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Immunohistochemical Analysis of the Spinal Cord Ischemia– Effect of Remote Ischemic Preconditioning in a Porcine Model

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ABSTRACT

Background: In experimental settings, remote ischemic preconditioning (RIPC) has shown a positive effect regarding spinal cord protection after local ischemia. In this study, we conducted spinal cord immunohistochemistry to demonstrate the protective effect of RIPC after 24 hours of the regional ischemia.

Methods: Twenty piglets were randomized into an RIPC group (n = 10) and a control group (n = 10). The RIPC group underwent transient left hind limb ischemia before systematic left subclavian artery and segmental artery occlusion at the level of the diaphragm. Twenty-four hours later, the thoracic and lumbar spinal cords were harvested, and the oxidative stress markers were immunohistochemically analysed.

Results: A total of 18 animals survived the 4-hour follow up (10 in the RIPC group, 8 in the control group) and 14 animals survived the 24-hour follow up (7 in each group). In the single sections of the spinal cord, the antioxidant pathway activation was seen in the RIPC group, as OGG1 and DJ-1/PARK7 activation was higher (P = .038 and P = .047, respectively).

Conclusions: The results indicate that the neuroprotective effect of RIPC on the spinal cord after local ischemic insult remains controversial.

INTRODUCTION

Spinal cord protection during extensive thoracoabdominal aortic aneurysm (TAAA) repair has been in the limelight for over half a century. Various neuroprotective strategies, including intraoperative motor-evoked potential monitoring, have decreased the spinal cord injury prevalence [Tanaka 2016]. In addition to the other adjuncts, one widely investigated method– remote ischemic preconditioning (RIPC)– has been beneficial in preventing spinal cord injury in experimental models [Herajarvi 2017; Haapanen 2016]. Remote ischemic preconditioning is an exciting concept, as it is inexpensive, feasible, and available for everyone. Although RIPC is intensively studied in various ischemia-sensitive organs, such as the heart, kidney, and liver, in the broad spectrum of clinical trials, the exact mechanism of RIPC is still unclear [Robertson 2017; Hausenloy 2015; MacAllister 2015; Meybohm 2015; Thielmann 2013]. Additionally, it is a possibility that the RIPC triggers several independent protective cascades, which might not be active in every organ simultaneously [Anttila 2016].

The primary endpoint was the immunohistochemical analysis of the ischemic spinal cord. We focused on the critical factors after ischemic DNA damage. Reactive oxygen species (ROS) leave a specific footprint, 8-hydroxydeoxyguanosine (8-OHdG), and 8-oxoguanine glycosylase (OGG1) removes 8-OHdG from the damaged DNA [Abedin 2013; Valavanidis 2009]. Ischemic damage to the mitochondria is due to an excessive release of cytochrome C, which activates several death cascades, including caspase-dependent pathways [Zhan 2001]. The antioxidant pathways, like nuclear erythroid-related factor-2 (Nrf-2), are activated and transformed in the nucleus, and cause the transcription of multiple cytoprotective proteins [Mitsuishi 2012]. DJ1/Parkinson disease protein 7 (DJ-1) is multifunctional redox-regulating protein, which is associated with the mitigation of oxidative stress injury [Mitsumoto 2001].

In our previous studies, RIPC has been beneficial regarding spinal cord protection [Herajarvi 2017; Haapanen 2016]. In addition, the early immunohistochemical analysis of the spinal cord showed a significantly higher activity of antioxidant pathways, though the hematoxylin-eosin staining findings were rather modest at six hours after the local ischemia. Interestingly, approximately half of all patients with permanent paraplegia after TAAA repair seemed to occur in a delayed fashion [Coselli 2016]. Most cases of delayed spinal cord injury appeared within 12-24 hours after the surgery, and usually, the deficit is expected to recover if appropriate treatment is performed at the early stage. Theoretically, RIPC might increase the ischemic tolerance of the neurons over the most vulnerable period. Therefore, we wanted to see whether RIPC has the potential neuroprotective effect on the spinal cord at 24 hours after the ischemic insult from the immunohistochemical point of view. Moreover, the immunohistochemical analysis could shed light on the potential mechanism of RIPC.

