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<https://hdl.handle.net/2324/4474997>

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出版情報 : Kyushu University, 2020, 博士 (医学), 課程博士

バージョン :

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# Hypothermia Inhibits the Expression of Receptor Interacting Protein Kinases 1 and 3 After Transient Spinal Cord Ischaemia in Rabbits

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## WHAT THIS PAPER ADDS

This study suggests that transient normothermic ischaemia induces necroptosis, a type of regulated necrosis, in the spinal cord as a potential factor in delayed paraplegia, and that hypothermia may inhibit necroptosis. In a transient spinal cord ischaemia rabbit model, the expressions of receptor interacting protein kinase 1, 3 and cellular inhibitor of apoptosis protein 1/2 were increased. Hypothermia decreased their expression. The inhibition of necroptosis has been reported to reduce the severity of many diseases, such as cerebral ischaemia, amyotrophic lateral sclerosis (ALS), and traumatic spinal cord injury, suggesting its therapeutic potential for delayed paraplegia via transient spinal cord ischaemia.

**Objectives:** Necroptosis, a form of regulated necrosis, might be a potential mechanism of delayed paraplegia; therefore, its role in transient spinal cord ischaemia was investigated by immunohistochemical analysis of necroptosis related protein receptor interacting protein kinase (RIP) 1, RIP3, and cellular inhibitor of apoptosis protein (cIAP) 1/2.

**Methods:** This study used rabbit normothermic ( $n = 24$ ) and hypothermic ( $n = 24$ ) transient spinal cord ischaemia models and sham controls ( $n = 6$ ). Neurological function was assessed according to a modified Tarlov score at 8 h, 1, 2, and 7 days after reperfusion ( $n = 6$  each). Morphological changes in the spinal cord were examined using haematoxylin and eosin staining in the sham, 2, and 7 day groups. Western blot and histochemical analyses of RIP1, RIP3, and cIAP1/2, and double label fluorescent immunocytochemical studies of RIP3 and cIAP1/2 were performed at 8 h, 1, and 2 days after reperfusion ( $n = 6$  each).

**Results:** There were significant differences in neurological function between the normothermic and hypothermic groups (median scores 0 and 5 at 7 days,  $p = .023$ ). In the normothermic group, most motor neurons were lost seven days after reperfusion ( $p = .046$  compared with sham), but they were preserved in the hypothermic group. Western blot analysis revealed the upregulation of RIP1, RIP3, and cIAP1/2 at 8 h in the normothermic group (RIP1,  $p = .032$ ; RIP3,  $p < .001$ ; cIAP1/2,  $p = .041$  compared with sham), and the overexpression of RIP3 was prolonged for two days. In the hypothermic group, the expression of these proteins was not observed. The double label fluorescent immunocytochemical study revealed the induction of RIP3 and cIAP1/2 in the same motor neurons.

**Conclusions:** These data suggest that transient normothermic ischaemia induces necroptosis, a potential factor in delayed motor neuron death, and that hypothermia may inhibit necroptosis.

**Keywords:** Delayed paraplegia, Necroptosis, Spinal cord ischaemia

Article history: Received 4 January 2019, Accepted 2 December 2019, Available online 26 December 2019

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## INTRODUCTION

Spinal cord injury after successful thoraco-abdominal aortic surgery is a disastrous complication. The mechanism of

acute spinal cord dysfunction is thought to be caused by ischaemic damage during cross clamping. However, patients undergoing thoraco-abdominal aortic aneurysm repair can sometimes develop delayed onset paraplegia, even when they wake without neurological deficits immediately after surgery.<sup>1</sup> Recent studies showed neuronal survival after spinal cord ischaemia was affected by apoptosis, disturbed ubiquitin–proteasome pathway, or autophagy under conditions of non-lethal stress.<sup>2–5</sup> In addition, local cooling during spinal cord ischaemia inhibited delayed and selective

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<https://doi.org/10.1016/j.ejvs.2019.12.004>

motor neuron death in rabbits,<sup>6</sup> although the exact mechanism of this delayed vulnerability is not fully understood.

Classically, cell death is classified into three types: apoptosis, autophagy, and necrosis.<sup>7</sup> Apoptosis is considered a regulated form of cell death and necrosis is thought to be passive and unregulated. However, regulated necrosis has emerged as a new form of cell death, termed necroptosis.<sup>8,9</sup> Necroptosis is controlled by defined molecular cascades, similar to apoptosis, and is characterised by swelling of the cell and its organelles leading to rupture of the plasma membrane, as in necrosis. Recent studies reported the induction of necroptosis in various disease models.<sup>10</sup> In addition, inhibition of necroptosis reduced disease severity, suggesting therapeutic potential.

Most necroptosis pathways start from tumour necrosis factor (TNF) production and signalling through TNF receptor (TNFR) 1. Binding of TNF to TNFR1 recruits complex I, which comprises receptor interacting protein kinase (RIP) 1, TNFR associated death domain (TRADD), TNFR associated factor 2 (TRAF2), and cellular inhibitor of apoptosis protein (cIAP) 1/2. When RIP1 is polyubiquitinated by cIAP1/2, nuclear factor kappa B (NFκB) survival pathways are activated. When RIP1 is de-ubiquitinated, complex I proteins dissociate from TNFR1. Complex IIa is then formed in the cytoplasm, consisting of RIP1, RIP3, Fas associated death domain (FADD), and caspase-8. Caspase-8 cleaves RIP1 and RIP3 leading to apoptosis. In the absence of caspase-8 activation, RIP1 and RIP3 form complex IIb (the necrosome) through their RIP homotypic interacting motif (RHIM) domains, to function as kinases that drive necroptosis.<sup>11–13</sup> Currently, necroptosis is defined as caspase independent cell death mediated by RIP1 and RIP3.

Previously it was revealed that apoptosis was involved in delayed motor neuron death after spinal cord ischaemia in rabbits.<sup>2</sup> Apoptosis and necroptosis share several signalling pathways. It was therefore hypothesised that some motor neurons, which eventually die from transient spinal cord ischaemia in this model, undergo necroptosis. In addition, differences in the expression levels of necroptosis related proteins in moderate hypothermia and normal conditions were assessed.

