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Inverse correlation of kidney interstitial cells expansion with hemoglobin level and erythropoietin expression in single and repeated kidney ischemic/reperfusion injury in mice

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Abstract: Ischemic/reperfusion injury (IRI) causes acute kidney injury (AKI) that may lead to chronic kidney disease (CKD). We investigated the correlation between kidney interstitial cells expansion, hemoglobin level, and erythropoietin expression as the chronic effects of single and repeated kidney IRI in mice. We created IRI model using male Swiss mice by clamping bilateral pedicle renal. Subjects were divided into 4 groups that contained 6 mice each: Control/sham operation (SO), single acute IRI (IR1), single chronic IRI (IR12), and repeated IRI (IR7-12). Our results showed that single chronic and repeated IRI significantly increased tubular injury score, decreased hemoglobin level, and increased erythropoietin expression compared to control. Lower hemoglobin level in all groups compared to control was associated with erythropoietin resistance. In single chronic and repeated kidney IRI, there were decreased creatinine level compared to control. Decreased creatinine levels from group IR1 to IR12 suggesting repair phase of IRI starting on day 7 occurred in group IR12. A macrophage marker, CD68, and an inflammatory mediator marker, MCP-1, significantly increased in all IR groups suggesting inflammation occurred due to IRI. In conclusion, chronic and repeated kidney IRI induced interstitial cells expansion and inflammation associated with anemia.

Keywords: anemia; chronic kidney disease; erythropoietin; fibrosis; ischemic/reperfusion injury

1. Introduction

Chronic Kidney Disease (CKD) is a major health problem in both developed and developing countries ((Prodjosudjadi & Suhardjono 2004)). Progression of this disease to develop into End Stage Renal Disease (ESRD) is difficult to avoid and the mortality rate still remains more than 20% per year even in the availability of dialysis therapy. Based on the data released by the National Kidney Foundation, the number of

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patients treated by dialysis and kidney transplantation has increased dramatically in the United States. More than 400,000 United States people suffered from ESRD and more than 300,000 people of those need dialysis therapy (Go et al. 2004).

CKD is known as one of the possible complications of Acute Kidney Injury (AKI). Kidney Ischemic/Reperfusion Injury (IRI) can cause AKI and more than 70% of AKI cases will develop into CKD (Bonventre and Yang 2011). Reactive Oxygen Species (ROS) can cause kidney insufficiency (Arfian et al. 2012) by inducing interstitial cells expansion and extracellular matrix production which inhibits tubular epithelial cell proliferation in kidney IRI models (Kim et al. 2010). Interstitial area expansion leads to kidney interstitial fibrosis which is known as the main characteristic of progressive kidney disease (Strutz and Zeisberg 2006).

Kidney fibrosis is mediated by fibroblast accumulation and dysfunction. Fibroblast dysfunction causes anemia due to erythropoietin (EPO) reduction, the hormone that stimulates erythropoiesis (Asada et al. 2011). One study using in situ hybridization and transgenic mice found that EPO was produced by interstitial fibroblast at the inner cortex and outer medulla (Obara et al. 2016). Patients with CKD also show increases of inflammatory molecules, for example, C-reactive protein (CRP), and groups of cytokines such as IL1, IL6 and TNF alpha (Bergstro and Lindholm 2000; Stenvinkel 2001). Proinflammatory cytokines can cause EPO resistance by disrupting iron metabolism (Donovan et al. 2005).

Subtotal Nephrectomy (SN) and Unilateral Ureteral Obstruction (UUO) are known as models for CKD, whereas IRI is known as a model for AKI in mice (Arfian et al. 2012; Asada et al. 2014). In this study, the IRI model was used to induce CKD, especially in the chronic period of IRI. IRI could also induce CKD and kidney fibrosis after 12 days based on the histopathological findings, as shown by fibroblast proliferation as well as myofibroblast expansion, which is also associated with anemia (Asada et al. 2014). Studies on the chronic effect of single and repeated kidney IRI and the process of how AKI develops into CKD in this model is still rare. Therefore, it is necessary to investigate the chronic effect of kidney IRI, especially interstitial cells expansion (fibroblasts and pericytes). Correlation of interstitial cells’ expansion with hemoglobin concentration and EPO expression in this model are also rarely investigated so it is equally important to be studied.
2. Materials and methods