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Table 1. Immunohistochemical Staining Methods for Oxidative Stress Markers

Antibody (clone)	Dilution	Retrieval	Detection Kit	Incubation time	Manufacturer
Nrf-2 (ab 62352)	1:300	Tris-EDTA	Envision	60 min	Abcam
Ogg-1 (NB 100-106)	1:1000	Citrate buffer	Envision	Overnight	Novus Biologicals
8-OhdG (N45.1)	1:75	Citrate buffer	Invitrogen, Hististain-Plus Bulk	45 min at 42°C	Japan institute For the Control of Aging
DJ-1 (ab 18257)	1:2000	Citrate buffer	Envision	60 min	Abcam
Cyt C (ab90529)	1:1500	Citrate buffer	Envision	30 min	Abcam
Casp-3 (AF835)	1:400	Citrate buffer	Envision	30 min	R&D Systems

Cyt C indicates Cytochrome C; Casp -3, cleaved caspase-3; 8-OHdG, 8-hydroxydeoxyguanosine; Ogg-1, 8-oxoguanine glycosylase; Nrf-2, nuclear erythroid-related factor-2; DJ-1, DJ1/Parkinson disease protein 7.

MATERIALS AND METHODS

Experimental Setting

Twenty female juvenile pigs from a native stock (19 to 22 kg) were randomized into two groups using sealed envelopes: an RIPC group (n = 10) and a control group (n = 10). Remote ischemic preconditioning was performed in the RIPC group before the segmental artery cutoff. The control group underwent a protocol identical to the RIPC group, excluding hind limb preconditioning. After 24 hours, the immunohistochemical analysis of the spinal cord was carried out.

Preoperative Management

All animals received humane care by the "Principles of Laboratory Animal Care," formulated by the National Society for Medical Research, and the "Guide for the Care and Use of Laboratory Animals" (http://www.nap.edu/catalog/5140.html). The study was approved by the Research Animal Care and Use Committee of the University of Oulu.

Anesthesia Protocol

Animals were sedated with an intramuscular injection of ketamine (350 mg), midazolam (45 mg), and medetomidine (1.5 mg). After endotracheal intubation, the subjects were ventilated with 50% oxygen and an end-tidal carbon dioxide concentration of 4.5% - 5.0%. Anesthesia was induced with fentanyl (50 μg/kg) and was maintained throughout the entire experiment by a continuous infusion of fentanyl (25 μg/[kg · h]) and ketamine (15 mg/[kg · h]). Sevoflurane 1.0% was used for inhalation anesthesia. Rocuronium (0.1 mg/kg) was used for muscle relaxation.

Hemodynamic Monitoring

An arterial line for arterial pressure monitoring and blood sampling was placed into the right femoral artery. A pulmonary artery thermodilution catheter (CritiCath 7F; Ohmeda GmbH & Co., Erlangen, Germany) was introduced through the right femoral vein for blood sampling and invasive hemodynamic monitoring. Rectal and blood temperatures and electrocardiograms were also monitored. A urinary catheter (10 Fr) was placed to control urine output, and the infused fluids were recorded to determine fluid balance. Mean arterial pressure was maintained over 60 mmHg throughout the experiment to avoid confounding factors in the experimental setting.

Remote Ischemic Preconditioning

A 9cm wide blood pressure cuff was placed around the left hind limb in the experimental group. The cuff was inflated to 250 mmHg for 5 minutes, followed by a 5-minute deflation (reperfusion). The RIPC consisted of four inflation-deflation cycles, and the total duration of RIPC was 40 minutes. Control animals were observed for 40 minutes without RIPC.

Surgical Procedures for Spinal Cord Ischemia

The fourth and seventh intercostal spaces were exposed from the left anterolateral thoracotomy. The left subclavian artery and upper thoracic segmental arteries were exposed and looped. The rest of the segmental arteries to the level of the diaphragm were exposed through the left eleventh intercostal space. Every animal had nine segmental arteries above the diaphragm. Fifteen minutes after RIPC (or control), the left subclavian artery and nine segmental arteries were ligated and cut consecutively at 5-minute intervals.