## MATERIALS AND METHODS

### *Animal models*

Animals were treated in accordance with the guiding principles for the care and use of animals during the experiments. The animal care committee of the Kyushu University School of Medicine approved the experimental and animal care protocols.

A total of 54 Japanese domesticated rabbits weighing 2.5–3.0 kg were used in this study and divided into three groups: normothermic ischaemia group (group N;  $n = 24$ ), hypothermic ischaemia group (group H;  $n = 24$ ), and sham control group (group S;  $n = 6$ ). Anaesthesia was induced by intramuscular administration of 50 mg/kg ketamine and maintained with 2% halothane inhalation and 100% oxygen. A 4 F paediatric catheter (CI-300; Harmac Medical Products,

Inc., Buffalo, NY, USA) was inserted through the femoral artery and advanced 15 cm into the abdominal aorta. Then, a balloon was inflated, and 15 min of transient ischaemia was performed, before the balloon was deflated and the catheter immediately removed. In rabbits, anterior spinal arteries are fed from lumbar arteries of the infrarenal aorta. Preliminary investigations confirmed the distal end of the catheter balloon was positioned approximately 0.5–1.5 cm distal to the left renal artery and that 15 min of transient spinal cord ischaemia was sufficient for selective and delayed motor neuron death.<sup>14</sup> In the S group, a catheter was inserted without balloon inflation. Aortic pressure was continuously monitored proximal and distal to the balloon during the experiment. When the balloon of the catheter was inflated in the abdominal aorta, the arterial pressure at the proximal end did not change, while the pressure at the distal end of the catheter decreased to almost zero, and no pulsation was recorded. The arterial blood pressure of this portion returned to normal levels after deflation of the balloon. Body temperature was maintained at 37 °C with a heating pad and monitored using a rectal thermistor during the procedure. Group H was treated using the same method but with a cooling pad,<sup>6</sup> attached to the lumbar region (L1–L5) on bare skin. The cooling effect by the temperature of the rectum was confirmed ( $N 36.92 \pm 0.71$  °C vs.  $H 33.24 \pm 1.35$  °C;  $p < .001$ ). Animals were killed using deep anaesthesia with potassium chloride (2 mmol/kg intracardiac administration) at 8 h and 1, 2, and 7 days after reperfusion ( $n = 6$  per group at each timepoint). In the S group, animals were killed 7 days after the procedure. Using the plunger of a 1 mL syringe, the spinal cords were quickly removed immediately after death. Tissue samples for western blot analysis and immunohistochemical studies were frozen and stored at –80 °C. Samples for histology were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer then stored at 4 °C for one week. Then, they were cut transversely at approximately the L2 or L3 level and embedded in paraffin.

### *Neurological assessment*

Neurological function was evaluated before the rabbits were killed and classified according to a modified Tarlov score<sup>15</sup> as follows: 0, no movement; 1, slight movement; 2, sitting with assistance; 3, sitting unaided; 4, weak hopping; and 5, normal hopping. Two individuals without knowledge of the treatment graded the neurological function independently.

### *Histological study*

To determine pathological changes in the spinal cord after ischaemia, haematoxylin and eosin staining was performed using light microscopy and the number of intact large motor neurons in the ventral grey matter region in five sections per animal were counted. An observer unaware of the animal groups and neurological outcomes examined each slide (magnification  $\times 100$ ). Neurons were considered “dead” if the cytoplasm was diffusely eosinophilic and “viable” if they

demonstrated basophilic stippling (e.g. contained Nissl substance).

### Western blot analysis

To investigate changes in RIP1, RIP3, and cIAP1/2 expression, western blot analysis was used. Tissue samples were homogenised in lysis buffer (1 µg/mL aprotinin, 0.01 mol/L Tris-HCl, pH 7.5, 0.1 mol/L NaCl, and 1 mmol/L ethylenediaminetetraacetic acid), and homogenates were centrifuged at 12 000*g* for 15 min at 4 °C. Assays to determine the protein concentrations of samples were performed by comparing the results with a known concentration of bovine serum albumin using a BCA Protein Assay Reagent Kit (#23225; Pierce Biotechnology, Rockford, IL, USA). Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed in a 12% polyacrylamide gel under non-reducing conditions. Briefly, protein samples were boiled at 100 °C in 5% β-mercaptoethanol and 2.5% SDS, and lysates equivalent to 20 µg of protein from each sample were run on the gel for 30 min at 20 mA, together with a size marker (dual coloured protein; Bio-Rad Laboratories, Hercules, CA, USA). The electrophoresis running buffer contained 0.1% SDS, 250 mmol/L glycine and 25 mmol/L Tris base. Proteins on the gel were transferred to a polyvinylidene fluoride membrane (Invitrogen, Carlsbad, CA, USA) using a transfer buffer consisting of 20% methanol, 0.4% SDS, 39 mmol/L glycine, and 48 mmol/L Tris base. Membranes were placed in 4% powdered milk in phosphate buffered saline (PBS) to block non-specific binding after transfer. Then, membranes were incubated for 20 h at 4 °C with primary antibodies: goat polyclonal anti-RIP antibody (sc-1169; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:500 dilution, mouse monoclonal anti-RIP3 antibody (sc-374639; Santa Cruz Biotechnology) at 1:1,000 dilution, and goat polyclonal anti-cIAP1/2 antibody (sc-12410; Santa Cruz Biotechnology) at 1:500 dilution. After washing in PBS, membranes were incubated with horseradish peroxidase conjugated anti-goat immunoglobulin G (IgG) (PI-9500; Santa Cruz Biotechnology) or horseradish peroxidase conjugated anti-mouse IgG (#7076; Cell Signalling Technology, Danvers, MA, USA), as appropriate, at 1:5000 dilution in PBS for 90 min. Blots were developed using the ECL Plus detection method (RPN2132; Amersham Bioscience, Little Chalfont, UK). Another membrane was similarly stained without primary antibodies to ascertain the specific binding of antibodies for the proteins. Images of western blots were quantified by plotting a two dimensional densitogram using ImageJ software, version 1.63 (Research Services Branch, National Institute of Mental Health, National Institute of Health, Bethesda, MD, USA).

