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Efficacy of remote ischaemic preconditioning for spinal cord protection against ischaemic injury: association with heat shock protein expression

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Abstract

Introduction: *We aimed to determine the efficacy of remote ischaemic preconditioning in the hind limb of rats for ischaemic damage of the spinal cord through neurological and histological investigation and examination of heat shock proteins (HSP).*

Material and Methods: *Thirty male Sprague-Dawley rats were divided into three groups as Group 1 (control group, n=10), Group 2 (ischaemia control group, n=10), and Group 3 (remote ischaemia preconditioning group, n=10). The right lower limb of the rats in the study group was compressed with a tourniquet for three cycles of ten-minute ischaemia followed by ten-minute reperfusion. After a period of 8 hours, the peritoneal cavity was accessed through a midline vertical incision. The abdominal aorta was clamped between the origin of the renal arteries and the iliac arteries for 45 minutes and spinal cord ischaemia was induced. The same procedure of abdominal aorta clamping was performed in the control group without creating leg ischaemia. The rats were evaluated for neurological parameters at 24 and 48 hours. At the end of this time period, all rats were sacrificed and the spinal cords were stained for determination of HSP and histopathological classification. For immunohistochemical evaluation, the samples were analyzed according to the degree of staining with HSP70 rabbit antibody.*

Results: *After completing the neurological examinations and histological evaluations, we determined the spinal cords of the animals in the sham group to be completely normal. The post-operative neurological examination scores of Group 3 at 24 and 48 hours were significantly higher than scores measured in the other two groups. There were seven rats with HSP expression and this was detected in animals pretreated with remote ischaemic preconditioning. There were also two rats in Group 2 with HSP expression.*

Conclusion: *Our results show that production of transient remote ischaemia preconditioning in the lower extremities reduces damage in the spinal cord secondary to ischaemia probably by the increase of HSP.*

Key words: *Spinal cord; Ischaemia; Hind limb; Preconditioning; Heat shock protein.*

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Introduction

Ischaemic preconditioning (IP) is a phenomenon of single or repeated periods of transient ischaemia that protect the cells and tissues from infarction and necrosis, in case of longer durations of ischaemic insult [21]. Heat shock proteins (HSP), which are intracellular chaperones, have gained importance in understanding the mechanism of IP effect and tolerance to ischaemia [4,29]. These proteins are synthesized during conditions of stress, such as ischaemia, hyperthermia, and hypothermia, and take part in cellular defence mechanisms against stress injuries.

In numerous studies, transient ischaemia has been induced in various organs by means of remote preconditioning (RP) and afterwards, it has been shown that the target organ has gained resistance to ischaemia. These mechanisms may be humoral, neural, or a combination of both, and involve adenosine, opioids, bradykinins, protein kinase C, and K-ATP channels, although the precise end-effector remains unclear [5,13,24,25].

The aim of this study was to demonstrate the efficiency of transient RP in the lower extremities of rats for the reduction of ischaemic damage to the spinal cord by an analysis of heat shock proteins.

Material and Methods

Thirty Sprague-Dawley rats weighing 310-430 g were used in the study. Rats were obtained from Istanbul University DETAM Institute and were kept at 20-22°C room temperature, with exposure to 12 hours of light, then 12 hours of darkness. The rats were fed with standard rat food and tap water. Ethical consent was obtained from Istanbul University Ethics Committee and procedures in the study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (NIH publication no. 85-23, revised 1996). Neurological examination was performed on all rats before initiating the study.

Preparation

The rats did not receive mechanical ventilatory support during the experiment. Respiratory support was given with an oxygen mask. Anaesthesia was induced with intraperitoneal injection of 40 mg/kg sodium pentothal. After infusion of 150 IU/kg heparin through the tail vein, ischaemia was induced by applying a tourniquet to the right lower limb of each rat with three cycles of 10-minute ischaemia followed by 10-minute reperfusion between each process. The blood circulation of the limb was stopped by increasing pressure on the trochanter with a rubber band. Occlusion and reperfusion were confirmed by Doppler ultrasonography.