Postoperative Management

Intercostal spaces were closed and the incision sutured after the ninth segmental artery cutoff. After a four-hour follow-up, each piglet was extubated and transferred to a recovery room. Analgesia (buprenorphine 6ug/kg intramuscular) was maintained until euthanasia. For continuous observation, each piglet was placed in a separate pen for 24 hours. Afterwards, the animal was euthanized with intravenous pentobarbital (60mg/kg), and the thoracic and lumbar spinal cord were harvested and immersed in 10% neutral formalin for two weeks. The spinal cord was dissected into the five predetermined areas: thoracic vertebrae nerve roots 1-3, 4-6, 7-9, and 10-13 (14), and lumbar vertebrae nerve roots 1-4 (5). The length of thoracic or lumbar spinal column varied by one vertebra among the animals. Immunohistochemical stains were examined by an experienced neuropathologist blinded to the grouping.

Table 2. Results of Immunohistochemical Analysis of the Spinal Cord 24 Hours After the Ischemia

	Th 1 – 2	Th 3 – 4	Th 5 – 6	Th 7 – 8	Th 9 – 10	Sum score
Ogg-1						
RIPC	2.0 (1.0 – 2.0)	3.0 (1.0 – 3.0)	2.0 (1.0 – 2.0)	2.0 (1.0 – 3.0)	2.0 (1.5 – 2.5)	9.0 (6.5 – 13.0)
Control	1.0 (1.0 – 1.5)	1.0 (1.0 – 1.0)	1.0 (1.0 – 2.0)	1.0 (1.0 – 1.5)	1.0 (1.0 – 2.0)	6.0 (5.0 – 8.5)
P	.232	.038	.430	.258	.337	.096
8-OHdG						
RIPC	3.0 (2.5 – 3.0)	3.0 (3.0 – 3.0)	3.0 (3.0 – 3.0)	3.0 (3.0 – 3.0)	3.0 (3.0 – 3.0)	14.0 (14.0 – 15.0)
Control	3.0 (3.0 – 3.0)	3.0 (3.0 – 3.0)	3.0 (3.0 – 3.0)	3.0 (3.0 – 3.0)	3.0 (3.0 – 3.0)	15.0 (15.0 – 15.0)
P	.147	1.0	.337	.356	.337	.096
NFR-2						
RIPC	1.0 (0.0 – 1.0)	1.0 (0.0 – 1.0)	0.0 (0.0 – 0.0)	1.0 (0.0 – 1.0)	1.0 (0.5 – 1.0)	3.0 (2.0 – 5.0)
Control	0.0 (0.0 – 0.5)	0.0 (0.0 – 1.0)	0.0 (0.0 – 1.0)	1.0 (0.5 – 1.0)	0.0 (0.0 – 1.0)	3.0 (1.0 – 4.5)
P	.232	.430	.175	.404	.218	.519
Caspase-3						
RIPC	1.0 (0.0 – 1.0)	0.0 (0.0 – 0.5)	1.0 (0.0 – 1.0)	0.0 (0.0 – 1.0)	1.0 (0.5 – 1.0)	3.0 (1.5 – 4.5)
Control	1.0 (0.5 – 1.0)	1.0 (0.5 – 1.0)	0.0 (0.0 – 1.0)	1.0 (0.0 – 1.0)	1.0 (0.0 – 1.0)	3.0 (2.0 – 4.0)
P	.611	.300	.626	.430	1.0	.582
Cyt C						
RIPC	2.0 (1.0 – 2.0)	2.0 (1.0 – 2.0)	2.0 (1.0 – 2.0)	1.0 (1.0 – 2.0)	1.0 (1.0 – 2.0)	7.0 (6.0 – 9.0)
Control	2.0 (1.5 – 2.0)	2.0 (1.0 – 2.0)	1.0 (1.0 – 2.0)	2.0 (1.0 – 2.0)	1.0 (1.0 – 2.0)	7.0 (7.0 – 8.0)
P	.404	1.0	.626	.626	1.0	.744
DJ-1/PARK7						
RIPC	1.0 (1.0 – 2.0)	1.0 (1.0 – 2.5)	2.0 (2.0 – 2.5)	2.0 (1.0 – 2.0)	1.0 (1.0 – 1.0)	8.0 (6.5 – 9.5)
Control	2.0 (1.5 – 2.5)	1.0 (1.0 – 1.5)	1.0 (1.0 – 1.0)	1.0 (1.0 – 2.0)	1.0 (1.0 – 2.0)	7.0 (6.0 – 9.5)
P	.337	.310	.047	.902	.218	.739

