

Detection of NeuN was used to quantify the survived nerve cells. Statistically significant decrease of NeuN positive neurons was observed in all experimental groups versus Sham group ($p < 0.001$). Among all experimental groups, the lowest number of NeuN positive neurons was found in both SC-ischemia groups. The PerC/24h, early PostC/24h, and late PostC/24h groups showed significant increase of NeuN positive neurons in comparison with SC-ischemia/24h group ($p < 0.001$). The number of NeuN positive neurons was significantly increased in PerC/72h, early PostC/72h, late PostC/72h groups compared to SC-ischemia/72h ($p < 0.001$) group. NeuN positive neurons were decreased in the PerC/72h, early PostC/72h, and late PostC/72h compared to PerC/24h ($p < 0.5$, $p < 0.001$), early PostC/24h ($p < 0.001$), and late PostC/24h ($p < 0.01$) groups. We evaluated significant increase of survived neurons – NeuN positive neurons in early PostC/24h group compared to PerC/24h ($p < 0.01$) group (Fig. 5).

Representative microphotographs (Fig. 6) are showing NeuN positive neurons visualised by immunohistochemical method using antibodies against NeuN (the neuron-specific nuclear antigen), which have been reported to selectively bind to nuclei and cytoplasm of mature neurons in adult animals and embryos (Lind et al. 2005).

Discussion

The study is focused on the investigation of the mechanisms leading to ischemic tolerance acquisition in the spinal cord neurons *via* application of non-invasive method of remote conditioning. We aimed on determination of the effect of remote conditioning on ubiquitin-mediated stress response in the nerve cells of rabbit spinal cord. Ischemic tolerance, an endogenous neuroprotective phenomenon, may be induced by sublethal ischemia (Gidday 2006; Liu et al. 2021). Several studies indicate that the underlying protective mechanisms of remote conditioning are associated with its ability to at-

tenuate production of free radicals, promote the cell survival pathway, modulate the immune system, or to inhibit the apoptotic cell signalling pathways (Xing et al. 2008; Wang et al. 2008; Zhao 2009; Hoda et al. 2012). Possible mechanism that participates in ischemic tolerance acquisition is restoration of proteosynthesis in the nerve cells (Liu et al. 2005; Liang et al. 2012).

The vulnerability of motor neurons of the spinal cord might be partially attributed to the different ubiquitin-mediated stress response after ischemia/reperfusion. Ubiquitination is vital to any eukaryotic cell under physiological conditions, but even more important under stress including oxidative, genotoxic, and heat stress, where ubiquitination levels are drastically increased (Hochrainer 2018). Ubiquitin proteasome system plays a decisive role in clearing the toxic metabolites in cells, abnormal damaged proteins and it is crucial to ensure cell survival and recovery from deleterious effect of ischemia (Yamauchi et al. 2008; Liang et al. 2012; Kumar et al. 2020). Ubiquitin is thought to be one of the stress response proteins involved in elimination of damaged proteins in the cells and its enhanced immunoreaction is observed under oxidative stress (Gubellini et al. 1997). Neuropathological studies showed an overexpression of ubiquitin in the hippocampus, cerebral cortex, and cerebellum, and in thalamic areas, in several neurodegenerative diseases indicating a positive role of ubiquitin in cell injury (Braten et al. 2012; Ciechanover 2013; Jankowska et al. 2013; Fagoe et al. 2014). Dysfunction of ubiquitin proteasome system is central to all the neurodegeneration mechanisms (Yamauchi et al. 2008; Bax et al. 2019).

