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Effect of Ischemia-Reperfusion injury and Preconditioning on Lung Parenchyma after Acute Limb Ischemia

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Abstract

Introduction. Acute limb ischemia, a sudden decrease of perfusion to the extremities, can compromise the survival of the limbs. Medical intervention and surgery are often needed to return perfusion. However, reperfusion injury can trigger oxidative stress and inflammatory response, leading to local and remote tissue damage, such as the lungs, which increases morbidity and mortality. This research aims to study the effects of hypothermia and remote ischemic preconditioning (RIPC) on the lung parenchyma after being exposed to reperfusion after acute limb ischemia in rabbits.
Method. Eighteen New Zealand White rabbits were divided into three groups of 6. The femoral artery was ligated to induce ischemia. The hypothermia group was given cooling pads to maintain a temperature of 28°C for 4 hours, and the RIPC group was assigned RIPC before ligation for 5 minutes in three cycles. Both groups underwent reperfusion for eight hours. Evaluation of histologic characteristics was performed independently by a pathologist.
Results. The mean scores for the control group, hypothermia group, and RIPC group were 12.03 $\pm$ 1.43 (severe injury), 8.03 $\pm$ 3.03 (moderate injury), and 4.80 $\pm$ 2.61 (mild injury), respectively. In addition, there was a significant difference between lung parenchymal damage in the control group and hypothermia group ($p = 0.015$) and between the control group and RIPC group ($p = 0.000$).
Conclusion. Both hypothermia and RIPC have a protective effect on lung parenchyma exposed to remote reperfusion injury after lower limb ischemia, where RIPC protects the lungs to a higher degree.

Keywords: *hypothermia, lungs, rabbit, remote ischemic preconditioning, reperfusion injury*

Introduction

Peripheral arterial disease (PAD) is a pathological condition that presents a public health problem. It has a broad spectrum of symptoms, from claudication to necrosis that leads to amputation. According to the Trans-Atlantic Inter-Society Consensus (TASC) in 2007, the prevalence of PAD is 10% in ages 55-60 years and increases as much as 5% for ages above 60.[1] Acute limb ischemia (ALI), an acute subset of PAD, defined as the sudden decrease of perfusion to the extremities that can compromise the survival of the limbs. The exact incidence of ALI remains undefined; it is estimated at 14 per 100,000 of the population and 10-16% of the vascular workload.[2] ALI is caused by several conditions such as arterial trauma or atherosclerotic thrombosis/emboli. Medical intervention and surgery were urgently needed to return perfusion. However, reperfusion injury after acute limb ischemia may lead to oxidative stress and trigger the inflammatory response leading to tissue damage, both local and in remote organs. A distant organ such as the lungs parenchyma is the target. Followed by increased morbidity and mortality due to a lower FiO_2/PaO_2 ratio and impaired oxygenation.[3]
Protecting the vascular endothelial cells is an option to prevent reperfusion injury. Some methods include remote ischemic preconditioning (RIPC) and induced hypothermia. RIPC produces resistance to reperfusion injury by repeating short episodes of ischemia to protect the tissue against subsequent ischemic insults. Studies have shown that RIPC can protect the brain, liver, and kidneys from further injury.[4–7] Hypothermia can also modify the apoptotic pathway by inhibiting caspase activation, preserving mitochondrial, and inhibiting the release of neurotransmitters. A study by Frink et al. showed that hypothermia could reduce tissue damage, even though apoptosis continued for three days. The modulation of the apoptosis cascade could be a therapeutic target in the early management of trauma to prevent complications.[8]
Thus, this study aims to challenge the hypothesis that hypothermia and RIPC may reduce parenchymal damage in a rabbit model of ALI and compare the effectiveness of both methods to find the best method to protect the lung from reperfusion injury.