After an interval of eight hours, the rats were anaesthetized for a second time. A short incision was made over the neck in the supine position. The left common carotid artery and external jugular vein were catheterized with a 24-gauge catheter for the measurement of proximal mean aortic blood pressure (PAP) and infusion of fluids, respectively. The peritoneal cavity was accessed through a vertical midline incision and the abdominal aorta was clamped between the origin of the renal arteries and the iliac arteries; ischaemia was induced in the spinal cord for 45 minutes. Body temperature was measured with a rectal probe and was kept at 37°C with heating pads. The isolated aortic ischaemic segment pressure (IASP) was monitored with a 24-gauge catheter throughout the occlusion in all groups and was kept at less than 15 mmHg. Arterial blood gases ($PaCO_2$ and PaO_2) and pH were measured at baseline, during aortic occlusion, and after reperfusion. In order to detect hypoxia, arterial oxygen saturation was followed continuously by use of a pulse oximeter placed on the ear in the preoperative and postoperative periods until the animals recovered from the anaesthesia.

Experiment protocol

Thirty rats were divided into three groups. Treatments were allocated according to the following groupings: Group 1 (n=10) served as the control group with only the peritoneal cavity accessed and no other invasive procedure performed; Group 2 (n=10) served as the ischaemia control group with anaesthesia given at 45 minutes, and at eight hours during the procedure, the peritoneal cavity was accessed and the abdominal aorta was clamped between the origin of the renal arteries and the iliac arteries for 45 minutes; Group 3 (n=10) served as the RP group and ischaemia was produced in the lower extremity for three cycles, with each cycle consisting of a 10-minute induction of ischaemia followed by 10-minute reperfusion. In Group 3, ischaemia was produced in the spinal cord by occluding the same segment of the abdominal aorta and after an 8-hour interval. The rats were given 150 IU/kg of heparin through the tail vein during each occlusion.

Neurological evaluation

All animals were closely monitored after the procedures. Neurological status was assessed postoperatively at 24 and 48 hours, by using the 15-point spinal cord performance scale (Appendix) [14,38]. Each animal's group assignment was blinded during neurological examination.

Appendix (Spinal cord performance scale (adapted from Zhang and Lemay) [8,9])

Spinal cord performance scale

Variable	Score
Lower extremity motor function	
Normal:	4
Mildly impaired walking:	3
Able to stand, but unable to walk:	2
Movement in lower extremity, but unable to stand:	1
Total paraplegia:	0
Horizontal rope	
Grasps rope and pulls up with lower extremity:	3
Grasps rope without pulling:	2
Cannot grasp rope:	1
Does not raise lower extremity:	0
45°C Bar	
Lower extremity grasps bar more than 10 seconds:	3
Lower extremity grasps bar more than 5 seconds:	2
Lower extremity grasps bar less than 5 seconds:	1
Lower extremity falls off bar:	0
Pain sensation	
Withdraws lower extremity:	2
Squeals but does not withdraw:	1
No reaction:	0
Metal screen (180°C)	
Lower extremity grasps screen more than 5 seconds:	3
Lower extremity grasps screen less than 5 seconds:	2
Lower extremity grasps screen less than 5 seconds, but not at 180°C:	1
Lower extremity falls from screen:	0
Total score: 15	