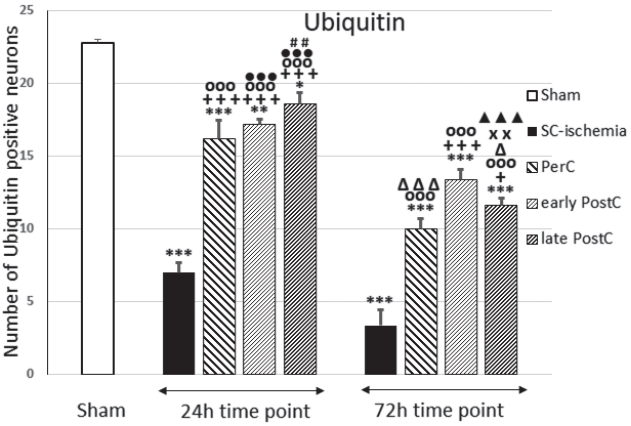

Figure 4. Graph illustrating the average number of ubiquitin positive neurons in anterior horns of rabbit spinal cord for each treatment condition. Note that ubiquitin positive neurons were increased in the PerC/24h, early PostC/24h, and late PostC/24h group compared to SC-ischemia/24h as well as SC-ischemia/72h group. At both time points decrease was present in all groups compared to Sham group. Ubiquitin positive neurons were decreased in the PerC/72h, early PostC/72h, and late PostC/72h group compared to PerC/24h, early PostC/24h, and late PostC/24h group Data are expressed as mean ± SEM; ANOVA and Tukey-Kramer tests were used. $^{+}$ $p < 0.5$, $^{+++}$ $p < 0.001$ *vs*. SC-ischemia/24h group; ^{ooo} $p < 0.001$ *vs*. SC-ischemia/72h group; * $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$ *vs*. Sham group; $^{\triangle}$ $p < 0.5$, $^{\triangle\triangle\triangle}$ $p < 0.001$ *vs*. PerC/24h group; ^{xx} $p < 0.01$ *vs*. early PostC/24h group; $^{\blacktriangle\blacktriangle\blacktriangle}$ $p < 0.001$ *vs*. late PostC/24h group; $^{\bullet\bullet\bullet}$ $p < 0.001$ *vs*. PerC/72h group; $^{\#\#}$ $p < 0.01$ *vs*. early PostC/72h group. For abbreviations, see Fig. 1.

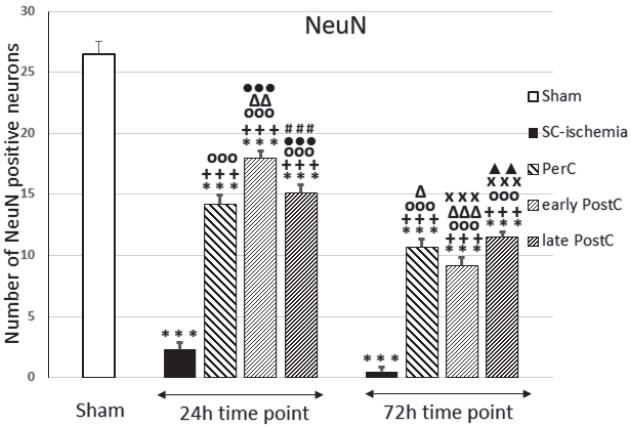

Figure 5. Graph illustrating the average number of neuronal nuclei (NeuN) positive neurons in anterior horns of rabbit spinal cord for each treatment condition. Note that NeuN positive neurons were decreased at both time points in all groups compared to Sham group, while increase was present in all conditioned groups compared to both SC-ischemia groups. NeuN positive neurons were decreased in the PerC/72h, early PostC/72h, and late PostC/72h groups compared to PerC/24h, early PostC/24h, and late PostC/24h group. Data are expressed as mean ± SEM; ANOVA and Tukey-Kramer tests were used. *** $p < 0.001$ *vs*. Sham group; $^{+++}$ $p < 0.001$ *vs*. SC-ischemia/24h group; ^{ooo} $p < 0.001$ *vs*. SC-ischemia/72h group; $^{\triangle}$ $p < 0.5$, $^{\triangle\triangle}$ $p < 0.01$, $^{\triangle\triangle\triangle}$ $p < 0.001$ *vs*. PerC/24h group; ^{xxx} $p < 0.001$ *vs*. early PostC/24h group; $^{\blacktriangle\blacktriangle}$ $p < 0.01$ *vs*. late PostC/24h group; $^{\bullet\bullet\bullet}$ $p < 0.001$ *vs*. PerC/72h group; $^{\#\#\#}$ $p < 0.001$ *vs*. early PostC/72h group. For abbreviations, see Fig. 1.

According to our results obtained in the SC-ischemia/24h group, the aggregates with strong ubiquitin immunoreaction in the cytoplasm of motor neurons were detected as well as the nuclei of motor neurons had detectable ubiquitin immunoreaction. Ubiquitin aggregates are one of the first signs of damaged, degenerated cells that have lost their ability of intracellular hydrolytic degradation of altered proteins (Mechírová et al. 2006). In the central area of anterior horns grey matter in SC-ischemia/24h group, damaged nerve cells without ubiquitin immunoreaction in the nucleus and cytoplasm were present. On the contrary, PerC/24h, early PostC/24h, and late PostC/24h group weak ubiquitin immunoreaction in the motor neurons cytoplasm was detected. The ischemia/reperfusion injury