### RIP3 and cIAP1/2 immunocytochemistry

To investigate the expressions of RIP3 and cIAP1/2 in neuronal cells, immunohistochemistry was performed on five sections per animal (magnification × 200). Spinal cord sections were rinsed for 20 min in 0.1 M PBS. Sections were

blocked in 2% normal horse serum for 2 h at room temperature and incubated with primary antibodies in 0.3% Triton-X 100 and 10% normal horse serum or 10% normal rabbit serum, as appropriate, for 20 h at 4 °C. Primary antibodies were the same as used for western blot analysis, as follows: anti-RIP3 at 1:200 and anti-cIAP1/2 at 1:100.

The sections were incubated in 10% methanol and 0.3% H<sub>2</sub>O<sub>2</sub> for 20 min to quench endogenous peroxidase activity, washed in PBS, and incubated for 3 h with biotinylated anti-goat IgG (PK-6105; Vector Laboratories, Burlingame, CA, USA) or biotinylated anti-mouse IgG (PK-6102; Vector Laboratories) at 1:200 dilution in PBS containing 0.018% normal horse or rabbit serum, as appropriate. Samples were then incubated with an avidin-biotin-horseradish peroxidase complex (PK-6102; Vector Laboratories), coloured with diaminobenzidine/H<sub>2</sub>O<sub>2</sub> solution and the cytoplasm was counterstained with haematoxylin. A set of sections was stained similarly without primary antibodies to determine specific antibody binding to proteins.

### Fluorescent double labelling of RIP3 and cIAP1/2

To investigate the co-expression of RIP3 and cIAP1/2 in neuronal cells, immunohistochemistry was performed on five sections per animal. Spinal cord sections were prepared as described above and blocked using 10% horse serum before the application of primary antibodies. Sections were then incubated with RIP3 mouse monoclonal antibody at 1:50 dilution and simultaneously with cIAP1/2 goat polyclonal antibody at 1:50 dilution overnight at 4 °C and detected using goat anti-mouse IgG linked with green fluorescent Alexa Fluor 488 at 1:500 dilution (A11001; Invitrogen) and donkey anti-goat IgG linked with red-orange fluorescent Alexa Fluor 555 at 1:500 dilution (A-2142; Invitrogen). Slides were mounted in aqueous mounting media with 1,4-diazabicyclo[2.2.2]octane and observed by fluorescence microscopy (magnification × 400).

### Statistical analysis

For quantitative analyses, the neurological score and cell numbers were analysed by Mann-Whitney *U* test. Optical density values of western blots were analysed by one way analysis of variance with *post hoc* Tukey's multiple comparison tests. Data normality was checked using the Shapiro-Wilk and Kolmogorov-Smirnov tests. Using the Shapiro-Wilk test, the relative optical densities of RIP1 were normally distributed, but those of RIP3 and cIAP1/2 were not. Using the Kolmogorov-Smirnov test, all values were normally distributed. Then, a Kruskal-Wallis test was performed followed by a Mann-Whitney *U* test with Bonferroni correction to assess RIP3 and cIAP1/2 densities. The results were similar to the results from ANOVA, therefore the ANOVA results were used. A *p* value < .050 was considered statistically significant. Parametric data are presented as mean ± standard deviation (SD). Non-parametric data are presented as the median.

**Table 1.** Neurological scores of rabbits two and seven days after normothermic and hypothermic transient spinal cord ischaemia

Groups	Sham	Normothermic ischaemia		Hypothermic ischaemia	
	Day 7	Day 2	Day 7	Day 2	Day 7
1	5	3	0	5	5
2	5	1	1	5	5
3	5	2	0	4	5
4	5	4	0	5	5
5	5	2	2	5	5
6	5	3	0	5	5
Median score	5	2.5*	0 <sup>†</sup>	5 <sup>‡</sup>	5 <sup>‡</sup>

Each score represents raw data from a single rabbit.

\*  $p = .027$ .

<sup>†</sup>  $p = .023$  compared with the scores observed for the sham group.

<sup>‡</sup>  $p = .045$ .

<sup>§</sup>  $p = .023$  compared with the score observed for normothermic ischaemia at the same time point.

## RESULTS

### Neurological assessment

The results of neurological assessment are shown in Table 1. There were significant differences in neurological function between the N and H groups seven days after reperfusion (median modified Tarlov score; N = 0, H = 5,  $p = .023$ ).

### Histological study

Results of cell counting in the ventral grey matter region of sections are shown in Table 2. In the S group, spinal cords were intact with many large motor neurons (Fig. 1A). In the N group, although most motor neurons were intact two days after reperfusion (Fig. 1B), approximately 65% of motor neurons were lost in the ventral grey matter seven days after reperfusion (Fig. 1C). In the H group, most motor neurons in the ventral grey matter were preserved two (Fig. 1D) and seven days after reperfusion (Fig. 1E). Dorsal horn neurons were intact after transient ischaemia (data

**Table 2.** The number of intact large motor neurons in the ventral grey matter region after normothermic and hypothermic transient spinal cord ischaemia in rabbits

Groups	Sham	Normothermic ischaemia		Hypothermic ischaemia	
	Day 7	Day 2	Day 7	Day 2	Day 7
1	20	23	9	19	19
2	23	20	7	24	19
3	22	16	6	17	18
4	19	14	6	19	17
5	23	15	7	21	19
6	18	19	7	16	15
Median score	21	17.5	7*	19	18.5 <sup>†</sup>

Each count represents raw data from a single rabbit.

\*  $p = .046$  compared with sham group.