Histopathological examination

Animals were sacrificed at the end of the last neurological assessment. All rats were anaesthetized with an intraperitoneal injection of sodium pentothal (50 mg/kg) and perfused with intracardiac 100 ml of 0.9% normal saline solution, followed by 750 µl of 4% paraformaldehyde in 0.1 M phosphate buffered saline solution (PBS, pH: 7.44). Following perfusion, eventually whole spinal cords and spinal ganglions were dissected and removed. The entire spinal cord specimen was post-fixed for 24 hours in the same fixative. At the end of 120 hours, the 3rd and 4th lumbar spinal cord segments were isolated and embedded in paraffin. Consecutive 5-µm thick sections were cut serially and mounted and stained with haematoxylin and eosin (HE) obtained for light microscopic examination. A neuropathologist, who was blinded to the experimental procedure, performed the histological evaluation with the light microscope. A score was given according to the extent and severity of histopathological changes in 3 sets of haematoxylin- and eosin-stained specimens in the mid-segment of the 4th lumbar cord. The grading of the acute grey matter injury was based on percent abnormal or dead neurons in the ventral horns: 0, severe neuronal injury or death (>50%); 1, moderate damage (10% to 50%); 2, mild damage (<10%); and 3, no damage. Three regions of the spinal cord grey matter were scored: the ventral horn with the large motor neurons (Rexed's laminae 8 and 9), the intermediate grey matter (laminae 7 and 10), and the dorsal horn (laminae 1 to 6) (Fig. 1). The white matter damage in the ventral and ventrolateral funiculi was assessed on the basis of the extent of microvacuolation: 0, severe damage (>50% affected with microvacuolations); 1, moderate damage (10% to 50% of area affected with microvacuolations); 2, moderate damage (<10% affected with microvacuolations); and 3, no damage (no microvacuolations) (Fig. 1). The score for the grey or white matter damage in each animal was the average of the right and left hemicords in 3 consecutive sets of specimens from each animal. Additionally, the number of neuronal cell bodies per microscopy field was counted in the ventral horn (laminae 8 and 9, the area with the large motor neurons and most of the adjacent part of lamina 7) (Fig. 1). The numbers obtained from the left and right hemicords were averaged for each animal. To avoid sampling errors, similar neuronal counts were also obtained from

specimens derived from the 3rd lumbar segments in the same fashion [12].

Immunohistochemical examination

Sections with a thickness of 5 µm were obtained from the 3rd and 4th lumbar spinal cord segments and mounted on poly-l-lysine coated slides. The sections were immersed in 0.3% H_2O_2 for 15 minutes and washed with PBS. The sections were then incubated in rabbit anti-HSP70 polyclonal antiserum (Abcam, UK) for 1 hour (1:100 dilution). After the primary incubation and three rinses in PBS, sections were incubated in biotinylated goat anti-rabbit IgG (Invitrogen, CA, USA) for 10 minutes (1:2000 dilution). Following the incubation in substrate chromagen solution for 10 minutes, all sections were washed in PBS and distilled water, mounted in glycerol and examined later under the microscope. The spinal cord sections stained positively for HSP were assessed and compared among groups.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed by one-way analysis of variance test with the post-hoc Tukey significant difference for comparison of physiological variables between groups. The comparison of neurological and histological scores was performed with Kruskal–Wallis test, whereas differences between the two groups were analyzed with Bonferonni-adjusted Mann-Whitney U-test. The comparison of neurological scores within a group was performed with paired-sample t test. Differences were considered statistically significant for a P-value of <0.05.

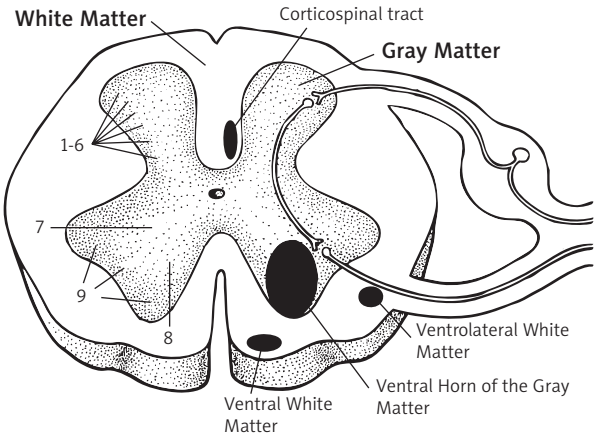

Fig. 1. Diagram showing a cross-section through the rat spinal cord at the level between the 3rd and 4th lumbar spinal cord segment. Numbers on the right refer to Rexed's laminae of the grey matter. Axonal counts were performed on the ventral and ventrolateral white matter and corticospinal tract based on immunohistochemistry. Neuronal counts were performed on Rexed's laminae