Method

This quasi-experimental cohort study proceeded to find out the association between RIPC and hypothermia regarding the severity of parenchymal lung injury as a complication of reperfusion injury from ALI, assessed histopathologically. Eighteen New Zealand White rabbits were divided into groups comprised of six, i.e., control, RIPC, and hypothermia group. The sample size was determined according to Federer's formula. The 5–6 months aged male rabbits of 2.0 and 2.5 kg weight were obtained and certified by the Livestock Research Institute of Indonesia were the subjects in the study. The rabbits were adapted for a week and fed with water *ad libitum*. Rabbits were cared for following the Helsinki Accords for Humane Treatment of animals during experimentation and the three Rs principle (replacement, reduction, and refinement).
The rabbits were anesthetized intramuscularly with 15-20 mg/kg ketamine and 0.5 mg/kg diazepam. After an hour, anesthesia was maintained with 10 mg/kg/h of intramuscular ketamine. The inguinal region of the right leg was shaved and disinfected with povidone-iodine

and 70% alcohol. Then, lidocaine for a local anesthetic, a longitudinal incision of the skin proceeded from the inguinal ligament to the proximal part of the knee. The femoral artery and branches were freed from the adjacent tissue and ligated. The incision was then approximated with sutures and covered with gauze. Successful ligation was confirmed by using a pulse oximeter. In the next four hours, ligation was released for the control group and was reperfused for eight hours. In the hypothermia group, cooling pads were placed covering the limb after ligation. To maintain a temperature at 28°C, water was continuously circulated to the cooling pads using a water pump. After inducing hypothermia for 4 hours, the leg was reperfused for 8 hours. Then, the ischemia was generated in the limb for 5 minutes in 3 cycles in the RIPC group, continued by ligating the femoral artery for 4 hours and reperfusion for 8 hours. Eight hours after reperfusion, the rabbits were euthanized using 100 mg/kg of sodium pentobarbital.

The left hilum was ligated, and the lung was taken from all groups through a lateral thoracotomy. A specimen of the lung parenchyma was collected and stored in 10% formalin for fixation. Following fixation, the lung tissue was embedded in a paraffin block, sectioned, and stained with hematoxylin and eosin. The hemorrhage, alveolar edema, vascular congestion, PMN leukocyte infiltration, and intracellular hyaline membranes were evaluated under a light microscope with 100 times magnification and scored in 5 randomly selected microscopic fields for each slide (Table 1). Parenchymal changes were assessed histologically for the five variables above. Each variable was scored on a 0 to 3, as in Table 1. A mean score of 0-3 indicates normal to minimal injury, 4-7 indicates a mild injury, 8-11 indicates a moderate injury and 12-15 indicates a severe injury. Evaluation of histologic characteristics proceeded independently by a pathologist who was blinded to grouping.

Table 1. Scoring system of parenchymal lung damage

Score	Alveolar wall	Haemorrhage	Vessel wall	PMN leukocytes	Hyaline membrane
0	No thickening	No haemorrhage	Congestion in less than 25% of the field	No leukocyte infiltration	No hyaline membrane
1	1-2x the normal thickness	Haemorrhages in 26-50% of the field	Congestion in 26-50% of the field	1-5 leukocyte infiltrations	1-10 hyaline membranes
2	3-4x the normal thickness	Haemorrhages in 51-75% of the field	Congestion in 51-75% of the field	6-10 leukocyte infiltrations	11-20 hyaline membranes
3	More than 4x the normal thickness	Haemorrhages in 76-100% of the field	Congestion in 76-100% of the field	More than ten leukocyte infiltrations	More than 20 hyaline membranes

Statistical analysis was performed using Statistical Procedures for Social Sciences (SPSS) version 16.0. The findings were expressed in mean and standard deviation. Next, the normality and homogenous data distribution test proceeded using the Shapiro-Wilk test, where a p-value of more than 0.05 indicates normal data distribution. Finally, a T-test was performed for normally distributed data to compare the mean score between the three groups. A p-value of less than 0.05 was considered significant.

The ethics committee of the Faculty of Medicine, Universitas Indonesia, approved this study (protocol number: 842/UN2.F1/ETIK/2014).

Results

The score of the control group ranged from 10 to 14, with a mean of 12.03 $\pm$ 1.43, indicating a severe injury. The microscopic findings of the control group are shown in Figure 1. The score of the hypothermia group ranged from 5 to 12, with a mean of 8.03 $\pm$ 3.03, indicating a moderate injury. Representative microscopic findings of the hypothermia group are shown in Figure 2.