<sup>†</sup>  $p = .044$  compared with normothermic ischaemia at 7 days.

not shown). Therefore, the selective loss of motor neurons and the protection of motor neurons by hypothermia were confirmed, as previously reported.<sup>6</sup>

### Western blot analysis

Results of western blot analysis are shown in Table 3 and Fig. 2. In the S group, RIP1, RIP3, and cIAP1/2 were detectable as weak bands of 74, 57, and 70 kDa, respectively. These bands were strongly enhanced 8 h after reperfusion in the N group (Fig. 2A). The enhancement of RIP3 was preserved until two days after reperfusion. In the H group, the levels of these bands were at similar to those in the S group until two days after reperfusion (Fig. 2B). Control membranes without primary antibodies exhibited no bands (data not shown). Analysis of variance indicated the levels of RIP1 (Fig. 2C), RIP3 (Fig. 2E), and cIAP1/2 (Fig. 2G) were significantly increased 8 h after reperfusion in the N group (relative optical densities; RIP1,  $1.75 \pm 0.40$ ,  $p = .032$ ; RIP3,  $2.81 \pm 0.26$ ,  $p < .001$ ; cIAP1/2,  $2.40 \pm 1.08$ ,  $p = .041$  compared with sham), and the level of RIP3 was significantly increased until two days after reperfusion compared with the S group (relative optical density  $2.54 \pm 0.32$ ,  $p < .001$  compared with sham). In addition, in a comparison between the N and H groups, each protein in the N group had significantly increased expression levels 8 h after reperfusion (relative optical densities of the H group; RIP1 (Figs. 2D),  $0.96 \pm 0.35$ ,  $p = .022$ ; RIP3 (Figs. 2F),  $1.33 \pm 0.14$ ,  $p < .001$ ; cIAP1/2 (Figs. 2H),  $0.81 \pm 0.65$ ,  $p = .016$  compared with the N group).

### Histochemical study

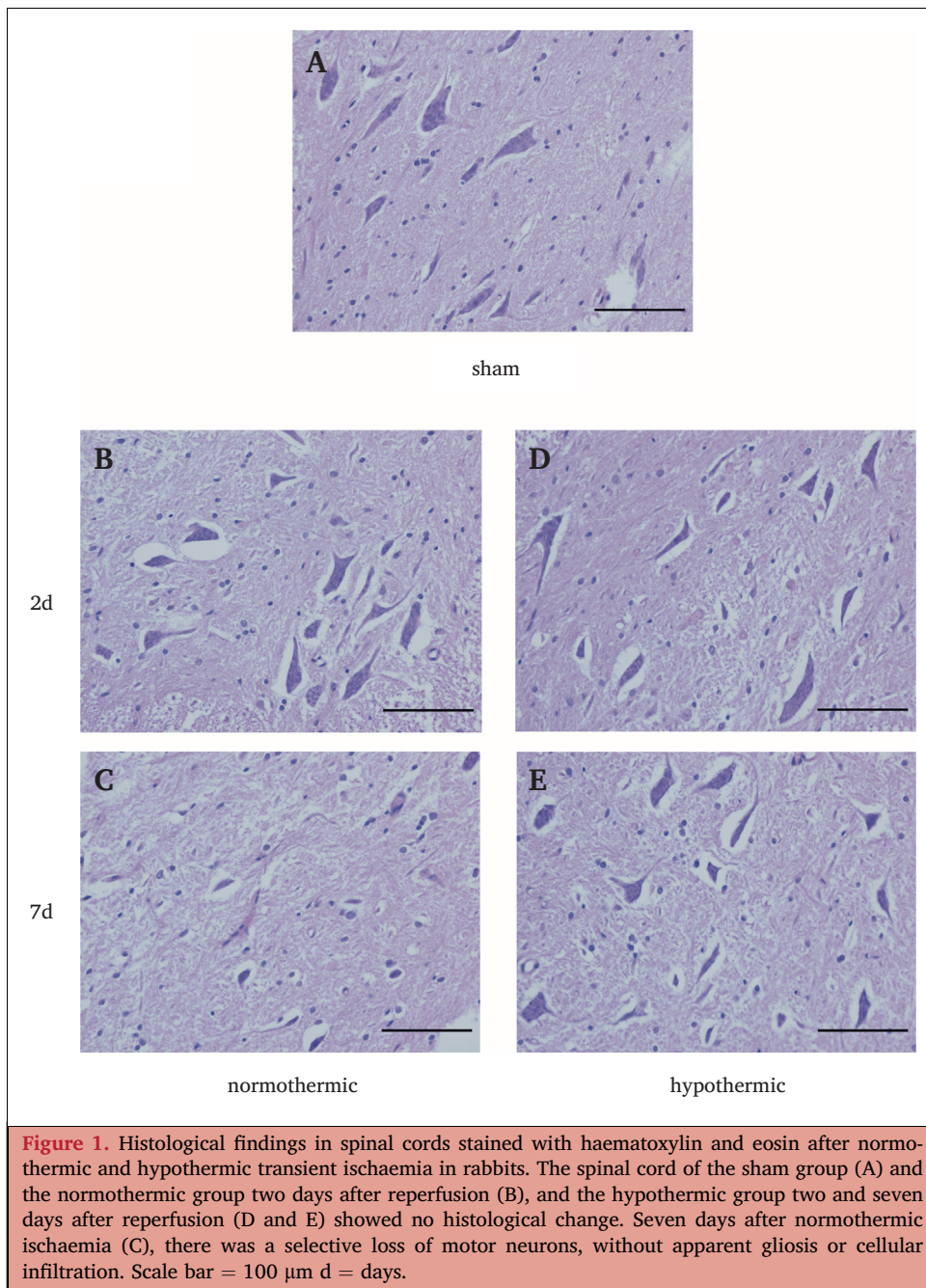
Immunoreactivity of RIP3 and cIAP1/2 in spinal cords is shown in Fig. 3. Spinal cords of the S group seven days after reperfusion did not show RIP3 (Fig. 3A) or cIAP1/2 (Fig. 3H) immunoreactivity in any cells. In the N group, motor neurons selectively exhibited strong immunoreactivity for RIP3 (Fig. 3B) and cIAP1/2 (Fig. 3I) 8 h after reperfusion and the RIP3 immunoreactivity in motor neurons was preserved at one and two days (Fig. 3C and D). In the H group, RIP3 and cIAP1/2 immunoreactivity was negligible in motor neurons (Fig. 3E–G and 3L–N).