Results

The mortality rate for all groups was 10% (3/30). One rat in each group was excluded from the study; one died because of haemorrhage, one had cerebral ischaemia due to carotid occlusion after repair, whereas no cause could be identified for the third one. The rectal temperature did not differ between the groups throughout the operative period (p=0.8). No significant difference was noted among groups in terms of mean IASP during aortic occlusion (p=0.08). During the occlusion and reperfusion periods, we tried to keep PAP at 75±5 mmHg. To overcome systemic acidosis and possible blood pressure depression, we used a mixture of 0.9% normal saline solution mixed with sodium bicarbonate following relief of the clamps in all animals. Arterial blood gases were similar in all groups and hypoxia was not detected during the operation or postoperative period (Tab. I).

Table I. Physiological parameters recorded during the operation

	Groups			
	Group 1 (*n*=9)	Group 2 (*n*=9)	Group 3 (*n*=9)	p-value
PAP (mmHg)				
baseline	81.5±6.4	84.2±4.5	82.7±3.9	0.72
ischaemia	81.5±6.4	115±7.5	112±5.3	0.43
reperfusion	81.5±6.4	73±1.1	71±4.3	0.4
O_2 saturation (%)				
perioperative	97±2.5	98±2.8	97±3.9	0.3
postoperative	96±2.3	97±3	96±2.8	0.47
IASP (mmHg)				
	NA	10.9±1.8	10.5±2.0	0.08
RT (°C)				
	36.3±0.5	36.5±0.4	36.7±0.3	0.8

Group 1 – control group; Group 2 – ischaemia control group; Group 3 – remote ischaemic preconditioning group. PAP – proximal aortic pressure; IASP – aortic ischaemic segment pressure; NA – not applicable; RT – rectal temperature.

Neurological outcome

Neurological score was significantly reduced at 24 and 48 hours in the control and the study groups when compared to the sham group. Additionally, the difference between the neurological scores of Group 2 and Group 3 was significantly higher in the remote ischaemic preconditioning group (Figs. 2-3) at both time points (24 hours: Group 2, 7.5 ± 2.3 vs. Group 3, 10.7 ± 2.8, p=0.04; and 48 hours: Group 2, 5.2 ± 1.5 vs. Group 3, 10.1 ± 1.9, p=0.04).

The decrease in the neurological score between 48 hours and 24 hour levels was significantly lower in Group 2 (7.5 ± 2.3 vs. 5.2 ± 1.5, p=0.03). On the other hand, the continuing decrease in the neurological score between 24 and 48 hours in Group 3 was not statistically significant (10.7 ± 2.8 vs. 9.1 ± 1.9, p=0.67) (Tab. II).

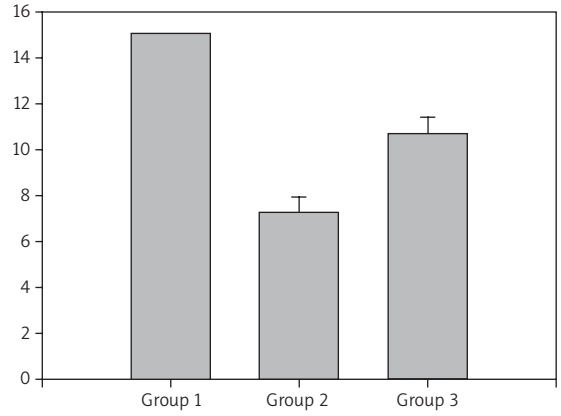

Fig. 2. Neurological score at 24 hours (vertical axis)

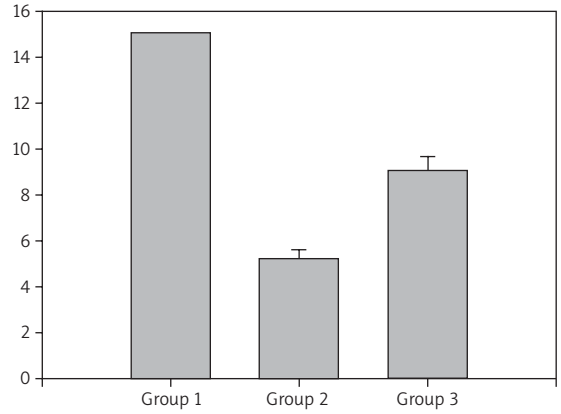

Fig. 3. Neurological score at 48 hours (vertical axis)