RIPC n = 7, Control n = 7. Values are shown as medians and 25th and 75th percentiles.

RIPC indicates remote ischemic preconditioning; Cyt C, Cytochrome C; Casp -3, cleaved caspase-3; 8-OHdG, 8-hydroxydeoxyguanosine; Ogg-1, 8-oxoguanine glycosylase; Nrf-2, nuclear erythroid-related factor-2; DJ-1/PARK7, DJ1/Parkinson disease protein 7.

Histopathological Analysis

Immunohistochemical staining was used for more detailed analysis of oxidative stress and cell death (Table 1). Six factors related to oxidative stress were determined: Nrf-2, OGG1, 8-OHdG, DJ-1, cytochrome C, and activated (cleaved) caspase-3.

Sections (4.5 µm) cut from paraffin-embedded specimens were deparaffinized in xylene and rehydrated through graded alcohols. For antigen retrieval, the sections were pretreated with either Tris–EDTA (pH 9) or with citrate buffer (pH 6) in a microwave oven. After neutralizing the endogenous peroxidase activity, the sections were incubated at room temperature with diluted antibodies. Bound antibodies were detected using the EnVision, EnVision FLEX (Dako, Agilent Technologies, Santa Clara, CA, USA), or Invitrogen systems (Thermo Fischer Scientific Inc., Waltham, MA, USA). Diaminobenzidine was used as the chromogen, and hematoxylin as the counterstain.

Five predetermined areas in the spinal cord were sampled: Th 1 – 2, Th 3 – 4, Th 5 – 6, Th 7 – 8, and Th 9 – 10. These samples were then stained with hematoxylin-eosin and immunohistochemical stains, and scored by a neuropathologist blinded to the grouping. Immunohistochemical analysis was based on four grades as follows: 0 = negative, 1 = mild, 2 = moderate, 3 = strong staining intensity.

Enzyme-Linked Immunosorbent Assay

The serum values of 8-OHdG were measured from pulmonary artery blood samples. The serum samples were stored in polypropylene tubes at -80-88°C until analysis. Highly sensitive 8-OHdG Check enzyme-linked immunosorbent assay (ELISA) kits were used to analyze the samples (from the Japan Institute for the Control of Aging, Fukuroi, Japan), following the manufacturer's instructions.

Table 3. Results of 8-OHdG Concentration during the Four-Hour Follow-Up

Group	Baseline	Post RIPC	Post op 1 h	Post op 4h
RIPC	1.46 (1.24 – 1.67)	1.38 (1.37 – 1.61)	1.34 (1.33 – 1.57)	1.34 (1.10 – 1.40)
Control	1.53 (1.48 – 1.98)	1.51 (1.27 – 1.88)	1.58 (1.26 – 1.93)	1.27 (1.07 – 1.67)
P	.413	.342	.314	.570

RIPC n = 7, Control n = 7. Values are shown as medians and 25th and 75th percentiles.
RIPC indicates remote ischemic preconditioning.

