Hepatic Reperfusion Injury following Remote Ischemia: 
Experimental Study on Oryctolagus cuniculus

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Abstract

Introduction. Ischemia/reperfusion (I/RI) injury following limb ischemia is realized to be targeted for remote organs injury which is found in vary, commence with mild injury to a severe one. Nevertheless, liver is an organ susceptible to such an injury. There were studies on IRI, where ischemia in those studies were induced by direct ligation of hepatic vessels. However, study of remote ischemia was infrequently found. Thus, we run a study aimed to find out hepatic injury following ischemia induced by ligation of an artery with a significant anatomical distance. 

Method. An experimental study was conducted on New Zealand white rabbit. Ischemia was induced by ligation of right common femoral artery under anesthesia. Ligation was maintained for four hours period. Afterwards, ligation was released, and rabbit was set free in the cage for eight hours period. Laparotomy was carried out to take liver specimens of three different area, namely central, midzonal, and peripheral. These specimens were subjected to study histopathology and biochemical examination for malondialdehyde as well as HIF–1α. In addition, liver function test was carried out for serum bilirubin and transaminases. 

Results. The study on histomorphology showed hepatic injury of central, midzonal and peripheral of the ischemic/reperfusion injury group, which was mostly sinusoidal dilatation. There was a significant statistical different of the three hepatic–zones (central, p = 0.028, midzonal, p = 0.012, and peripheral, p = 0.030). MDA levels showed a significant increase in the ischemic/reperfusion group (p = 0.012, sig α <0.05). Tissue HIF–1α level increased denoted tissue hypoxia in the treatment group. Liver function test showed no abnormality. 

Conclusion. Oxidative stress and sinusoidal changes were found in three zones, i.e. central, midzonal and peripheral following ischemic of a significant anatomical distance.

Keywords: hepatic ischemia/reperfusion injury, sinusoidal dilatation, oxidative stress, HIF–1α

Introduction

Ischemia/reperfusion injury (I/RI) referred to a serious problem found following hypoxia. A condition lead to cells and/or tissue damage locally and those located far apart to the ischemic zone (namely, remote organ). In hypoxic cell, a normal metabolic activity turns to anaerobic due to mitochondrial distress let there is no high energy phosphate (adenosine triphosphate, ATP) produced in tricyclic acid cycles, otherwise those for cells survival.1 With distressed mitochondria, production of injurious reactive oxygen species (ROS) is about to increased.2 Thus, should arterial flow be restored, these toxic material released even to systemic circulation lead to cellular damage locally and those of a significant anatomical distance. This referred to the pathogenesis of ischemia/reperfusion injury.3 Cellular antioxidant defense of which are synthesized internally in normal cells and plays an important role against ROS were found attenuated in hypoxic cells.3 Imbalance of ROS and internal or primary antioxidants known as oxidative stress.4 Should a condition continue, cells injury encountered let cells structures damage. Cells membrane as well as cytoplasmic changes were just aggravating the cells injury let ionic compartmentalization. Cellular homeostasis is no longer maintained, apoptosis and/or necrosis is a logic consequence.1 Nowadays, there are known biomarkers of oxidative stress, one is malondialdehyde (MDA) of which is a surrogate marker in detection.5–6

Nevertheless, cellular injury of those tissue at a significant anatomical distance is an issue found following ischemia. This injury might be found in vary, commence with a mild injury to a severe one. Tissue/organ involved of most are alveoli, renal tubules, intestinal mucosa, and liver. Liver is an organ susceptible to IRI.7 The possible cause is somewhat due to delivery system of systemic circulation through portal vein. In contrast, the nature of anaerobic glycogen metabolism that take place in liver provide a unique protective impact of ischemia. However, liver dysfunction as the impact of reperfusion is common, for instance, those following systemic hypoxemia as found in hemorrhagic shock and in septic shock. Activated xanthine oxidase in hypoxic cells and released to circulation as the perfusion restored will activate Kupffer cells and provokes the release of inflammatory mediators. This may explain the possible pathogenesis of liver damage to a remote ischemia.7–9