### Fluorescence double labelling study

Results of RIP3 and cIAP1/2 double staining immunohistochemistry in the N group are shown in Fig. 4. RIP3 (Fig. 4A) was detected using green fluorescence and cIAP1/2 (Fig. 4B) was detected using red orange fluorescence; these molecules were preferentially expressed in the cytoplasm of motor neurons. Merged images show cells were double positive; in motor neurons, RIP3 and cIAP1/2 were expressed and colocalised in the cytoplasm (Fig. 4C).

## DISCUSSION

In this study, normothermic transient spinal cord ischaemia induced selective delayed motor neuron cell death and increased the expressions of RIP1, RIP3, and cIAP1/2 8 h



**Table 3.** Relative optical density of proteins after normothermic and hypothermic transient spinal cord ischaemia in rabbits vs. sham (6 animals per group)

Proteins	Normothermic ischaemia			Hypothermic ischaemia			p value
	8 h (n = 6)	1 d (n = 6)	2 d (n = 6)	8 h (n = 6)	1 d (n = 6)	2 d (n = 6)	
RIP1	1.75 $\pm$ 0.40*	1.20 $\pm$ 0.49	0.87 $\pm$ 0.39	0.96 $\pm$ 0.35 <sup>§</sup>	1.21 $\pm$ 0.33	0.80 $\pm$ 0.33	.004
RIP3	2.81 $\pm$ 0.26 <sup>†</sup>	2.75 $\pm$ 0.28 <sup>†</sup>	2.54 $\pm$ 0.32 <sup>†</sup>	1.33 $\pm$ 0.14 <sup>  </sup>	1.06 $\pm$ 0.26 <sup>  </sup>	1.45 $\pm$ 0.50 <sup>  </sup>	<.001
cIAP1/2	2.40 $\pm$ 1.08 <sup>‡</sup>	1.72 $\pm$ 0.70	1.86 $\pm$ 0.90	0.83 $\pm$ 0.62 <sup>¶</sup>	0.81 $\pm$ 0.65	1.09 $\pm$ 0.32	.004

Data are presented as mean  $\pm$  standard deviation; h = hours; d = days; RIP = receptor interacting protein kinase; cIAP = cellular inhibitor of apoptosis protein.

\*  $p = .032$ .

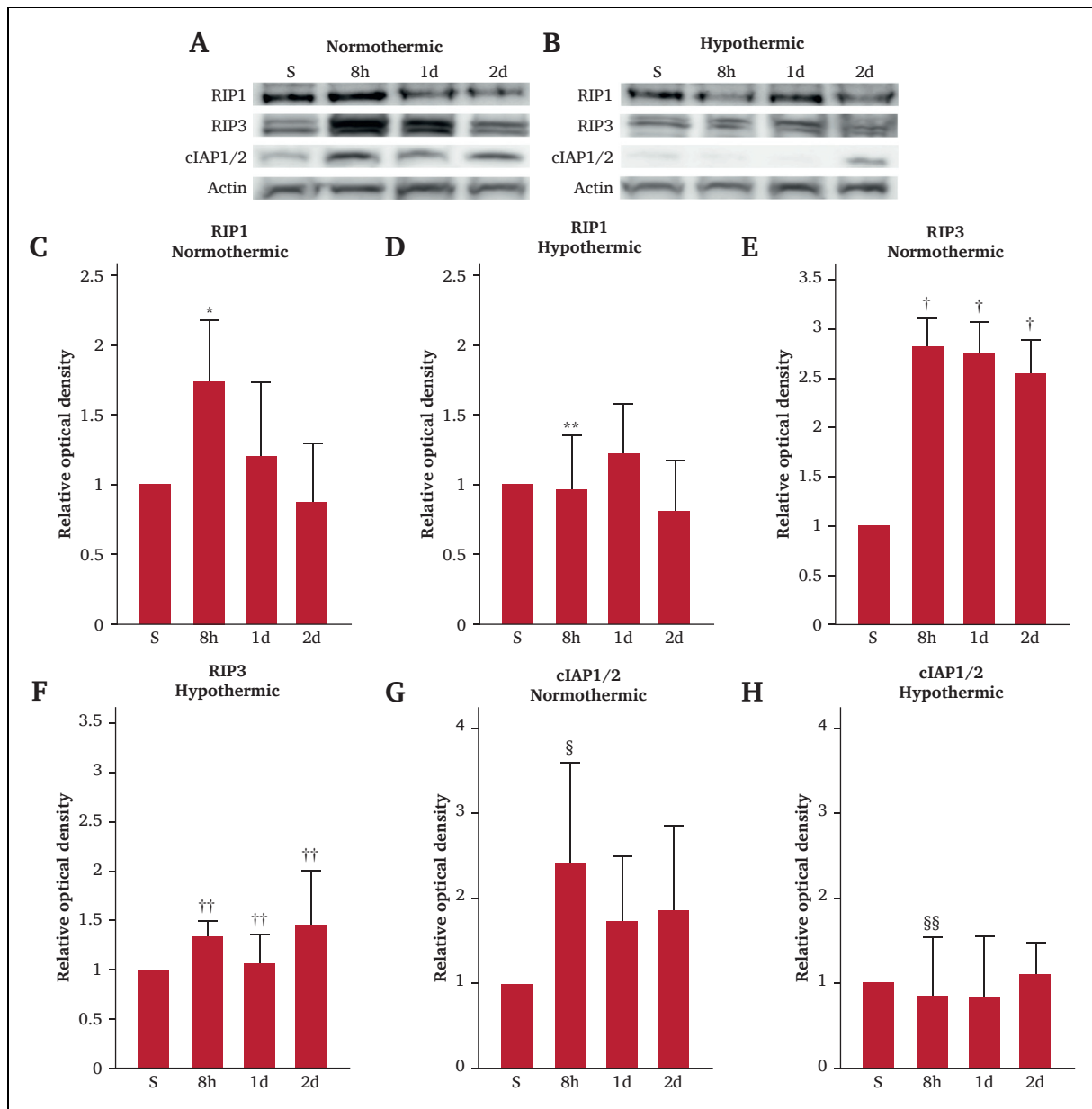
<sup>†</sup>  $p < .001$ .