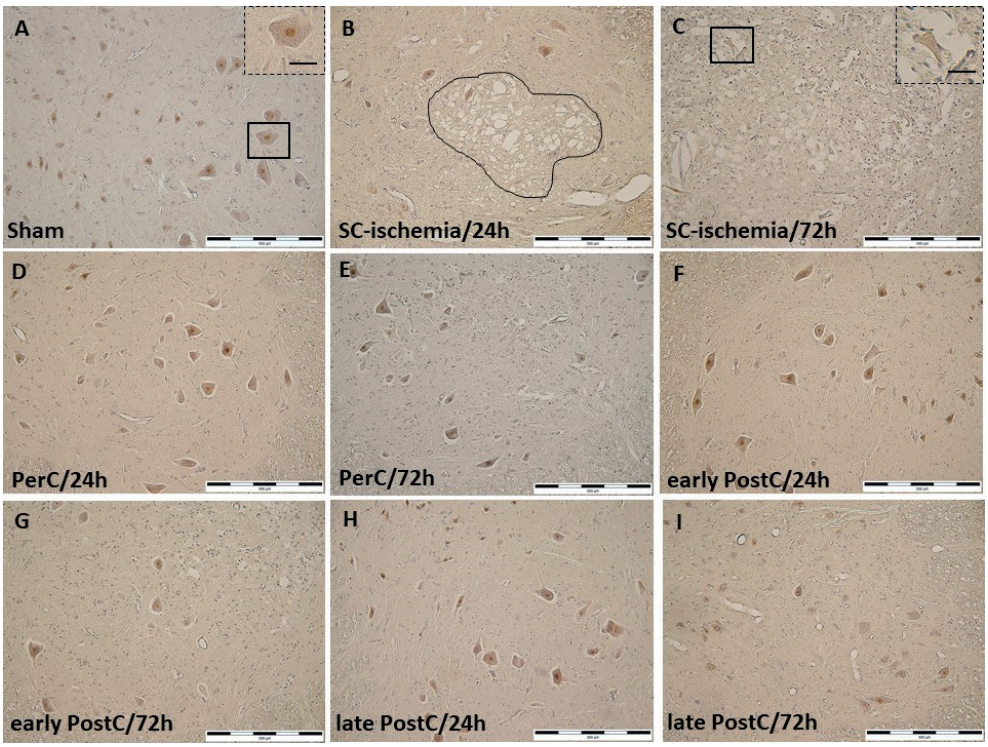

Figure 6. Neuronal nuclei (NeuN) immunoreaction in the neurons of spinal cords. Micrographs of representative regions of the grey matter of the anterior horns in spinal cords in the Sham (**A**), 24 h after ischemia in the SC-ischemia (**B**), PerC (**D**), early PostC (**F**) and late PostC (**H**) conditions and 72 h after ischemia in the SC-ischemia (**C**), PerC (**E**), early PostC (**G**), and late PostC (**I**) conditions. Scale bar = 500 μm (in insets, scale bar = 100 μm). Sham group with NeuN positive nuclei of nerve cells (A); the damaged central area of grey matter in spinal cord ventral horns in the SC-ischemia/24h (B, area determined by line); damaged grey matter of spinal cord ventral horns in SC-ischemia/72h and detail of neuron without NeuN positivity in the nucleus (C); NeuN positive neurons in experimental groups with remote conditioning at different time points (D–I). For abbreviations, see Fig. 1.

of spinal cord grey matter has fully developed in the SC-ischemia/72h group, where the majority of the nerve cells had pale cytoplasm without immunoreaction in contrary to their damaged, shrunken and irregular shaped nuclei, where intensive ubiquitin immunoreaction was detected. On the other hand, in PerC/72h, early PostC/72h and late PostC/72h groups, the majority of motor neurons showed ubiquitin immunoreaction in their cytoplasm, the nuclei were without marked ubiquitin immunoreaction. Only, in some motor neurons, the ubiquitin immunoreaction was found in both, in the cytoplasm and nuclei in contrary to damaged nerve cells in SC-ischemia/72h group. According to the study of Risuleo et al. (2003) ubiquitin is promptly synthesized in the cytoplasm and transduced to the nucleus where it mediates removal of denatured proteins and chromatin rearrangements. The ubiquitin-chromatin interaction could mediate transcription of specific DNA regions. The upregulation of ubiquitin in the nucleus may mediate the stress response to ischemia/reperfusion injury by influencing the activation of ischemic tolerance. In the nuclei, damaged proteins increase after transient ischemia and it is important to eliminate those proteins to survive ischemic insult. Therefore, higher immunoreaction of ubiquitin in nuclei of grey matter nerve cells might play an important role (Yamauchi et al. 2008). On the contrary, the lack of ubiquitin positivity in nuclei could be considered to be great disadvantage for nerve cells in the grey matter (Giffard et al. 2004; Noor et al. 2013). If ubiquitin system fails and it is not possible to restore full cell function, the neuron may undergo either apoptosis or necrosis (Risuleo et al. 2003). Yamauchi et al. (2008) reported that under normal conditions ubiquitin was slightly expressed in the cytoplasm of motor neurons and interneurons, but its positivity was not observed in nuclei.

