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Effect of Preconditioning and Hypothermia in Ischemia–Reperfusion Injury to the Endothelial Cells of Blood Vessels in *Oryctolagus cuniculus*

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Abstract

Introduction. Ischemia/reperfusion injury (I/RI) remain a problem in post–hypoxia period, leading to remote organ injury. Studies showed that ischemic preconditioning (IPC) and hypothermia (HI) let destructive effect of ischemia to be minimized. The aim of study was to find out the impacts of interventions such as IPC and HI on morphology and function of the endothelial distal to ligation (ischemia) and contralateral vessel (I/RI).

Method. An experimental study carried out by ligation the right common femoral artery of *Oryctolagus cuniculus* to induce ischemia. Endothelial cells distal to ligation and contralateral side was subjected to investigation. The effect of IPC and HI were investigated and compared to those in I/RI.

Results. Morphological study showed significant difference scores between endothelial damage in ipsilateral vessels in interventional subjects with control, and intervention with I/RI group ($p < 0.05$). Tissue MDA level increased in all interventional groups and were not significantly differed to control ($p > 0.05$).

Conclusion. Ischemia may lead to remote endothelial dysfunction; IPC and HI showed the efficacy to minimize the impact of reperfusion.

Keywords: *Ischemia/reperfusion injury, ischemia–reperfusion preconditioning, hypothermia during ischemia–reperfusion injury, hypoxia, endothelial cells, malondialdehyde*

Introduction

Ischemia/reperfusion injury (I/RI) remain challenging issue surgical care following shock patients This issue referred to serious problem encountered in post–hypoxic period leading to cellular and tissue injury at the distance (remote organ injury).^{1,2,3} The ischemia leads to ineffective cellular aerobic metabolism with negative impacts such as increased lactic acid, deranged xanthine metabolism that produce reactive oxygen species (ROS), activation and release of inflammatory mediators. As the circulation restored, these toxic compounds enter the systemic circulation lead to multiorgan injury; brain, lungs, kidneys, intestines, and liver.^{1,2,3} Injured endothelial cells due the ischemia play an important role as the basic in remote organ injury during reperfusion. These cells which have a relatively low tolerant to hypoxia (ischemic time of 4 hours) known to play an important role in various body responses to injurious matter and conditions especially during hypoxia.^{4,5,6} In addressing the issues, there were studies conducted to find out the solution of this entity, namely ischemia/reperfusion preconditioning (IPC) and hypothermia (HI). When tissues are exposed to repeated hypoxic stimulus before subjected to prolonged severe ischemic environment, mitochondrial respiratory dysfunction saves up to 50%, production of ROS up to 38%, and mitochondrial lipid peroxidase up to 36%. In relatively low temperature, cellular metabolic system including cellular response to ischemia is saving up.^{5,7,8,9}

Many studies showed beneficial effects both of IPC and HI to prevent organ damage due to hypoxia. However, those studies addressed an

organ distal to ischemia. As there's a lack of study focused on the effect of I/RI, IPC, and HI to endothelial changes, thus the study was run to investigate.

Method

An experimental study on *Oryctolagus cuniculus* (New Zealand White rabbits) was carried out. Twenty–four of 3–4 kg weight rabbits were enrolled to a study, which were certified and adapted for a week prior to the investigation. Subjects were divided into 4 groups, consist of control group (3 subjects) and three interventional groups (each consist of 7) i.e. ischemia–reperfusion injury (I/RI), ischemia–reperfusion preconditioning (IPC), and hypothermia (HI). All the procedures were carried out under sedation using ketamine IM 15–20 mg/kg and diazepam 0.5 mg/kg. The sedative effects were maintained using ketamine IM 10 mg/kg. In I/RI group, ligation of the right femoral artery preceded using 3–0 silk which was maintain for 4 hours. To monitor the ischemia, a pulse oximetry was used. After such a period, the ligation was released, and subjects were set free in the cage for 8 hours. In IPC group, a 2 minutes ligation and 3 minutes release protocol were proceeded for 2 cycles; and set free in the cage for 8 hours. In HI group, the right lower extremity wrapped with ice. The temperature was maintained between 31–33°C, then they were treated as the first two groups.