This was a quasi-experimental study with post test only controlled group design using twenty-four Swiss male mice, 3 months old, weighing 30-40 grams. Mice were obtained from the Integrated Research and Analytical Laboratory (Laboratorium Penelitian dan Pengujian Terpadu), Universitas Gadjah Mada. The animals were randomized and maintained under standard laboratory conditions and given access ad libitum to an animal diet and tap water. All experimental procedures were conducted according to the Medical and Health Research Ethics Committee, Faculty of Medicine Universitas Gadjah Mada with expediency number KE/FK/845/EC.

2.1. Kidney Ischemic/Reperfusion Injury Model

Animal subjects were divided into 4 groups that contained six mice each: Control/Sham Operation (SO), Ischemic/Reperfusion 1 (IR1), Ischemic/Reperfusion 12 (IR12), and Ischemic/Reperfusion 7-12 (IR7-12). Sham Operation was used as the control, IR1 was used as the AKI model, IR12 was used as the chronic single IRI model, and IR7-12 was used as the repeated IRI model.

The kidney IRI model was performed under general anesthesia. Intraperitoneal injection of pentobarbital solution 1:10 (0.1mg/10 gramBW) was used as an anesthesia agent. In this model, we performed clamping of right and left renal pedicles using non-traumatic vascular clamp (Karl HammacherGmbh, Solingen, Germany) for 30 minutes. After 30 minutes, the clamp was released and followed by reperfusion. The incised skin and peritoneum were then closed using surgical thread silk 3/0 (OneMedHealthcare, Surabaya, Indonesia).

In the SO group, no pedicle clamping was performed, only abdominal incision followed by the closing of the incised skin. This group was euthanized on day 12. In IR1 and IR12 groups, bilateral pedicle renal clamping was performed on day 0 and subjects were euthanized on day 1 and 12, respectively. In group IR7-12, bilateral pedicle renal clamping was performed on day 0 and 7 followed by euthanasia on day 12.

2.2. Blood Serum Analysis

Whole blood was obtained from the retro-orbital vein for hemoglobin and creatinine levels measurements. Whole blood samples for hemoglobin measurement were transferred into an EDTA tube, whereas whole blood samples for creatinine measurement were transferred into a 1.5mL tube followed by
centrifugation on 10,000 rpm for 10 minutes. Supernatants were obtained as the blood serum and all samples were brought to Clinical Pathology Laboratory, Faculty of Medicine, Public health, and Nursing, Universitas Gadjah Mada for hemoglobin and creatinine measurement.

2.3. Microscopic Analysis

2.3.1. Tubular Injury Score

The kidneys were embedded in paraffin block and 4µm sections were stained with Periodic Acid Schiff (PAS), examined with light microscope Olympus CX22 (Olympus Corporation, Tokyo, Japan) and portrayed with Optilab software with 100x magnifications at the corticomedullary junction area to measure the tubular injury score. Tubular injury scores were determined using a semi-quantitative scoring system. Fifteen fields per kidney were examined and injuries were graded from 0-4 (0, normal; 1, injury affecting <25%; 2, injury affecting 25-50%; 3, injury affecting 50-75%; 4, injury affecting >75%), according to these variables: renal tubular atrophy and dilatation, intraluminal cast formation (reddish color inside the lumen in PAS staining), loss of brush border, accumulation of inflammatory cells and intraluminal cast (Park and Kim 2013).

2.3.2. Immunohistochemical (IHC) staining

The kidneys were embedded in paraffin blocks and 4 µm sections were made, deparaffinized and rehydrated using 100%, 90%, 80%, and 70% alcohol, followed with the heating process in citrate buffer pH 6 for antigen retrieval and blocking endogenous peroxidase using H₂O₂ 3% in PBS solution. The slides were then incubated using Background sniper, rabbit 1st monoclonal antibody PDGFRβ with 1:200 dilution (Abcamab32570, Cambridge, UK), TrekAvidin-HRP, 2nd antibody anti-rabbit Trekkie Universal Link (Biocare MedicalSTUHRP700, CA, USA), and diaminobenzidine tetrahydrochloride (DAB) (Biocare, STUHRP700H L10). The results were analyzed using ImageJ software, examined with a light microscope (Olympus CX22) and portrayed with Optilab software with 400x magnification.
2.4. Reverse Transcriptase PCR Analysis