<sup>‡</sup>  $p = .041$  compared with sham group.

<sup>§</sup>  $p = .022$ .

<sup>||</sup>  $p < .001$ .

<sup>¶</sup>  $p = .016$  compared with normothermic ischaemia at the same time point.



**Figure 2.** Representative western blots for receptor interacting protein kinase (RIP) 1, RIP3, and cellular inhibitor of apoptosis protein (cIAP1/2) in rabbits (A) In the normothermic transient ischaemia group, RIP1, RIP3, and cIAP1/2 were strongly enhanced at 8 h and the enhancement of RIP3 was preserved until two days (d) after reperfusion (B) In the hypothermic transient ischaemia group, RIP1, RIP3, and cIAP1/2 were not enhanced after reperfusion. Quantitative analysis showed that normothermic transient ischaemia significantly increased RIP1 (C), RIP3 (E), and cIAP1/2 (G) expression ( $n = 6$ ; RIP1,  $*p = .032$ ; RIP3,  $†p < .001$ ; cIAP1/2,  $§p = .041$  compared with the sham group (S)). In the hypothermic transient ischaemia group, the expression of RIP1 (D), RIP3 (F), and cIAP1/2 (H) were not increased ( $n = 6$ ; RIP1,  $**p = .022$ ; RIP3,  $††p < .001$ ; cIAP1/2,  $§§p = .016$  compared with the normothermic ischaemia group at the same time point). The bars above each staple represent the standard deviation.

after reperfusion. The prolonged expression of RIP3 was observed until two days after reperfusion in the N group, while selective delayed motor neuron cell death was not induced and the expressions of these proteins were unaltered after reperfusion in the H group.

Delayed paraplegia sometimes occurs after thoraco-abdominal aortic surgery, with an incidence of 1.4%.<sup>1</sup> Clinically, hypotension, extensive aneurysm repair, coexistence

of atherosclerotic disease, and fewer intercostal arteries reattached are possible factors in delayed paraplegia. It was reported that apoptosis disturbed ubiquitin–proteasome pathways, and autophagy affected neuronal survival after transient ischaemia in the spinal cord. In the brain, ischaemia induces necrosis. Around the necrotic core, adjacent tissues (penumbra) are perfused and can be rescued. Programmed cell death occurs in this area and

cerebral infarction expands.<sup>16</sup> The same phenomenon is assumed to occur after spinal cord ischaemia.

Recently, necroptosis has emerged as a novel type of programmed cell death that is involved in various disease models.<sup>8–10</sup> The serine/threonine kinase activity of RIP1 and its interaction with RIP3 are necessary for necroptosis.<sup>11</sup> In addition, RIP3 is considered an essential mediator of necroptosis.<sup>17,18</sup> In cerebral ischaemia, necroptosis contributed to delayed ischaemic brain injury, and necrostatin-1, which inhibits the kinase activity of RIP1, reduced the infarct volume after middle cerebral artery occlusion in mice.<sup>9</sup> Ischaemic insults upregulated RIP1 and RIP3 and necroptotic cell death in hippocampal cells following oxygen–glucose deprivation in an *in vitro* model of global cerebral ischaemia.<sup>19</sup> After focal cerebral ischaemia, RIP3 expression was increased in the ischaemic penumbral area, in which secondary cerebral injury occurs.<sup>20</sup> Taken together, necroptosis correlates with delayed neuronal death after cerebral ischaemic injury. Given the similarity between cerebral and spinal cord ischaemia, necroptosis may correlate with neuronal cell death after transient spinal cord ischaemia.

Recent studies demonstrated a relationship between necroptosis and the onset of amyotrophic lateral sclerosis (ALS). Death of motor neurons triggered by ALS astrocytes occurred via necroptosis involving RIP1.<sup>21</sup> The loss of optineurin, mutations, which are implicated in sporadic and familial ALS, led to progressive dysmyelination and axonal degeneration in the central nervous system through necroptotic machinery, including RIP1 and RIP3.<sup>22</sup> Inhibiting RIP1 may provide an axonal protective strategy for the treatment of ALS.<sup>23</sup> In addition, necroptosis plays a role in a novel molecular mechanism associated with secondary neuronal tissue damage following traumatic spinal cord injury.<sup>24,25</sup> The results suggest the mechanism of motor neuron death in the spinal cord after transient ischaemia may have features similar to those of ALS and spinal cord injury.

As the mechanism of necroptosis has been revealed, the inhibitory process of necroptosis has been found. cIAP1/2 was initially identified as a direct inhibitor of caspases. It is a component of Complex I, and an E3 ubiquitinase of RIP1 that induces the NF $\kappa$ B survival pathway stimulated by TNF. Degradation of cIAPs sensitises cells to TNF induced killing.<sup>26</sup> cIAP1/2 limited macrophage necroptosis by inhibiting RIP1 and RIP3 activation<sup>27</sup> and protected cells from necroptosis by preventing RIP1/RIP3 dependent reactive oxygen species production.<sup>28</sup> Moreover, hypothermia led to decreases in RIP1 and RIP3 and attenuated tissue damage in rats in a traumatic brain injury model, suggesting hypothermia inhibited necroptosis.<sup>29,30</sup> Hypothermia has multifocal mechanisms that protect cells against ischaemia. Hypothermia preserves metabolic substrates, alters cerebral blood flow, prevents excitatory amino acid accumulation and depresses free radical production. Hypothermia also