In next eight hours subjects were sacrificed and dissected to obtain the samples, i.e. samples of artery and vein distal to ligation (ischemic area) and contralateral side (remote organ). Samples were divided into two. The first section kept in tube with sterile saline and stored in

a refrigerator for biochemical analysis. The second section fixed with 10% formaldehyde buffer for morphological examination. Embedded samples in paraffin block were stained using hematoxylin

and eosin and subjected to investigation under light microscope. The extent of injury was measured using modified Moenadjat criteria.

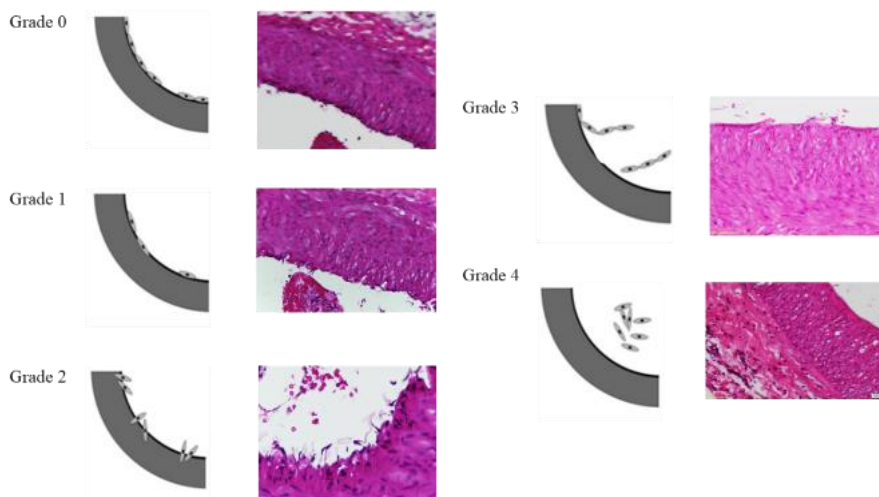


Figure 1. Modified Moenadjat's score of endothelial cells injury in blood vessels.

Table 1 Modified Moenadjat's criteria for endothelial lining injury

Score	Description
Score 0	No specific changes in blood vessels. All tissue structures (mucosal cells, basement membrane, tunica intima, tunica muscularis, and tunica adventitia) are intact and unchanged.
Score 1	Mucosal epithelial cells of blood vessels begin to be lifted, and remain adhered each other. All tissue structures (mucosal cells, basement membrane, tunica intima, tunica muscularis, and tunica adventitia) are still intact and unchanged.
Score 2	Desquamation of mucosal epithelial cells and mild necrosis in basement membrane. Hyalines in basement membrane remain adhered each other and were not fragmented. Tunica muscularis and tunica adventitia are remain intact and unchanged.
Score 3	Desquamation of mucosal epithelial cells and necrosis of basement membrane, tunica intima, and adventitia. Hyalines in basement membrane are fragmented. Erythrocyte leakage in tunica muscularis.
Score 4	Severe necrosis in blood vessels and the blood vessels structures are difficult to be identified. Injury to the tunica muscularis and adventitia.

For examination of oxidative stress, malondialdehyde (MDA) assay kits was used to measure tissue MDA. Each test proceeded in duplo.

Data was subjected to statistical analysis using SPSS ver. 20. Normality test was carried out and hypothetical test for the injury of femoral artery and vein in all groups carried out using Kruskal–Wallis, while as independent analysis for each interventional group preceded using Mann–Whitney test. Hypothetical test for the biochemical analysis of all group preceded using one–way ANOVA, while as hypothetical test comparing each interventional group preceded using unpaired T test. Significance is met if $p < 0.05$. The committee of ethic Faculty of Medicine, Universitas Indonesia approved the study.

Results

These 24 of 5 months old subjects were in average of 3.146 kg. weighted. Oxygen saturation (SaO₂) in the ipsilateral side prior to ligation was in average of 98.42% and of 68.67% following ligation (ischemia). After 4 hours the ligation released, mean SaO₂ was 98.48% (showing an improved saturation denoting the reperfusion). On endothelial morphological study, the score in I/RI group found was 4 in all members and differed to control with $p < 0.05$. the score distribution as shown in figure 2. However, only the ipsilateral artery

samples from the IPC and HI had significant injury compared with control ($p < 0.05$). Additionally, samples from the IPC and HI group had less injury compared with the I/RI group in both ipsilateral and contralateral samples ($p < 0.05$).