Finally, the score of the RIPC group ranged from 1 to 8, with a mean of 4.80 $\pm$ 2.61, indicating a mild injury. Representative microscopic findings of the RIPC group are shown in Figure 3.

A Shapiro-Wilk test showed a normal data distribution for the control group ($p = 0.856$), hypothermia group ($p = 0.821$), and RIPC group ($p = 0.922$). A T-test showed a significant difference in histopathological scoring of parenchymal damage score between the control and hypothermia groups, with a p-value of 0.015 (Table 2) and between the control group and RIPC group, with a p-value of 0.000 (Table 3).

Table 2. Comparison between the score of the control group and hypothermia group

	Lung parenchymal damage			
	n	Mean	SD	p-value
Control group	6	12.03	1.43	0,015
Hypothermia group	6	8.03	3.03	

Table 3. Comparison between the score of the control group and RIPC group

	Lung parenchymal damage			
	n	Mean	SD	p-value
Control group	6	12.03	1.43	0.000
RIPC group	6	4.80	2.61	

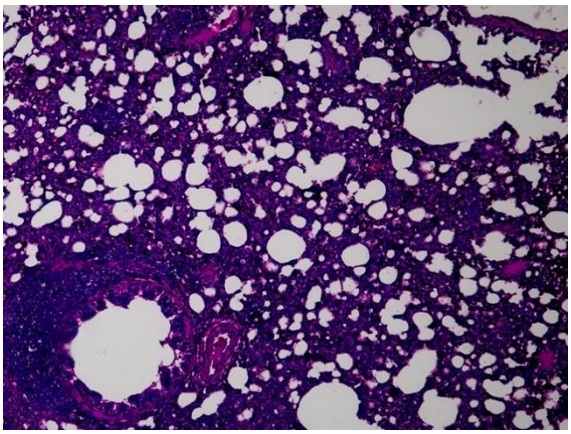

Figure 1. Representative of lung parenchyma in the control group. Microscopic findings include alveolar wall thickening by four times the normal thickness (3 points), hemorrhage involving 76% of the field (3 points), congestive affecting 76% (3 points), more than 10 PMN leukocytes found per field (3 points), and the presence of 10-20 hyaline membranes (2 points), which adds up to 14.

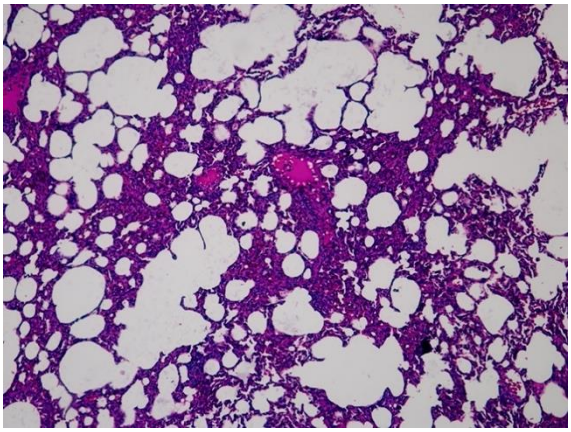

Figure 2. Representative of lung parenchyma in the hypothermia group. Microscopic findings include alveolar wall thickening by 3-4 times the normal thickness (2 points), hemorrhage involving 51-76% of the field (2 points), congestive blood vessels involving 51-76% of the field (2 points), more than 10 PMN leukocytes found per field (3 points), and the presence of 1-10 hyaline membranes (1 point), which adds up to 10.