2.4.1. RNA Extraction and cDNA Synthesis

Total RNA was extracted using Genezol (GeneaidGZR100, Geneaid Biotech Ltd, New Taipei City, Taiwan), followed by quantification of RNA concentration using spectrophotometry. We used 3,000 ng RNA for making cDNA. The cDNA was made using Rever Tra Ace® (Toyobo Cat.No. TRT-101, Osaka, Japan) and random primer (Toyobo Cat.No. 3801), with PCR conditions: 30°C for 10 minutes (denaturation), 42°C for 60 minutes (annealing) and 99°C for 5 minutes (extension).

2.4.2. Reverse Transcriptase PCR and Electrophoresis

Reverse Transcriptase PCR was done to amplify the following specific cDNAs: Erythropoietin/EPO (F: AGGAATTGATGTCGCCTCCA and R: AGCTTGCAGAAAGTATCCACTGTG); CD68 (F: CATCAGAGCGAGTACAGTCTACC and R: AATTCTGCGCCATGAATGTCC); Monocyte chemoattractant protein-1/MCP-1 (F: GCCATCACAGTCCGAGTCACAC and R: CTACAGACAACCACCTCAAGCATTCTGT), and GAPDH (F: GCCACAGTCAAGGCTGAGAATG and R: TCTCGCTCCTGGAAGATGGTGA).

Reverse Transcriptase PCR was performed by mixing 2μL cDNA, 12.5μL of Tag master mix (Bioron, Germany, Cat. No. S101705), 0.6μL of forwarding primer and reverse, and 9.3μL of PCR water. cDNA was amplified to the following conditions: 94°C for 2 seconds (initial denaturation), 94°C for 10 seconds (denaturation), 60°C for 20 seconds (annealing), 72°C for 1 minute (extension), and 72°C for 10 minutes (last extension) for 35 cycles. The PCR products were analyzed in 2% agarose gel along with a 100bp DNA ladder (Bioron Cat. No.306009, Germany). Expressions of the gene were quantified with densitometry analysis using ImageJ software. GAPDH was used as the housekeeping gene.

2.5. Results analysis

Data obtained were analyzed using the Shapiro Wilk test for distribution analysis. Pearson correlation and Spearman correlation tests were used if the data was normally and abnormally distributed respectively. p<0.05 was used to determine the level of significance. Multiple comparisons among the groups were done by one-way ANOVA and followed by post hoc LSD tests if the data were normally distributed. If
the data were abnormally distributed, Kruskal Wallis and post-hoc Mann Whitney tests were used. $p<0.05$
was used to determine the level of significance.

3. Results

3.1. Blood serum analysis

Single chronic and repeated kidney IRI induced decrease of hemoglobin level as shown by significant lower in groups IR12 and IR7-12 compared to SO group ($p<0.01$). Single acute kidney IRI, represented by IR1 group, also induced significant lower hemoglobin level compared to SO, but in smaller decreases compared to IR12 and IR7-12 groups (Figure 1A). This result suggested the more chronic and more severe conditions caused greater decreases in hemoglobin level. Decrease of hemoglobin level in all groups compared to SO was associated with EPO resistance.

Compared to SO group, single acute and single chronic kidneys IRI induced significantly higher creatinine level as shown in IR1 and IR12 groups. However, repeated kidney IRI (IR7-12 group) did not show a significant difference compared to SO. Furthermore, single chronic (IR12 group) and repeated (IR7-12) kidney IRI had lower creatinine level compared to single acute (IR1 group) kidney IRI (Figure 1B). The decrease of creatinine level from group IR1 to IR12 suggesting repair phase of IRI started in day 7 occurred in IR12 group.