Data from the MDA assay from ipsilateral artery showed statistically significant difference between control and interventional group including IRI, IPC, and HI ($p < 0.05$), which was showing that all subjects in interventional groups underwent endothelial dysfunction compared with control. However, statistically there was no significant difference of tissue MDA levels between ipsilateral veins, contralateral arteries and veins from IPC and HI group with control. In contrast, there was significant difference in all subjects of I/RI group compared with IPC and HI group ($p < 0.05$) where both of IPC and HI were found less injured than I/RI group.

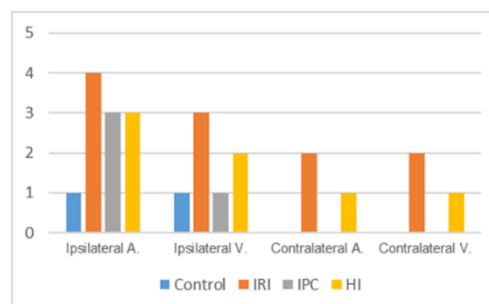


Figure 2. Diagram showing the score of endothelial lining morphological changes

Table 2 Relationship between morphological changes in endothelial lining of blood vessels in ipsilateral and contralateral common femoral arteries and veins in the control, I/RI, IPC, and HI group.

Histomorphology		Ipsilateral artery	Ipsilateral vein	Contralateral artery	Contralateral vein
Control	I/RI	0.012*	0.010*	0.006*	0.028*
	IPC	0.012*	0.121	0.199	0.789
	HI	0.014*	0.059	0.056	0.450
I/RI	IPC	0.010*	0.001*	0.002*	0.006*
	HI	0.040*	0.001*	0.002*	0.040*
IPC	HI	0.775	0.298	0.258	0.475

* $p < 0.05$ with Mann–Whitney test

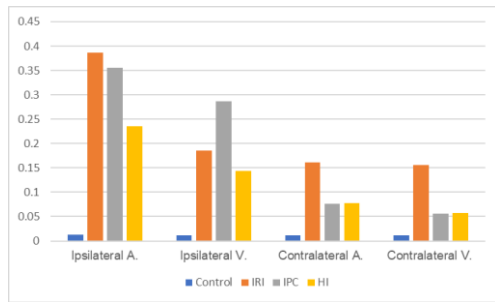


Figure 3. MDA levels in ipsilateral and contralateral blood vessels

Table 2 relationship between MDA levels changes in ipsilateral and contralateral common femoral arteries and veins in control, IRI, IPC, and HI group

MDA level		Ipsilateral artery	Ipsilateral vein	Contralateral artery	Contralateral vein
Control	IRI	0.025*	0.002*	0.001*	0.023*
	IP	0.003*	0.001*	0.046*	0.039*
	C				
IRI	HI	0.000*	0.008*	0.026*	0.004*
	IP	0.787	0.073	0.497	0.966
	C				
IPC	HI	0.133	0.254	0.359	0.983
	HI	0.146	0.17	0.970	0.952

* p < 0.05 with unpaired t-test

Discussion

Limb ischemia in the study induced by ligation of right common femoral artery; oxygen saturation on the monitor showing 68.67%, which was met the criteria of ischemia. Endothelial morphological changes were found both in ipsilateral (distal to ligation) and contralateral side. It was clear that endothelial morphological changes in artery distal to ligation referred to direct impact of ischemia, while as endothelial changes of vein were a kind of reperfusion injury. It is shown in ischemia–reperfusion injury (I/RI) that ligation of common femoral artery has triggered inflammatory cascade as a body response to ischemia affecting local and systemic organ (remote or at a distance to ligation site). In ischemic–preconditioning (IPC), cells were adapted to withstand a long period of ischemia, which prepared the cells during the ischemic condition and reperfusion. In hypothermia (HI), the cells were preserved from ischemia to suppress cell metabolism by enzymes, inflammatory mediators, and free radical inactivation during therapeutic hypothermia.^{9,10,11,12,13}