So far, study of liver damage following ischemia/reperfusion injury of distant ischemia is less frequent one, otherwise studies on liver transplant, which is a kind of direct I/RI is quite often carried out. Thus, a study focused on the impact of reperfusion injury on liver following distant ischemia was carried out using rabbits as a model.
Method

An experimental study focused on histopathological changes as well as oxidative stress were carried out using New Zealand White (NZW) rabbits (Oryctolagus cuniculus) of five months old and of 3–3.5 kg weight. There were 10 rabbits enrolled in the study, seven of treatment and 3 of control. These rabbits were scrutinized and adapted for a week prior to investigation. Ischemia was induced by ligation of right common femoral artery under 15–20 mg/kg body weight ketamine given intramuscular in addition to diazepam 0.5 mg/kg body weight. Ligation carried out using 2.0 silk through an incision in the right inguinal and ischemia was confirmed by arterial oxygen saturation (SaO2). Ligation was maintained for four hours period, and after for hours the ligation was released. Rabbits were set free in the cage for next eight hours period and afterwards these rabbits were sacrificed. Through laparotomy, specimens were taken from central–, midzonal–, and peripheral zones. Each od specimens were divided into two parts, one for study on histomorphology and the other for oxidative stress.

Study on histomorphology were carried out on hematoxylin and eosin stained samples under light microscope with objective magnification of 40 and 100 times. Histomorphologic changes were scored and classified into categories in accordance with classification of Knudsen et al,10 whereas grade 0 if no injury found, grade 1 should there were dilatation of hepatic sinusoidal vessels, grade 2 as there were focal necrosis on hepatic parenchyma, grade 3 if there were necrotic area in hepatic parenchyma of >10 high power fields, and grade 4 if there were injuries and necrosis on almost all area of hepatic parenchyma.

Whilst, study on oxidative stress was carried out using tissue malondialdehyde (MDA) as a surrogate marker, instead of serum. Tissue level MDA were investigated using polymerase chain reaction (PCR) in accordance to Livak method.11 In addition, liver function tests i.e. serum bilirubin, aspartate aminotransferase (AST, or serum glutamic oxaloacetic transaminase, SGOT) and alanine aminotransferase (ALT, or serum glutamic pyruvate transaminase) were carried out.

These data were subjected to statistical analysis using SPSS version 20. Normality test proceeded for descriptive analysis by treatment group. Grade of hepatic damage and tissue MDA level were presented in numerical data. Hypothetical test carried out using Kruskal–Wallis test, and post hoc test proceeded using Mann–Whitney test. Tissue MDA level were analyzed using paired t-test. Those were significance if p value of <0.05.

The use of experimental rabbit in this study proceeded in accordance with the principles of research principles using experimental animals, namely 3Rs: i.e. replacement, reduction and refinement. This research has been approved by the Committee of ethics, Faculty of Medicine, Universitas Indonesia No. 495b/H2.F1/ETIK/2014.

Results

Ten NZW rabbits with median weight of 3.225 kg (2.5–3.5 kg) were enrolled in the study. Following ligation, mean SaO2 of 68.67% ± 0.02 confirmed the ischemia distal to ligation. Following release of ligation mean SaO2 98.48% ± 0.04 denoted a restored perfusion. Study on histomorphology showed differences between I/RI group with control. The most grade of hepatic damage in such a group was of grade 3 of midzonal area, whereas in central– and peripheral zone were of grade 2. Statistical analysis with non-parametric test of Kruskal–Wallis test showed the difference between central–, midzonal–, and peripheral zones to control were significant with p value of 0.028, 0.012, and 0.030, respectively.

Tissue MDA level increased in I/RI group (0.032 nmol/mg ± 0.007), showed a significant difference to control (0.005 nmol/mg ± 0.002) with p value of 0.012 (sig α <0.05). There was increased tissue HIF–1α of 0.808 ng/mL (control 0.143 ng/mL), representing hypoxic hepatic tissue. In this study, serum AST, ALT, bilirubin in ischemia/reperfusion showed no significant difference to control (independent t-test) with p value of 0.453, 0.259, and 0.690, respectively.