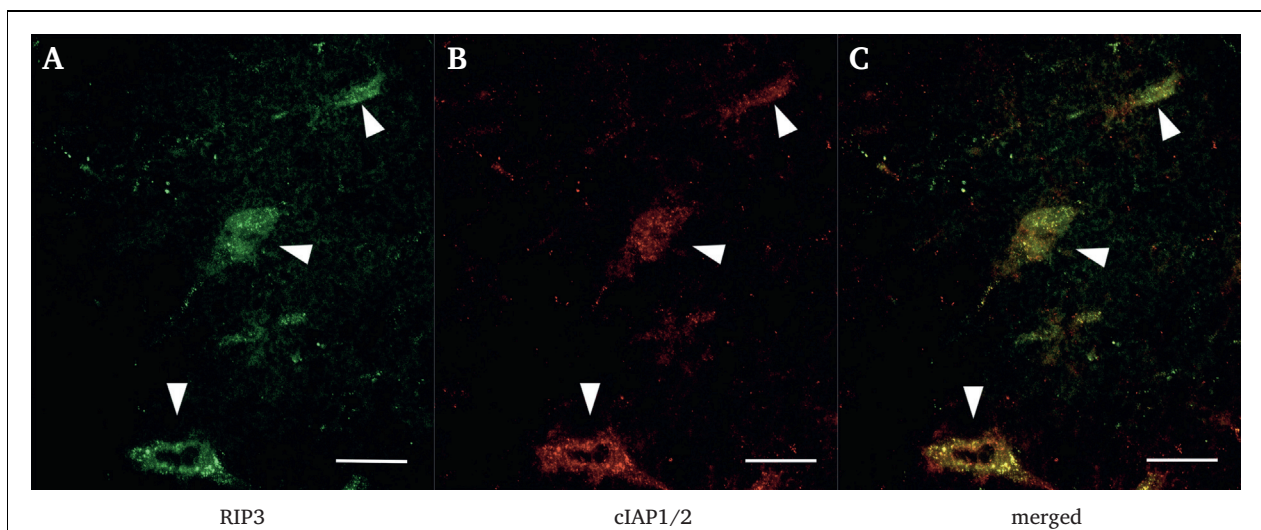
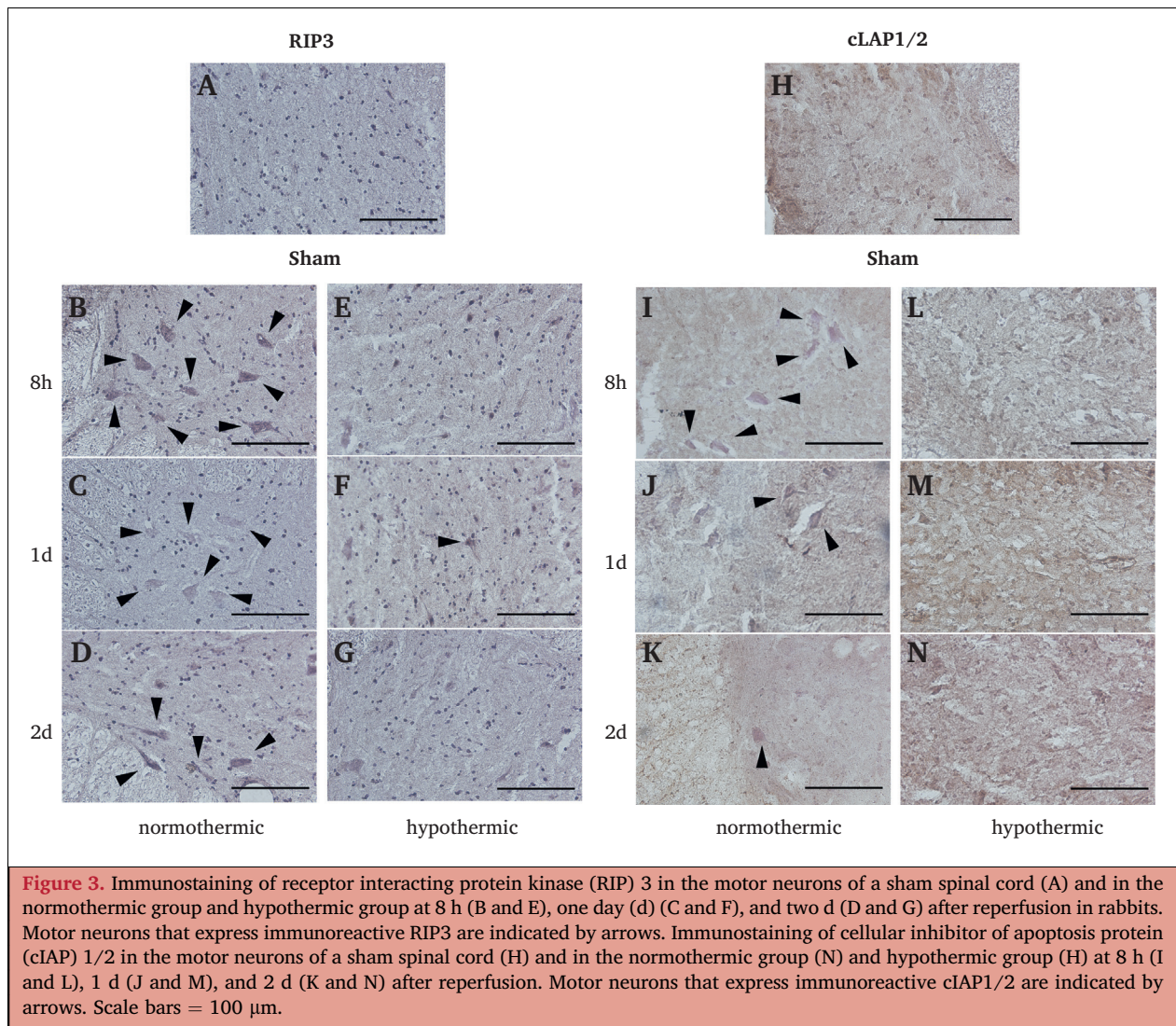
suppresses inflammatory responses by inhibiting NF $\kappa$ B pathways.<sup>31</sup> In the study, the expression of cIAP1/2 was increased after normothermic spinal cord ischaemia. The selective induction of cIAP1/2 in motor neurons indicates a stress response after normothermic spinal cord ischaemia. Hypothermia reduced the levels of many inflammatory mediators and pro-inflammatory cytokines such as TNF $\alpha$ . It suppressed the expressions of RIP1, RIP3, and cIAP1/2, resulting in neuronal cell survival. Thus, hypothermia might suppress the function of cIAP1/2. Further studies are needed to elucidate the *in vivo* role of cIAP1/2 in necroptosis.