The main objective of this study is to find out any morphological change of endothelial lining and the evidence of oxidative stress represent by tissue MDA level as metabolic stress response to I/RI, but not the serum. Increased of cellular hypoxic metabolites during ischemia which is released to systemic circulation as the circulation restored (‘reperfusion’) is the main concept to explain the pathophysiology of I/RI. Systemic endothelial dysfunction triggers multisystem organ dysfunction through multiple pathways. Interventions such as preconditioning and hypothermia may be applied to minimize injury due to reperfusion.^{9,14,15}

The etiology of endothelial injury may be the direct effect of ischemia or due to reperfusion. In the study on endothelial morphology, the difference of scores in ipsilateral (distal) and contralateral to the ligated blood vessels is shown. The most injured one found in common femoral artery distal to the ligation which is a consequent of ischemia. This kind of injury was observed to be more severe in the I/RI, IPC in the second place, and HI group in the third place compared to control. This is suggesting that the process of apoptosis might be activated during ischemia in all conditions; any intervention prior to or following ischemia did not prevent the process of endothelial apoptosis.^{9,16,17,18,19}

After the reperfusion, injured structures of blood vessels were also denoted in remote organ, such as the contralateral blood vessel in I/RI group. However, protective effects were observed in the contralateral blood vessels in IPC and HI group. The morphological injury in the contralateral arteries and veins of interventional groups had no significant difference compared with control. The preconditioning may induce free radicals and NO production during repeated ischemia–reperfusion period and were activated to produce peroxynitrites which may reduce the number of interactions between neutrophil–endothelial cells and the increase endothelial cells adhesion. Hypothermia will give protective effect to I/RI by reducing oxygen consumption, decreasing cellular metabolism, chemokines and adhesion molecules inhibition, and decreasing mitochondrial gene expression which will inhibit apoptosis.^{19,20,21,22,26}

The increase in tissue MDA level found in a study is showing a response to metabolic stress due to ischemia or reperfusion injury. The increased number ROS production and circulating ROS during reperfusion will somehow lead to phospholipid peroxidation of remote cell’s membrane, resulted in increased level of tissue MDA in ischemic as well as in re–perfused area. In study of tissue MDA, a significant difference is found between all groups compared with control in ipsilateral and contralateral arteries and veins as the impact of ischemia–reperfusion injury. However, there was no significant difference in tissue MDA level between I/RI, IPC, and HI group. Thus, it was concluded that ischemia is a destructive process leading to local and systemic metabolic stress, and could not be prevented by external interventions.^{18,19,26,27,28}

In this study, it was observed that increased tissue MDA level in IPC and HI group is not accompanied by endothelial injury. This finding suggesting that both of IPC and HI may prevent further organ damage due to ischemia and reperfusion. Increased tissue MDA level during ischemia and reperfusion in these group representing a physiological biochemical response to hypoxia of which might not be prevented.^{18,19,29,30}

Clinical implication of IPC and HI have not been applied in humans frequently. To date, the principle of shock management is to restore cellular perfusion as quick as possible to prevent further failure of cellular functions; with an insight to shorten the ischemic period. Nevertheless, aggressive restoration is like induce reperfusion injury. Preconditioning for organ proximal to ischemic area had been done during a lot of procedures, such as during the ligation of radial artery to prevent cardiac myocyte apoptosis when preceding cardiac bypass and during temporary ligation of portal vein before hepatic segmentectomy in liver transplantation, and had been shown to prevent post–transplantation liver failure. Preconditioning let the cell adapted of which prepare the cells for a prolonged ischemia, and to prevent cell injury after reperfusion. Preconditioning can be performed before revascularization procedure in patient with acute limb ischemia by continuously ligating large peripheral arteries such as radial and femoral artery before the procedure to prevent reperfusion injury in ischemic and target organ. Side effects such as compartment syndrome, acute respiratory distress syndrome, and acute kidney injury may also be prevented. However, further studies are needed to provide more evidence.^{18,19,26,30}

Conclusion

Preconditioning and hypothermia showed an efficacy to provide protective effect against destruction due to ischemia–reperfusion injury in endothelial cells of remote organs. The study showed that protective effect of preconditioning is better than hypothermia.

However, metabolic stress as a physiological response of cells to ischemia and reperfusion were not intervened.

Disclosure

This study has no conflict of interest

Acknowledgment

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