Histopathological outcome

HE staining was used to analyze the degree of ischaemic neuronal cell injury. At the time of sacrifice, according to the histopathological grading scale, Groups 1, 2 and 3 had scores of 2.8 ± 0.2, 1.5 ± 0.4 and 2.3 ± 0.5, respectively (Group 1 vs. 2, p<0.01; Groups 1 vs. 3, p=0.04; Groups 2 vs. 3, p<0.01; Fig. 4). The histological evaluation as well as neurological evaluation of the sham group was accepted as normal.

There were 7 rats with HSP70 expression in ependymal and endothelial cells (Fig. 5) and in neurons of the spinal cord and spinal ganglion in animals pretreated with remote ischaemic preconditioning, i.e. Group 3 (Figs. 6-7). In concordance, HSP70 expression was observed in the spinal cords of 2 rats in Group 2. The production of HSP70 in Group 3 was significantly higher than HSP production in Group 2 (p<0.05).

Discussion

Operations involving the thoracoabdominal aorta may lead to paraplegia/paraparesis secondary to clamping of the aorta and resultant ischaemic spinal cord damage [33]. The pathophysiological mechanisms of neuronal damage involve the production of free radicals during ischaemia-reperfusion, lipid peroxidation, intracellular calcium deposition, and apoptosis [17,18,22,36]. In order to protect the spinal cord from the ischaemic damage due to distal aortic perfusion, drainage of the cerebrospinal fluid, reim-

Tab. II. Neurological scores

Groups	Neurological score 24 h	p-value[a]	Neurological score 48 h	p-value[a]
Group 1	15	Group 1 vs. 2, p<0.01	15	Group 1 vs. 2, p<0.01
Group 2	7.5±2.3	Group 2 vs. 3, p=0.01	5.2±1.5	Group 2 vs. 3, p=0.01
Group 3	10.7±2.8	Group 1 vs. 3, p=0.04	9.1±1.9	Group 1 vs. 3, p=0.04
	**p-value <0.001		**p-value <0.001	

***p-value obtained with Kruskal-Wallis test. [a] p-value obtained with Bonferonni-adjusted Mann-Whitney U-test.*

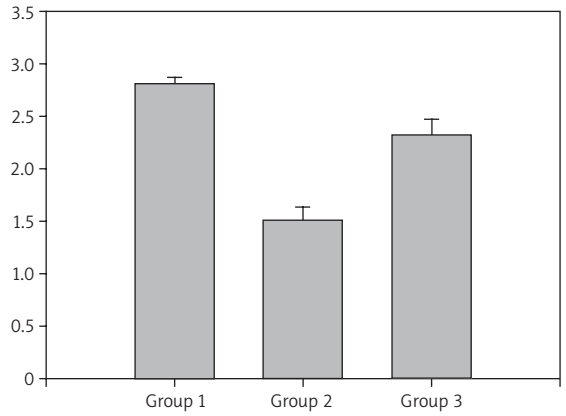

Fig. 4. Histopathological score (vertical axis)

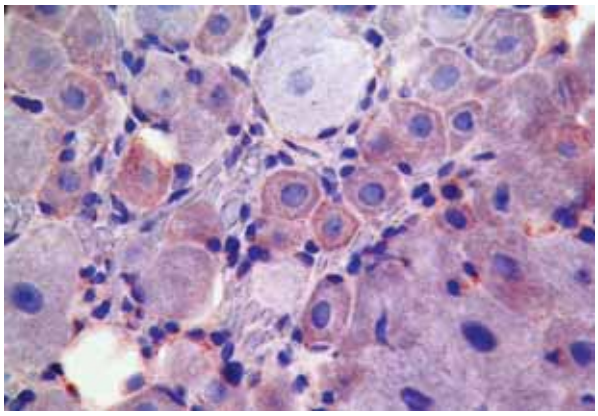

Fig. 5. Cytoplasmic HSP70 expression in spinal ganglions in Group 3 (×40)

plantation of the intercostal arteries, and pharmacological treatments have been used [27,28,34].