Figure 1. Results for mean hemoglobin level (A) and mean creatinine level (B). Control/Sham Operation (SO), Ischemic/Reperfusion (IRI) 1 (IR1), Ischemic/Reperfusion 12 (IR12), and Ischemic/Reperfusion 7-12 (IR7-12). Data were analyzed by One-way ANOVA ($p=0.000$) and Kruskal Wallis ($p=0.000$) tests. Asterisks show significance between SO and IRI groups (***, $p<0.001$; **, $p<0.01$; *, $p<0.05$). The elbow connectors show significances between IRI groups (##, $p<0.001$; ±, $p<0.01$; #, $p<0.05$).
3.2. **Microscopy analysis**

Kidney IRI induced tubular injury which was characterized by intraluminal cast formation, dilatation, epithelial effacement, and brush border loss (Figure 2A). Higher magnification of PAS staining revealed repair of tubular epithelial occur in IR12 group as shown by brush border formation. However, the regeneration and tubular repair did not occurred in repeated IR (IR7-12 group). Tubular injury score quantification showed significant higher tubular injury score in IR1, IR12, and IR7-12 groups compared to SO group (*p*<0.001). IR1 and IR 7-12groups demonstrated higher tubular injury compared to IR12 group (Figure 2A, B, D). This condition suggested that the repair phase happened in group IR12. Microscopically, IR12 group revealed a regeneration process of tubular epithelial (Figure 2A, B, C).

Kidney IRI induced a significant increase in the number of positive PDGFRβ (fibroblast) cells in IR1, IR12, and IR7-12 groups compared to SO group. This condition was associated with severe injury that leads to interstitial cell (fibroblasts and pericytes) expansion (Figure 2C, E).
Figure 2. Histological quantification of tubular injury and PDGFRβ-positive cells. (A) Representative figures of Periodic Acid Schiff (PAS) staining. Control/Sham Operation (SO) group represented normal tubules with brush border (black triangles); tubular injury showed damage of tubular with cast formation (red color), brush border loss (white arrow) and epithelial cells effacement (black arrow) in Ischemic/Reperfusion (IRI) 1 (IR1), IR12, and IR7-12 groups. Brush border regeneration could be found in IR12 group. Scale bar = 100µm. (B) Microscopic figures of kidney tissue. Scale bar = 100µm. (C) Microscopic figures of PDGFRβ-positive cells. PDGFRβ-positive cells were shown by the black arrow. Scale bar = 100µm. (D) Results of Tubular injury score and (E) number of PDGFRβ-positive cells. Tubular injury scores were analyzed using Kruskal Wallis test (p=0.001). The number of PDGFRβ-positive cells were analyzed by One-way ANOVA (p=0.000). Asterisks show significance between SO and IRI groups (***, p<0.001; **, p<0.01; *, p<0.05). The elbow connectors show significances between IRI groups (##, p<0.001; ±, p<0.01).

3.3. RT-PCR Analysis

Kidney IRI induced a significantly higher mRNA expression of EPO in IR1, IR12, and IR7-12 groups compared to the SO group. This condition suggested that hypoxia, either in an acute or chronic condition, induced production of HIF and subsequently leads to EPO production (Figure 3A, B). RT-PCR of CD68 as macrophage marker and MCP-1 as inflammatory mediator demonstrated inflammation with macrophage infiltration occurred in IR1, IR12, and IR7-12 groups. It revealed significantly higher expression of CD68 mRNA in IR1, IR12, and IR7-12 groups compared to the SO group (Figure 3A,B,C) as well as MCP-1 (Figure 3A,B). Repeated IRI as represented by IR7-12 group revealed significant higher MCP-1 mRNA expression compared to IR1 and IR12 groups. These results suggested that chronic inflammation might be associated with EPO resistance in all groups thus inducing anemia.
Figure 3. The mRNA and protein expression of pro-inflammatory mediators. (A) Gel electrophoresis figures of RT-PCR analyses of MCP-1, erythropoietin (EPO), CD68, and GAPDH from kidney tissue. (B) Bar charts showing relative quantification for mean EPO/GAPDH, CD68/GAPDH, and MCP-1/GAPDH mRNA expressions. Data were analyzed by One-way ANOVA test (p=0.000). Asterisks show significances between Control/Sham Operation (SO) and Ischemic/Reperfusion (IRI) groups (***, p<0.001; **, p<0.01). The elbow connectors show significances between IRI groups (#, p<0.001; #, p<0.05). (C) Immunohistochemistry showing CD68 as macrophage marker to demonstrate inflammation (white arrows) in IR1, IR12, and IR7-12 groups. Scale bar = 100μm.