Discussion

Through this experimental study we learned a lot, a ligation of artery leads to ischemia which is confirmed by arterial oxygen saturation 68.67%, as the criteria <70% is fulfilled,12 and afterwards, arterial oxygen saturation of 98% confirmed that perfusion was restored. Through study on histomorphology, we found that the most pathologic findings were sinusoidal dilatation and followed by midzonal necrosis. Such changes described as the impact of reperfusion of a distant ischemia, in this case limb ischemia. Dilated sinusoid found with distribution of mononuclear cells denoting the first phase of hepatic injury (1–4 hours period) which in turn will

Figure 1. Sinusoidal dilatation of grade 1 in hematoxylin – eosin stained specimen under 40 times objective lens magnification.

Figure 2 Extensive necrotic area (hepatic injury of grade 3) in hepatic specimen (hematoxylin–eosin stained) under microscope with 40 times objective lens magnification (arrows).

| Table 1. Histomorphologic changes relationship between three hepatic zones of ischemia/reperfusion group |
|-----------------------------------------------|---|---|
| Central zone | Control | I/RI | *p* |
| Midzonal zone | 0 (0–1) | 2 (1–2) | 0.028 |
| Peripheral zone | 0 | 1 (0–2) | 0.030 |

activate both hepatic endothelial– and Kupffer cells to release ROS and proteases that injurious to endothelial– and parenchymal cells. Dilatation sinusoid reflects hypo-perfused sinusoid (in other literature described as sinusoidal obstruction). Degree of sinusoidal hypoperfusion depends on period of ischemia, paralleled to hepatic cells changes in hypoxia, hepatocellular integrity and function of hepatic parenchyma. MacPhee showed their findings that velocity of sinusoids was heterogeneous in pathologic conditions.16 Vollmar et al. showed a greater heterogeneous change in perfusion changes of sinusoid in rats with ischemia/reperfusion. Our findings in accordance with such theories, particularly in hepatic ischemia/reperfusion injury following limb ischemia, in the absence of systemic shock, and paralleled to those findings of Brock et al. Thus, hepatocyte apoptosis or necrosis following hypoperfusion is a logic consequence. Necrosis, particularly found in midzonal area in IRI group. However, in other two zones, most samples in the treatment group shows no necrotic area. This suggesting the possible apoptosis that should be proven furthers. The role of cytokines released following ischemia might be responsible to this hepatic injury through activation of Kupffer cells as shown in study on mice whereas IL6 in systemic was much higher than the portal system. IL6 is somewhat activates Kupffer cells with furthers lead to hepatic injury. This might be describing the study’s finding though a study was not focused on interleukin.

Tissue malondialdehyde (MDA) as the product of lipid peroxidation representing increase of superoxide radical anion (O2−) leading to cellular oxidative stress. In the study we noted increased of tissue MDA level were found in ischemia/reperfusion group with statistically significant difference to control. This finding is supported by increased tissue HIF–1α level representing cellular hypoxia, in this case, hepatic cells; though this HIF–1α level were not measured in all samples due to cost constraints. Hypoxia of hepatic cells occurred secondary to oxidative stress. Furthers, HIF–1α may induce apoptosis in hypoxic hepatic cells with ischemia/reperfusion. Cursio et al, showed that increased HIF–1α level were correlated to hepatic apoptosis in mice with ischemia/reperfusion, and may be found in other hepatic pathologies. Serum bilirubin, AST, ALT, and albumin was found normal, in other words there were no defect in liver function at a time. Studies shown increased hepatic transaminase enzymes (AST and ALT) were related to muscular disorders following limb ischemia, but not a reflect of liver injury. Further, Yamaguchi et al (1996) showed that bilirubin increased three times in ischemia/reperfusion, may acts as an antioxidant against ROS.

The limitation of the study was factors that may leading to a bias were not fully controlled, as the study enrolling a small sample size and cost constrains.

Conclusion
We concluded that oxidative stress and sinusoidal changes were found in three zones, i.e. central, midzonal and peripheral following ischemic of a significant anatomical distance.

Conflict of interest
Author disclose there was no conflict of interest.

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