Statistical Analysis

Statistical analysis was performed using SPSS (version 22.0; SPSS Inc, Chicago, III) and SAS (version 9.2; SAS Institute, Cary, NC) statistical software packages. Continuous and ordinal variables are expressed as the median and 25th and 75th percentiles in the tables and figures. The repeated measurements were analyzed using a linear mixed model with animals fitted at random, and the best covariance pattern was chosen according to Akaike's information criteria. Complete independence was assumed across animals (by random statement). Reported *P* values are as follows: *P* between groups (P_g) indicates the level of difference between the groups, *P* time · group ($P_{t \cdot g}$) indicates behavior between the groups over time. The Shapiro-Wilk test was used to assess the distribution of variables between the study groups. In turn, the differences at a single time point were assessed by using Mann-Whitney U test, or if the values were normally distributed, the Student T test was conducted. Two-tailed significance levels are reported; $P < .05$ was considered statistically significant.

RESULTS

Mortality

Two experiments were terminated because of intraoperative bleeding, and excluded from the analysis. Four animals were lost during observation due to respiratory problems. A total of 18 animals survived the 4-hour follow-up (10 in the RIPC group, 8 in the control group) and 14 animals survived at 24-hour follow- up (7 in each group).

Comparability of Study Groups

The mean weight of the RIPC group was 20.3 kg, and 21.7 kg for the control group ($P = .083$). Blood ($P_g = .45$) and rectal ($P_g = .18$) temperatures were similar in both groups. Hemoglobin did not differ between groups ($P_g = .20$). There was no difference in mean arterial pressure throughout the duration of the experiment ($P_g = .78$). Experimental and metabolic data has been published previously [Haapanen 2016].

Histology of Spinal Cord

Hematoxylin-eosin staining did not reveal any differences between the groups [Haapanen 2016]. The antioxidant markers OGG1 and DJ1 were more activated in the RIPC group in the single sections, although the overall scores in these factors were not statistically significant (Table 2). The oxidative stress marker, 8-OHdG, did not differ significantly between groups, but the expression was somewhat active in both groups. We found it surprising that the nuclear erythroid-related factor 2 (Nrf2), which regulates the appearance of a large pool of antioxidant and cytoprotective genes, was only mildly activated in both groups, as our previous study showed stronger activation six hours after the spinal cord ischemia [Herajarvi 2017]. In addition, we observed at least mild dysfunction of the mitochondria since there was a cytochrome C release in both groups, but no activation of caspase-3 was seen in either of the groups.

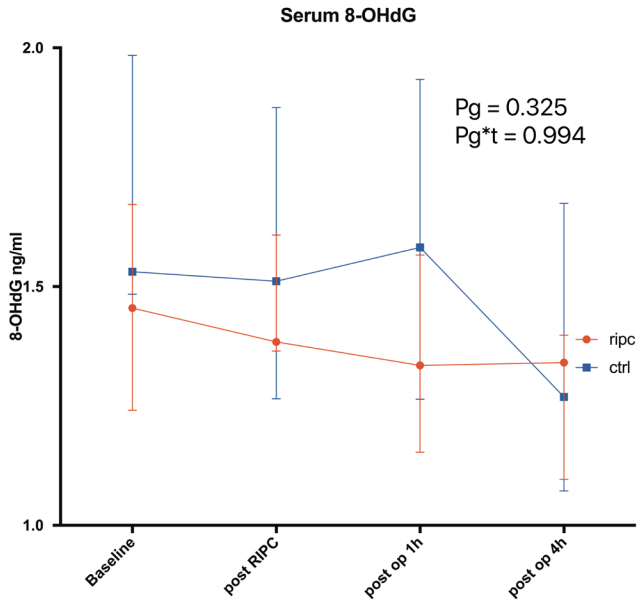
8-OHdG Concentration

The difference of 8-OHdG concentration was not statistically significant at any time point between groups (Figure and Table 3).

DISCUSSION

The most devastating complication of aortic aneurysm surgery is the permanent spinal cord injury, which has been shown to be related to increased mortality [Coselli 2016; Svensson 1993]. In the experimental models, RIPC has been beneficial against local ischemic insult to the spinal cord, but the exact mechanism of RIPC is still unclear [Herajarvi 2017; Haapanen 2016]. In this study, we analyzed the effect of RIPC on the spinal cord after local ischemic insult from the immunohistochemical point of view. The hematoxylin-eosin analysis revealed that ischemic insult to the spinal cord was rather modest, as the main findings were edema and hemorrhage [Haapanen 2016]. Thus, we performed further analysis to specify the ischemic injury and to shed light on the possible RIPC mechanism. After 24 hours, there were some findings favorable to RIPC, though they were not conclusive.