Consistently, obtained data in number of ubiquitin positive nerve cells showed that increased number of ubiquitin positive neurons was in all groups with remote conditioning at 24 h time point in comparison to SC-ischemia/24h group. The highest number of ubiquitin positive neurons was found in anterior horns of grey matter in late PostC/24h group. The smallest number of ubiquitin positive neurons was detected in SC-ischemia/72h group and this decrease was significant versus all groups with remote conditioning at 72 h time point. Of note, the elevated ubiquitination was detected transiently in neurons that return to a normal morphology after the insult, whereas the presence of ubiquitin aggregates remained high in the cytoplasm of neurons that appear to die (Hu et al. 2000, 2001). Kahles et al. (2021) found that cerebral ischemia-reperfusion increases intraneuronal levels of ubiquitinated proteins and it may promote cell survival by

engaging beneficial stress-response pathways. Vulnerability of motor neurons of the spinal cord can be partially attributed to differences in ubiquitin-mediated stress responses after ischemia (Sakurai et al. 1998; Yamauchi et al. 2008). The loss of ubiquitin homeostasis is one of a mechanism that can lead to aggregation of misfolded proteins in the nerve cells and neuronal dysfunction (Hallengren et al. 2013). In early reperfusion period endogenous protective mechanisms are induced in first hours after ischemia, usually reach the peak of activity at 12 h, and then declined at 24 h (Fang et al. 2013; Zhang et al. 2013). Taking into consideration that protein synthesis is reduced 24 h after ischemia (Xie et al. 2018), we hypothesize that application of second stress did not interfere with endogenous protective mechanisms after ischemia and probably triggers another defence mechanism to ensure cell survival.

Results showed that the remote conditioning significantly promoted neuronal survival in all groups *versus* both SC-ischemia groups. In both SC-ischemia groups, a statistically significant reduction in the number of NeuN positive nerve cells was detected *versus* all groups with remote conditioning. According to our findings, in animals in early PostC/24h group the best effect of remote conditioning to neuronal protection was observed, as demonstrated by increased neuronal survival and trends towards improvement of neurological status. Values scored for animals in this early PostC/24h group were also significantly increased compared to the SC-ischemia/72h group. Trends towards improvement of neurological status have been preserved also at 72 h time point. On the other hand, also in other groups with remote conditioning the application of sublethal stress resulted in neuroprotection.

Obtained results showed that remote conditioning leaded to the neuronal protection and to influence ubiquitin-mediated stress response in production and distribution of ubiquitin into cytoplasm or nuclei of motor neurons. One of the possible mechanisms involved in neuroprotection after ischemia/reperfusion is ubiquitin activity preservation for elimination of misfolded proteins induced by ischemia/reperfusion. Our findings suggested that ubiquitination was occurred specifically in cells with survival potential. The post-ischemic ubiquitination could be an adaptive response to cell stress related to the nature of the ubiquitination process (Kahles et al. 2021). Perhaps, this less studied effect of ubiquitination could be useful in understanding the relationships between ubiquitination, aggregate formation, and cell death.

Presumably, remote early postconditioning starts the processes of the neuroprotection, but remote late postconditioning is more beneficial, as it showed more stable numbers of survived neurons at both the 24 and 72 h time points. Intermittent remote perconditioning in the last minutes of aortic occlusion in rabbits also showed possible therapeutic window neuroprotection, despite the fact that the number of survived neurons at 24 h time point were lower than in remote postconditioning.

The results of this study indicated that a sublethal insult by application of remote conditioning, as a trigger to changes of ubiquitin immunoreaction in the nerve cells, plays probably an important role in the formation of the tolerance against spinal cord ischemia/reperfusion injury.

Conclusions

Remote conditioning, through activation of ischemic tolerance, was involved in preserving the integrity and survival of nerve cells but also participated in influence of ubiquitin-mediated stress response. This study showed the effectiveness of remote conditioning as a neuroprotective strategy, evidenced by significant reduction of degenerated neurons in grey matter anterior horns of spinal cord of remote conditioned rabbits compared to both SC-ischemia groups. Application of remote conditioning by transient limb ischemia is a simple, easily feasible, non-invasive, inexpensive, effective, safe and clinically relevant stimulus that provides potent neuroprotection. This simplicity and non-invasive nature of applied remote conditioning model as well as the flexibility of the timing of remote conditioning stimulus, may have promising clinical implications. However, further investigation is necessary to clarify the underlying molecular mechanism that could fully explain the vulnerability of motor neurons against ischemic/reperfusion injury.

Conflict of interests. The authors declare that they have no conflicts of interest.

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