Despite these methods, ischaemic jeopardy of the spinal cord is still encountered. Therefore several studies involving the IP method have been conducted aiming to minimize ischaemic spinal cord damage. Ischaemic preconditioning is a procedure of single or repeated periods of transient ischaemia that triggers protective mechanisms of the tissue by challenging the cells with transient ischaemia/reperfusion without producing sustained damage [21].

It was shown that the infarct size was reduced with IP in some studies. IP was first tried on dog hearts by Murry et al. [20]. In this study, 5-minute periods of brief coronary ischaemia followed by reperfusions reduced necrosis formation by 75% during 40 minutes of coronary occlusion. In the literature, different durations of IP production were noted for various tissues in rats, rabbits and human heart [3,15].

Similarly to our study, Thaveau et al. produced ischaemia, consisting of three cycles of 10-minute ischaemia followed by 10-minute reperfusion, in the hind limbs of rats, and stated that the IP mechanism resulted in resistance to ischaemia and tissue damage [32]. Several studies have shown that IP has a protective effect which follows two different time scales classified as short-term and long-term effects of IP [6,23].

The effect of short-term IP lasts for 2-3 hours. During this period, adenosine, bradykinin, norepinephrine and opioids are released. These compounds prevent damage by activation of the potassium channels and increasing ATP stores [2,31]. The protective

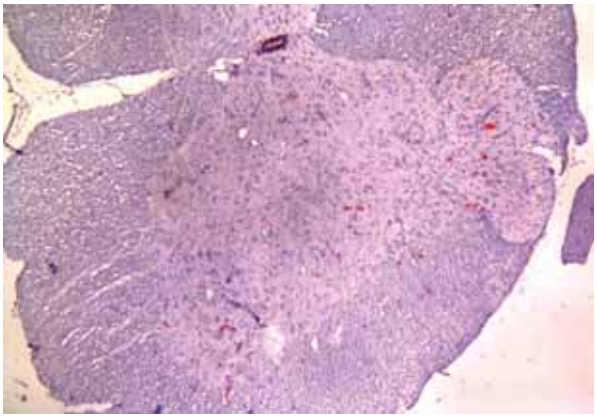

Fig. 6. Ependymal and endothelial cells showing ischaemic injury in neurons (×4)

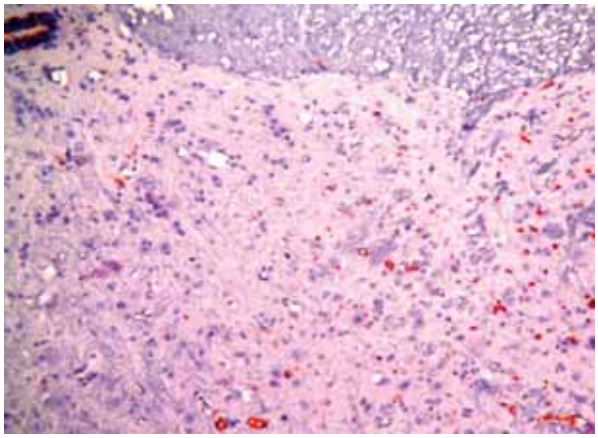

Fig. 7. Ependymal and endothelial cells showing ischaemic injury in neurons (×20)

mechanism that begins 12-24 hours after ischaemia is known as long-term IP, which involves nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and heat shock proteins [23]. The reason for producing the transient ischaemia, 8 hours ahead in this study, was to benefit from the long-term effect of IP.

