Effect of kidney ischemia/reperfusion injury on proliferation, apoptosis, and cellular senescence in acute kidney injury in mice

Finsa Tisna Sari1,2,3, Nur Arfian1, Dwi Cahyani Ratna Sari1

1Department of Anatomy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia; 2Postgraduate Programs of Biomedical Science, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia; 3Faculty of Dentistry Universitas Gadjah Mada, Yogyakarta, Indonesia

ABSTRACT
Introduction: Kidney ischemia/reperfusion injury (IRI) is the leading cause of acute kidney injury (AKI). Kidney IRI demonstrated apoptosis of epithelial cells in acute phase followed by proliferation of interstitial cells in chronic episode, and cellular senescence may contribute to development of AKI, however, its occurrence within acute or chronic episodes is still not completely understood.

Methods: Kidney IRI was performed with bilateral pediculus clamping in Swiss Background mice (3 months, 30-40g). Mice were euthanised on day one (IR1, n=6), day eight (IR8, n=6), and day twelve (IR12, n=6) to examine acute and chronic episodes. Sham operation procedure was performed in the control. Tubular injury was assessed based on periodic acid-Schiff (PAS) staining. Reverse transcriptase PCR (RT-PCR) was done to quantify mRNA expression of Bax, Bcl-2, and p16. Immunohistostaining (IHC) was performed to examine localisation of apoptosis (p53) and proliferation (Bcl-2).

Results: RT-PCR analysis showed upregulation of mRNA expression of Bcl-2, Bax, and p16 (p<0.05). The data showed that ischemia/reperfusion induces upregulation of Bax (p=0.20), Bcl-2 (p=0.45), p16 (p=0.18). Apoptosis and proliferation occurred in the epithelial cells in acute episodes, but occurred in interstitial areas in chronic episodes.

Conclusions: Ischemia/reperfusion injury induces upregulation proliferation, apoptosis, and cellular senescence in acute kidney injury. Apoptosis reached its peak on day 1, proliferation on day 8, and cellular senescence on day 12.

KEY WORDS:
kidney ischemia/reperfusion injury, proliferation, apoptosis, cellular senescence

INTRODUCTION
Kidney ischemia/reperfusion injury (IRI) is a sudden restriction of blood flow to the kidneys followed by restoration of blood flow and reoxygenation. IRI induced acute kidney injury (AKI) is a clinical syndrome with rapid kidney dysfunction and contributes to a high morbidity and mortality rate in a wide range of injuries. Ischemia primarily affects the structure and function of tubular epithelial cells and also causes interstitial inflammation and microvasculopathy. Both inflammation and microvasculopathy are particularly important in terms of post-ischemic kidney repair. These alterations can delay restoration of renal function which potentially worsens the prognosis of patients with ischemic AKI.

Ischemic AKI is categorised into four phases, i.e., initiation, extension, maintenance, and recovery. The initiation phase occurs when cellular injury results from reduction in renal blood flow, particularly the renal tubular epithelial cells, and there is continued decline in GFR. Renal tubular epithelial cell injury is a key feature of the initiation phase. Apoptosis of renal tubular epithelial increase after severe injury. Vascular and inflammatory processes contribute to further cell injury and a further decline in GFR occurs in the extension phase. During the maintenance phase, GFR reaches a stable level as cellular repair processes are initiated in order to maintain and re-establish organ integrity. Cells undergo repair, migration, apoptosis and proliferation in order to maintain cellular and tubule integrity. The recovery phase is marked by a return of normal cell and organ function that results in an improvement in GFR. Renal function can be directly related to the cycle of cell injury and recovery.