3.4 Correlation test

Pearson correlation test between the number of PDGFRβ-positive cells and hemoglobin level showed significant, strong, negative correlation (p=0.000; r=-0.822), whereas Pearson correlation test between the number of PDGFRβ positive cells and EPO expression showed a significant, strong, positive correlation (p=0.000; r=+0.795).

4. Discussion

This study showed a reduction of hemoglobin level occurred in kidney IRI which might associate with inflammation and macrophage infiltration. Kidney IRI causes an imbalance between vasoconstriction and vasodilation mediators which contributes to microvascular and tubular injury. Microvascular injury represents with an increase of vasoconstriction and decrease of vasodilation response, endothelial and vessel
smooth muscle cells injury, leucocyte-endothelial interaction, and inflammation. Tubular injury induces
cytoskeleton damage, loss of polarity, apoptosis, and necrosis (Bonventre and Weinberg 2003).

The tubular injury might occur in acute and chronic time after Kidney IRI model. Morphologic changes
of kidney microstructure demonstrated tubular injury characterized by loss of brush border at proximal
tubules; intraluminal cast accumulation, tubular renal dilatation and atrophy, and accumulation of
inflammatory cells in all IRI groups. The tubular injury also induces degradation of cellular debris into the
tubular lumen and contributed to casting formation ((Biophysics et al. 1990), tubular lumen obstruction, and
an increase of tubular pressure ((Mason et al. 1977).

Tubular injury scores quantification showed IR1, IR12, and IR7-12 groups had higher significant
compared to the SO group. The highest tubular injury score was in group IR7-12 followed by IR1 group.
Mechanisms and process which happened in IR1 group showed that tubular epithelial cells injury is the main
mechanism in the initiation phase of kidney injury (Sutton et al. 2002). However, based on our result in
day12 after IRI (IR12 group) and repeated kidney IRI (IR7-12 group), the tubular injury still occurred which
represented delayed epithelial cells and in-adequate of the regeneration process. This result also revealed that
there was a repairing process in the recovery phase of kidney IRI. In the recovery phase, cells differentiation
continues, epithelial polarity regained, and organ functions normally return (Hagmann et al. 2014; Harris
1997; Schena 1998). IR7-12 group represents the model for maladaptive repair response. Second IRI in IR7-12
represented the disruption of maintenance phase, which leads to maladaptive repairing process.

Repeated injury in IR7-12 group also demonstrated the highest number of PDGFR β positive cells, then
followed by IR12 group. We chose PDGFR β as the marker for fibroblast and pericyte in this study although
there are other markers, such as NG2, that can also be used to examine these two kinds of cells. Both
fibroblast expansion and myofibroblast formation do not only change the structure of fibroblast, but also the
function of fibroblast (Asada et al. 2014). More hypoxic-induced chronic hypoxia which leads to
tubulointerstitial injury (Bonventre and Yang 2011). Maladaptive repair leads to fibroblast activation and
tubulointerstitial fibrosis, chronic and excessive profibrotic cytokines production by activated macrophages.
IL13 and TGF β-1 are some of the profibrotic cytokines produced (Bonventre and Yang 2011). Hypoxic
Inducible Factor 1α (HIF-1α) may also contribute to the chronic effect of hypoxia that may lead to fibrosis.
HIF increases in the acute phase of IRI and provides protective effects in the acute phase of ischemia injury.
However, in renal fibrosis model, deletion of HIF-1α may prevent epithelial to mesenchymal transition and tubulointerstitial fibrosis (Higgins et al. 2007). Prolonged activation of HIF-1α could also induce the upregulation of profibrotic substances, such as connective tissue growth factor (CTGF). This upregulation stimulates fibrosis and destruction of renal prenchyme (Haase et al. 2013). Examining fibroblast to myofibroblast formation in this study may give some point of view to observe the EPO-producing cells, especially the periods of kidney IRI.