One crucial part of ischemia-reperfusion injury is the generation of reactive oxygen species from several sources, including the mitochondrial electron-transport chain. Oxidative DNA damage exacerbates instantly after ischemia, inducing abundant DNA lesions consisting of hydroxyl radical-modified bases, such as 8-OHdG [Oka 2008]. A group of DNA repair enzymes (base excision repair, BER), including OGG-1, are essential for attenuating the ischemic damage in neurons. In an experimental study, the ischemic preconditioning enhanced the DNA repair capacity by increasing the



Serum 8-OHdG measures during the 4 hours follow-up. Reported *P* values are as follows: *P* between groups (P_g) indicates a level of difference between the groups, *P* time · group ($P_{t \cdot g}$) indicates behavior between the groups over time. No statistically significant *P*-values were recorded at any time point.

OGG-1 activity [Li 2006]. In another experimental stroke model, OGG-1-deficient mice had significantly more vulnerable neurons to death [Liu 2011]. In our study, remote ischemic preconditioning showed a similar effect on the spinal cord in a single spinal cord section.

DJ-1, a multifunctional protein known to have a protective role against oxidative stress, is proposed to be independently able to handle the ischemia-induced oxidative damage in neurons [Aleyasin 2007]. DJ-1 modifies the transcription of the other antioxidant enzymes, and one suggested way of action is through the regulation of Nrf2, which is another critical antioxidant transcription factor [Clements 2006]. We have previously shown RIPC increases Nrf2 levels in spinal cord at six hours after the ischemic insult [Herajarvi 2017]. In this study, DJ-1 levels were increased in one section, but Nrf2 levels did not differ between groups. The Nrf2 levels were indeed surprisingly low in both groups.

In our experimental spinal cord ischemia model, the left subclavian and segmental arteries above diaphragm were occluded to achieve more extensive spinal cord ischemia. The ischemic injury, defined as an increased 8-OHdG level in the spinal cord, was comprehensive, and observed in both the anterior and posterior horns, and on both sides. Therefore, the occlusion of left subclavian artery did not "weaken" the collateral network in a way that the insult would be detectable only on the one side of the spinal cord. It is possible that RIPC performed by using blood pressure tourniquet may cause temporary injury to the hind limb nerves as the motor-evoked potential recordings were diminished in the RIPC–limb, and when RIPC was performed by occluding the common iliac artery, such a difference was not seen [Herajarvi 2017]. One may also speculate the oxygen supply was the most compromised in the middle of the spinal cord, as the collateral network might diminish the ischemic insult proximally and distally. Therefore, the protective effect of RIPC might also be more evident in the vulnerable area.

Additionally, the behavior of the serum 8-OHdG did not differ between groups during the four-hour follow-up. We have previously demonstrated RIPC diminished serum 8-OHdG levels after global ischemia, but similar results were not seen in this study [Arvola 2016]. One possible explanation might be the local ischemia on the spinal cord, and therefore, no systemic effect of RIPC was seen.

The study carries several limitations. The experimental model was acute, therefore, we do not have long-term surveillance analysis. Also, there is a possibility that the local ischemic insult to the spinal cord was too modest; thus, the collateral network covers the blood supply sufficiently, and eventually the beneficial effects of RIPC are not seen in immunohistochemical analysis. Lastly, the reported *P*-values of the immunohistochemical analysis should be treated with caution due to the multiple analyses performed.

CONCLUSION

In conclusion, the anti-ischemic effect of RIPC on the spinal cord remains controversial. One explanation for the results might be ischemia that was too mild. In further experimental studies, exposure of all segmental arteries and median sacral artery through thoraco-phreno-laparotomy might be a more optimal approach.

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