The nature of the recovery response is mediated by the degree to which sublethal cells can restore normal function and promote regeneration. The successful recovery from AKI depends on the degree of repair processes. Furthermore, there is maladaptive repair occurring when IRI can lead to chronic injury by myofibroblast formation in tubulointerstitial area. Recent data suggest that CKD is the chronic consequence of ischemia injury and considered to be related to glomerulointerstitial fibrosis and persistent kidney dysfunction. In maladaptive IRI repair, proliferating cells can initiate an additional response by adopting a state of permanent cell-cycle arrest that is termed cellular senescence. Senescent cells can remain metabolically active and adopt a senescence-associated secretory phenotype, which is associated with the release of connective tissue growth factor and TGF-β, both contributors to chronic inflammation, collagen deposition and vascular rarefaction. Senescent cells are also documented to produce IL-8, which may potentiate cells entering G2/M arrest.
Although the pathophysiology of IRI is not completely understood, several important mechanisms resulting in kidney failure have been elucidated. Kidney IRI promotes apoptosis of epithelial cells in the acute phase followed by proliferation of interstitial cells in the chronic episode, and cellular senescence may contribute to development of AKI, not completely understood. Better understanding of the cellular pathophysiological mechanisms underlying kidney injury will hopefully result in the design of more targeted therapies to prevent and provide better treatment for kidney injury.

MATERIALS AND METHODS

Animal subjects

This study used 24 male Swiss-Webster mice aged 3-4 months old with 30-40g body weights (BW). Mice were obtained from the Animal Model Care Unit of, the Integrated Research Testing Laboratory, Universitas Gadjah Mada, Yogyakarta and were divided into three groups, with six mice in each group, i.e., sham operation (SO) as control group, kidney ischemia/reperfusion day one (I/R1) as AKI model group, kidney ischemia/reperfusion after eight days (I/R8) and kidney ischemia/reperfusion after twelve days (I/R12). Mice were maintained based on standard laboratory conditions and provided diet and water ad libitum before use. Protocol of this study was approved by the Ethics Committee Integrated Research Testing Laboratory (LPPT), Universitas Gadjah Mada, number 0016/04/LPPT/XII/2018.

Kidney ischemia/reperfusion injury model

The mice were administrated general anaesthesia with intraperitoneal injection of ketamine cocktail (0.1mg/g body weight [BW]). Kidney IRI model was performed by clamping both of the renal pedicles, using non-traumatic vascular clamp (Hammacher®) for 30 minutes. Both clamps were then released and followed by reperfusion. The incision site was then closed using silk surgical thread 3/0 (One Med®).

Kidney harvesting

Subjects in I/R1, I/R8, I/R12, SO groups were then euthanised. Prior to opening the abdomen and thorax, mice were anaesthetised with intraperitoneal injection of pentobarbital sodium (60mg/kg BW, Somnopentyl®). Perfusion of the organ was done from the left ventricle, using 0.9% NaCl solution. Both perfused kidneys of mice were harvested, one kidney was kept in RNA later® for RNA extraction and the others fixated into 4% PFA in PBS for 24 hours, then paraffin was used for the embedding tissue process.

Histology and immunohistostaining

The kidney was embedded in paraffin block with 4µm sections. Paraffin sections were deparaffinised and rehydrated using serial xylene and alcohol. Specimens were then stained with Periodic Acid-Schiff (PAS) to determine tubular injury. For immunohistochemical staining, after deparaffinised and rehydrated, followed antigen retrieval, next blocking peroxidase using H₂O₂ 3% in PBS solution, and then blocking non-specific antigen using background sniper. The slides were incubated with p16 (forw ard TGAAGCTGAA CTGAC, reverse TG ACCATC T), Bcl-2 (forw ard TGA A CCGGG CATCT, reverse GCA TCCAGC CTGTTT) and GAPDH forward GGC AGTGTC AAGCTG AATG, reverse TCT CGTC TGG GAA TGGTA were used as reference. The gene expressions were quantified using densitometry analysis (ImageJ software) and GAPDH gene was used to normalize the gene expressions (housekeeping gene).