Heat shock proteins (HSP) are intracellular chaperone molecules that co-direct the correct structuring, stabilization and organization of intracellular proteins [22,33]. HSPs have been shown to play an important role in the protection of cells under stress conditions [14,38]. The 70kD subgroup of HSP (HSP70) is one of the most widely studied molecules. These chaperones exhibit similar properties in several species by supporting cellular regeneration and repair. The most important task for the chaperons is to stabilize the spatial structure of other intracellular proteins. HSPs gain in concentration in cellular stress conditions and derogate the stabilization of intracellular proteins, protect the cell from further damage and may help in the repair of damaged cells [9,29,33]. HSPs have also been reported to exert important effects in the cardiovascular system. The cardioprotective function of HSP70 has already been shown to exist under myocardial stress [1]. Further cardiovascular protective effects have been reported to be prevention and attenuation of atherosclerosis, and protection of endothelial functions in the case of vasculitis and hypertension [26].

Studies involving HSP70 have gained medical attention again in the last decade [16]. These molecules, which are synthesized during periods of stress such as ischaemia, hyperthermia, and hypothermia, are involved in the cellular defence mechanisms during these periods [9,30]. They are one of the molecules that can be investigated in studies on tissue protection as well as ischaemic injury in various organ systems.

Basaran et al. demonstrated the IP effect in the spinal cord with ubiquitin, which is a heat shock protein [1]. We conducted a similar method with the authors. Przyklenk et al. first reported that 4 cycles of 5-minute left circumflex coronary artery occlusion and 5-minute reperfusion reduced infarct size following 1-hour sustained left coronary artery occlusion and 4.5-hour reperfusion in anaesthetized dogs [26].

Further animal studies demonstrated the effect of transient ischaemia of different organ systems on the reduction of ischaemic damage in the target organ, which is known as 'remote preconditioning' [8,11,13]. There are several studies on the explanatory mechanisms of RP between various organs. In a study conducted by Gho et al., the RP created between the intestines and the kidney was abolished by the ganglion blocker hexamethonium [7]. The RP produced between the myocardium and the renal tissue involves adenosine receptors and ATP-sensitive potassium channels [25]. Patel et al. demonstrated the effect of RP by reduction in the infarct size during myocardial ischaemia between the intestinal tissue and the myocardium with the opioid antagonist naloxone [24]. Similarly, various studies have also suggested the effect of opioids on RP [5]. Moreover, studies have included the role of nitric oxide on the long-term protective effects of RP [35,37].

Gurcun et al. demonstrated the effectiveness of direct IP and RP on the prevention of spinal cord ischaemic injury in an experimental model with rabbits by occlusion in the abdominal aorta and renal artery [10]. They evaluated neuron-specific enolase, malondialdehyde, and nitric oxide levels. Neurological, clinical, and histopathological analysis revealed that IP and RP increased the tolerance of the tissue to ischaemia; however, the superiority of one method over the other has not been clearly identified. The duration of RP produced by occlusion of the renal arteries was 5 minutes, which was repeated twice with a 5-minute reperfusion interval between each process in the study, and this may be the reason for low efficiency of the RP method [10].

In our study, RP was produced 8 hours before the spinal cord ischaemia in animals under analgesic sedation to test the long-term effect. Thus, the duration of laparotomy was kept shorter. The efficacy of remote IP was measured with neurological scoring and production of HSPs in our study. Murphy et al. investigated RP by producing ischaemia by application of a tourniquet to the hind limbs of animals in an animal study. They showed that preconditioning with glutamine protected against local and distant organ damage in the setting of tourniquet-induced IR injury [19]. In our study, ischaemia-reperfusion was produced with similar cycles. The transient ischaemia produced in the limbs increased the heat shock proteins in the evolving spinal cord ischaemia.

In conclusion, the study supports the hypothesis that remote preconditioning increases the target tissue resistance to ischaemia. In our investigations it has been demonstrated that remote ischaemic preconditioning created at the lower extremity 8 hours

before spinal cord ischaemia exerted a protective effect, probably by the expression of tissue heat shock proteins. However, spinal cord protection still remains a challenge in the current era and requires further investigation in order to develop more sophisticated protective strategies.

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