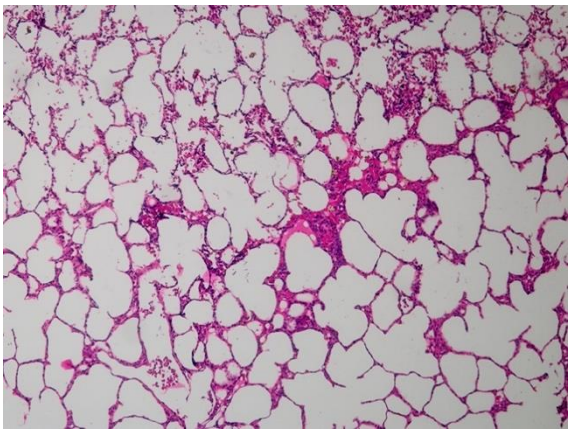

Figure 3. Representative of lung parenchyma in the RIPC group. Microscopic findings include alveolar wall thickening by 1-2 times the normal thickness (1 point), hemorrhage involving 26-50% of the field (1 point), congestive blood vessels involving 26-50% of the field (1 point), 1-5 PMN leukocytes found per field (1 point), and the presence of 1-10 hyaline membranes (1 point), which adds up to 5.

Discussion

The histopathological assessment showed severe injuries to the lung parenchyma. Such a condition shows a cause-effect relation between reperfusion injury after limb ischemia and lung parenchymal damage. Our finding is in accordance with a study by Mansour et al., showing reperfusion injury decreases the lungs' mitochondrial maximal oxidative capacity.[10] Due to their microvascular complexity, the lungs are vulnerable to remote organ injuries. A study by Klausner et al. shows reperfusion after lower torso ischemia can lead to increased pulmonary microvascular permeability due to sequestration of PMN leukocytes.[9,11]

Our findings showed that the RIPC group had a mean score of 4.800 (mild injury), much lower than the control group. This difference was statistically significant, with a p-value of 0.000. Ischemic preconditioning (IPC) is defined as short periods of sublethal ischemia on the target organ before a subsequent reperfusion injury aimed to increase the tissues' endurance and tolerance toward reperfusion injury, where it stimulates the release of nitric oxide and adenosine. Both play a major role in regulating endothelial function and maintaining blood flow, which may reduce organ damage. IPC was first introduced by Murry et al. in 1986, where multiple transient ischemic episodes show a cardioprotective effect on dogs with myocardial infarction.[12] However, this effect applies locally, and remote organs are also affected. Thus, the development of IPC is RIPC, in which transient episodes of ischemia were induced in non-target organs, followed by reperfusion injury of the target organ. Although RIPC is primarily used on the myocardium at first, RIPC provides similar protection to other organs, such as the brain and kidneys.[4,5] Thus, RIPC becomes preferable to IPC as IPC may also cause trauma to major blood vessels and stress to the target organ.[7]

Hypothermia controls the inflammatory response following reperfusion injury by reducing the circulation of inflammatory cytokines, as one mechanism of reperfusion injury includes the inflammatory cascade.[13] Hypothermia is also a protective agent to minimize injury and preserve organs by reducing metabolic rate and oxygen consumption. It also preserves phosphate storage and decreases excitatory neurotransmitter release by the central nervous system.[14] Our study found that the hypothermia group also had a lower mean score than the control group for lung parenchymal damage, which was 8.03 ± 3.03 (moderate injury). Statistical analysis showed a significant difference between the control and hypothermia groups, with a p-value of 0.015. These results are consistent with a study by Vinardi et al., where moderate hypothermia of the intestines after reperfusion protects the lungs by reducing infiltration of neutrophils.[15] Weng et al. also found that regional hypothermia on limbs that have been applied tourniquets relieves injuries in the lungs, where the histological assessment of the lungs showed less hemorrhage.[16]

Limitations of this study include our inability to assess the lung parenchyma of rabbits before giving any intervention for comparison. We also did not compare multiple temperatures to evaluate the effects of hypothermia and did not vary the number of cycles used to assess the effects of RIPC. Further studies are still needed to determine the exact temperature and duration for the use of hypothermia in preventing injuries of remote organs due to reperfusion injury and whether the use of both hypothermia and RIPC simultaneously is more effective in reducing remote organ injuries. We also suggest further research on the effects of reperfusion on other remote organs and the correlation between ischemic time and histopathological lung damage.

Conclusions

Both induced hypothermia and RIPC effectively preventing histopathological lung injury following reperfusion after ALI, whereas RIPC prevents lung injury superiorly. Therefore, we recommend using RIPC to mitigate lung injury from reperfusion after ALI.

Disclosure

Authors declare no conflict of interest

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