RESULTS

Histological changes in kidney IRI

Kidney ischemia/reperfusion injury was conducted to study renal cell response to injury in mice. As designed, this model was characterised by acute epithelial tubular damage. Tubular injury in kidney IRI was shown in I/R1, I/R8, and I/R12 groups detected by impairment cell polarity, cytoskeleton integrity, and loss of brush border. Additionally, intraluminal cast and lumen dilatation were found compared to the SO group (Figure 1).

p16 mRNA upregulation in chronic episode of kidney I/R injury

To investigate kidney ischemia/reperfusion injury that could induce apoptosis, Bax expression was examined by reverse transcriptase PCR and immunostaining was performed using p53 antibody. As shown in Figure 2 (A, D), expression of Bax was higher in I/R groups compared to SO (p=0.020). I/R1 had the highest mean±standard deviation (SD) (1.99±0.41). Immunopositive p53 antibody showed in epithelial cells in I/R1 and I/R8 groups, however in I/R12 group it showed in interstitial area. In early episode of kidney ischemia/reperfusion injury, apoptosis was incorporated into the DNA damage and caused cellular death in epithelial tubular cells. The cellular response to repair the damage that caused cellular death is proliferation. The aim of proliferation is to maintain normal kidney function. To determine renal ischemia/reperfusion injury could induced proliferation, Bcl-2 expression was examined by reverse transcriptase PCR and immunohistochemistry staining. As shown in Figure 2 (B, D), we found that Bcl-2 expression was significantly higher than normal control. Immunohistochemical staining showed that Bcl-2 positive in the epithelial tubular cells in I/R1 and I/R8 groups, but Bcl-2 positive in interstitial area in I/R12 group. Repair process can be adaptive to gain normal function without alteration in kidney structure. On the other hand, massive damage could lead to a maladaptive repair process indicated by an accumulation of senescent cells. Expression of cellular senescence marker, p16 was examined using reverse transcriptase PCR. As shown in Figure 2 (C), mRNA expression of p16 was higher in the I/R group, especially in the chronic episode (I/R12).
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DISCUSSION
Kidney ischemic/reperfusion injury causes complex interactions involving vascular and tubules. In the vascular area there is an increase in vasoconstrictors followed with decreased vasodilators, further damage to endothelial cell and smooth muscle cell structure. Furthermore, reperfusion injury increased leukocyte-endothelial adhesion, vascular obstruction, and inflammation. Damage to tubular cells occurs in the cytoskeleton, with loss of cell polarity, apoptosis, necrosis, cell desquamation, and tubular obstruction.¹

Kidney IRI effect on cell apoptosis
Kidney ischemia/reperfusion injury is characterised by impairment of cell polarity, cytoskeleton integrity, and loss of renal tubular brush border which can induce apoptosis and the necrotic process. Brush border and cell debris can cause intraluminal obstruction which forms an intraluminal cast in the distal tubules. These obstructions can promote the dilatation and atrophy of proximal tubules in AKI. Apoptosis becomes increasingly important over time after the initiation phase. Expression of pro-apoptotic members of the Bcl-2

![Fig. 1: Histological pictures of kidney injury after kidney IRI model based on PAS staining. Impairment cell polarity, cytoskeleton integrity, loss of brush border, intraluminal cast and lumen dilatation were shown in I/R1, I/R8, and I/R12 groups.](image1)

![Fig. 2: Showed upregulation of Bax, Bcl-2, and p16 mRNA expression in I/R groups compared SO (A-C). Representative pictures of Bcl-2 and p53 immunostaining showed positive in epithelial cell on I/R1 and I/R8 but showed in interstitial on I/R12 (D). *p<0.05 vs SO; # p<0.05 vs I/R1](image2)
family including Bax, Bak, and Bad, as well as caspases increased in ischemia/reperfusion injury. There is also increased expression of other apoptosis components from both intrinsic and extrinsic pathways. These pro-apoptotic factors are induced in response to DNA damage and production of ROS. In general, increases of expression occurred in proximal and distal tubules.

The highest Bax expression occurred in group I/R1, followed by groups I/R8 and I/R12. Increased proapoptotic protein expression are known in ischemic/reperfusion injury.

Proapoptotic proteins are induced in response to DNA damage, generation of ROS, and ceramide formation. High expression of Bax on day-1 indicates that injury enters the extension phase. The extension phase is a continuation of the initiation phase so that apoptosis predominantly occurs in this phase. On day 12, Bax downregulation indicates that the kidney injury enters recovery phase.