In clinical settings, thoraco-abdominal aortic operations are performed under systematic moderate hypothermia (32 °C–34 °C) induced by partial extracorporeal bypass. A few reports showed that a slight temperature difference established a protective effect against neuronal death after cerebral ischaemia.<sup>32,33</sup> A body core temperature of 33 °C gave excellent outcomes after stroke in clinical settings.<sup>34</sup> During thoraco-abdominal aortic surgery, spinal cord protection may therefore be achieved by allowing the patient to cool to 33 °C.<sup>35</sup> Furthermore, deep hypothermia with circulatory arrest during thoraco-abdominal aortic surgery was effective in preventing paraplegia.<sup>36</sup> In addition, epidural cooling is sometimes applied to avoid complications of deep hypothermia including coagulopathy, arrhythmia, and respiratory dysfunction.<sup>37</sup> According to these reports and the results, moderate hypothermia may reduce delayed motor neuron death after transient spinal cord ischaemia by inhibiting necroptosis, and deeper hypothermia might be even more effective.

There were some limitations in this study. First, the relationships between necroptosis and other forms of cell death were not assessed, and the function of cIAP1/2 in necroptosis was not fully elucidated. Apoptosis is an important cause of secondary cell death in the central nervous system after ischaemic injury. It was previously revealed that apoptosis was one of the responsible factors for delayed motor neuron death after transient spinal cord ischaemia in rabbits.<sup>2</sup> Apoptosis and necroptosis have several common pathways and their interactions remain unclear. Second, the results from the immunohistochemical and fluorescence double labelling study were not quantified. To clarify the extent to which necroptosis is involved in delayed motor neuron death, quantification of cell death is necessary.

In conclusion, this study demonstrated that immunoreactivity for RIP1, RIP3, and cIAP1/2 was induced at an early phase in motor neurons and confirmed differences in the expression profiles of these molecules between the normothermic and hypothermic groups. The prolonged induction of RIP3, necroptosis, might be responsible for selective motor neuron death after transient spinal cord ischaemia.





## CONFLICT OF INTEREST

None.

## FUNDING

This work was supported by JSPS KAKENHI Grant Number JP17K17935.

## ACKNOWLEDGEMENTS

We appreciate the technical assistance from The Research Support Centre, Research Centre for Human Disease Modelling, Kyushu University Graduate School of Medical Sciences. We also thank Ann Turnley, PhD for editing a draft of this manuscript.

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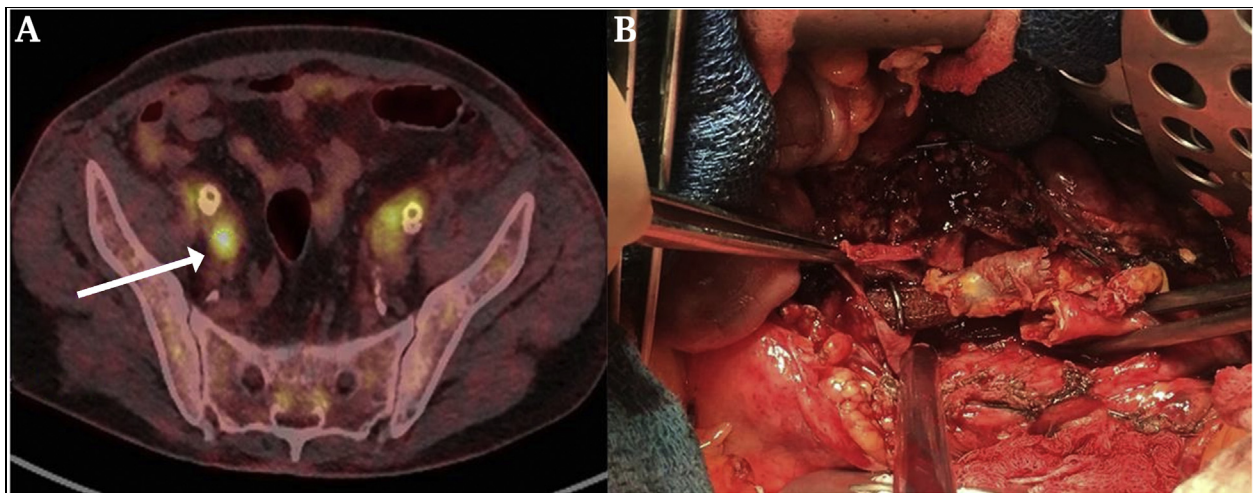
Eur J Vasc Endovasc Surg (2020) 59, 833

## COUP D’OEIL

# Explantation of Infected Iliac Stents: Old Tools Should Not Be Forgotten

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A 59 year old man with chronic lymphocytic leukaemia presented with a ruptured mycotic left common iliac artery aneurysm (*Streptococcus pneumoniae*) and pseudo-aneurysm of the right external iliac artery. He initially underwent bridging endobypass of both iliac arteries including Advanta V12 (Getinge, Goteborg, Sweden) and Viabahn (WL Gore & Associates, Flagstaff, AZ, USA) stent grafts. Despite appropriate antibiotic therapy, he developed stent graft infection three months later, presenting as septicæmia, confirmed by PET/CT (A, arrow). As severe iliac atherosclerosis precluded conventional retrieval, stent graft explantation was undertaken using a Vollmar ring via a femoral approach (B), followed by successful aortobifemoral allograft repair.

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<https://doi.org/10.1016/j.ejvs.2019.12.033>