RT-PCR analysis showed an increase of EPO mRNA expression in groups IR1, IR12, and IR7-12. Pearson correlation test between the number of PDGFR β positive cells and EPO expression showed a significant, strong, positive correlation ($p=0.000; r=+0.795$). This positive correlation showed the higher number of PDGFR β cells positive, the more EPO which was produced. Pearson correlation tests between the number of PDGFR β positive cells and hemoglobin concentration showed significant, strong, negative correlation ($p=0.000; r=-0.822$). It can be concluded that the greater the number of PDGFR β cells positive, the more hemoglobin level was decreased. This study shows that correlation between the number of PDGFR β positive cells with hemoglobin concentration and creatinine level trend in opposite directions. It can be induced by chronic inflammation which becomes the basis for EPO resistance in CKD models. This condition may need other examination about the proliferation of fibroblast. Proliferated fibroblast may transform into myofibroblast, thus reducing the EPO production from fibroblast (Asada et al. 2014). In this study, we found that anemia still occurred in spite of the increased mRNA expression of EPO. Further analysis of the phenotype of fibroblast is necessary for the completion of this study. Double immunostaining to examine fibroblast to myofibroblast transition or EPO immunostaining may be considered important to continue this study. We consider this matter as one of the limitations of this study. In CKD cases, anemia can be due to EPO deficiency and resistance, and also disruption in iron metabolism (Hörl 2013), and it may relate to inflammation.

Inflammation play roles in the pathogenesis of kidney injury. Inflammation occurs in both acute and chronic phases of kidney injury, and further contribute to maladaptive responses (Akcay et al. 2009). This process activates proinflammatory cytokines and chemotactic agents, such as TNF-α, MCP-1, IL-8, IL-6, TGF-β, regulated on activation, normal T expressed and secreted (RANTES) protein and epithelial neutrophil-activating protein 78 (ENA-78), then induces macrophage infiltration (Basile and Yoder 2014).
We elucidated macrophage infiltration through the expression of mRNA and protein CD68 as macrophage marker. CD68 expression was increased in groups IR1, IR12, and IR7-12 and was significantly different from group SO. Immunostaining revealed infiltration of macrophage in IR groups which associated with upregulation of MCP-1 and CD68 mRNA expression. This result could be due to chronic inflammations in groups IR12 and IR7-12, which leads to chronic and excessive production of profibrotic cytokines. Anemia in CKD is a type of Anemia of Chronic Disease/ACD(Weiss and Goodnough 2005). The ACD is characterized by blunted EPO response, decrease of red blood cells age, and defects in iron metabolism, consisting of decreases of iron absorption and iron retention by macrophage(Weinstein et al. 2002).

Macrophage infiltration leads to macrophage polarization and M1 formation due to inflammatory mediator stimulation. M1 plays the main role in inflammation modulation and inducing tissue damage(Cao et al. 2015). Prolonged inflammation also induces production on hepcidin in liver. Hepcidin can degrade transmembrane protein ferroportin (Fpn) in M1. This condition prevents releasing of iron to the extracellular environment, thus iron will undergo sequestration in M1 and inflammation area. Chronic increase of hepcidin and iron sequestration reduces the iron level in the blood and finally induce anemia (Cairo et al. 2011). Kidney IRI upregulates inflammatory cytokines production, then induces M1 formation and iron sequestration. Proinflammatory cytokines, such as TNFα, IL-1βand IL-6, may also upregulate hepcidin (Gamella et al. 2014). Elucidating proteins that contribute to iron metabolisms, such as hepcidin or transferrin, may provide a better understanding about the effect of IRI in anemia and EPO production. In the future, it also needs to examine the possibility of iron entrapment and deposition in the kidney after IRI.

5. Conclusions
In conclusion, chronic effects of single and repeated IRI cause interstitial cells expansion and EPO mRNA expression elevation. The decrease of hemoglobin level could be due to chronic inflammation that inhibits EPO effectiveness. Therefore, our study revealed the chronic and repeated kidney ischemic/reperfusion injury induced fibroblast expansion and inflammation associated with anemia.

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Authors’ contributions
NA, DP and DC designed the study. NA, DP and WA carried out the experimental works in the laboratory, such as the surgical procedure to the mice subjects, immunohistochemistry staining, RNA extraction and PCR analysis. MR and NA analyzed the data. DP and NA wrote the manuscript. All the authors read and approved the final version of the manuscript.

Competing interests
The authors declared there is no conflict of interest.

References