Immunopositive p53 cells were seen in groups I/R1, I/R8, and I/R12. In groups I/R1 and I/R8, immunopositive was found in the epithelial cell nucleus, whereas in group I/R12 it was in the kidney interstitial tissue. In the initiation and extension phases, epithelial cells also experienced damage. During the extension phase, ischemic/reperfusion injury model in mice showed the S3 segment of the proximal and medullary tubules thick ascending limb was the most affected part of the injury. The changes in the proximal tubule parallel with the process comes from metabolic interactions between epithelial cells and endothelial cells. This shows that apoptosis occurred in the epithelial cells in acute phase. Injury will activate the inflammatory response resulting from interaction of epithelial and endothelial cells. The consequence of this phenomena is production of proinflammatory cytokines and infiltration of neutrophils into interstitial areas. Neutrophils will invoke other inflammatory cells such as macrophages, NK cells, and T lymphocyte cell subtypes. Increased inflammatory cells to interstitial tissue are known to aggravate injury in the interstitial area.

Kidney IRI effect on cell proliferation

The I/R group showed higher Bcl-2 mRNA expression than normal mice. Apoptosis that occurs in the previous phase will induce kidney tubule epithelial cells to proliferate. Proliferation can replace tubular epithelial cells that undergo apoptosis. Under normal conditions, tubular cells show low proliferation activity rate. But in this condition, the proliferation rate increased significantly. According to Hammerman, in 2000, tubular cell proliferation in ischemic/reperfusion injury occurs due to an increase in growth factors such as epidermal growth factor (EGF), insulin-like growth factor, and hepatocyte growth factor which aims to improve growth. However, in addition, activation of survival pathways involves a large number of anti-apoptotic effects. The results of this study are also in accordance with another Sutton et al., which found that in the maintenance phase high proliferation rate occurred with cellular migration and differentiation. When I/R8 has entered the maintenance phase, it has the highest Bcl-2 expression. Although when the I/R12 group entered the repair phase the proliferation decreased. Events that occurred in the repair phase are redifferentiation and repolarisation. In IHC staining, cells that are immunopositive Bcl-2 are characterised by a brownish colour seen in groups I/R1, I/R8, and I/R12. In group I/R1 and I/R8 the brownish colour is seen in the cytoplasm of tubular epithelial cells, whereas in group I/R12, brownish colour is seen in the interstitial areas of the kidney. Proliferation that occurs shows the process of repairing the injury which aims to replace cells that experience apoptosis at the extension phase. At I/R1 and I/R8, the injury is still in the extension phase so that cellular proliferation occurs in epithelial cells. However, in the chronic phase, cells that proliferate are cells at the interstitial. This is in accordance with the conditions of apoptosis which also occurs in the interstitial area in the extension phase.

Kidney IRI effect on cellular senescence

The expression of p16 mRNA in the I/R group also had differences compared with the control group. The mean of p16 mRNA expression was higher in both I/R8 and I/R12 groups, different significantly compare with SO. According to Basnakian et al., DNA damage is the main signal to upregulate p53 expression. DNA damage that occurs in ischemic/reperfusion injury is in the form of oxidation and endonuclease mediated breaking of single strand DNA. More chronic the phase of ischemic/reperfusion injury, the higher the p16 mRNA expression. In contrast to Bax mRNA expression was high on I/R8, but decreases on I/R12 suggesting the accumulation of cells that experience cellular aging is increasing in the chronic phase. The accumulation of senescent cells shows the repair process leads to a maladaptive response. Cells that experience cellular ageing cannot experience proliferation and apoptosis. Repair in renal ischemia/reperfusion injury can continue to be a maladaptive response indicated by a persistent inflammatory process. According to Canaud et al., maladaptive repair results in a decrease in proliferation with G2/M cell cycle arrest.

CONCLUSION

Kidney ischemia/reperfusion injury induces upregulation of p16 mRNA expression, especially in chronic episode (day 8 and 12) after I/R injury. This may associate with proliferation of interstitial cells which induce chronic effects after I/R injury. Elucidating interstitial cells which in the senescence state due to I/R injury is important for continuing this study.

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