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Prolonged Kidney Ischemia-Reperfusion Injury Associates with Inflammation, Vascular Remodelling, and Myofibroblast Formation

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ABSTRACT

Prolonged kidney ischemia-reperfusion injury (IRI) is the important risk factor for leading to chronic kidney disease (CKD). Persistent hypoxia and inflammation are considered as the main pathogenesis of chronic injury, followed by myofibroblast expansion and fibrosis process. Tubular injury, cell proliferation, and vasoconstriction, as acute compensatory responses, are restored in chronic phase. The aim of the study was to investigate the relation between inflammation, vascular remodeling, and myofibroblast formation as response to ischemia injury after prolonged kidney ischemia-reperfusion (I/R). Fifteen male Swiss mice aged 3-4 months were used as kidney I/R injury model after bilateral pedicle renal clamping. Rats were divided into 3 groups with five rats in each group i.e. control group (sham operation/SO), acute I/R model (IR1), and chronic I/R model (IR12). PAS staining was used for scoring tubular injury. Fibrosis was assessed using sirius red and α-SMA immunostaining for myofibroblast expansion. PCNA and CD68 immunostaining were used for identifying cell proliferation and macrophage infiltration. RT-PCR was conducted for assessing MCP-1, HIF-1α, and ppET-1 expression, which were quantified using ImageJ software. Data were analyzed using one way ANOVA and Kruskal-Wallis test with significance level of $p<0.05$. Significantly increase of tubular injury score ($p<0.001$) and PCNA positive cell ($p<0.001$) in IR1 group compared to SO were observed, otherwise HIF-1α of IR12 enhanced ($p<0.05$). Macrophage cell count ($p<0.01$) and MCP-1 expression ($p<0.05$), were significantly increase in IR1 and IR12 injury, compared to SO. Wall thickness of arteries was significantly increase ($p<0.05$) as well as decrease of vascular lumen area ($p<0.05$), followed by enhancement of ppET-1 expression ($p<0.01$) in IR1 group and restored significantly ($p<0.05$) in IR12 group. Fibrosis fraction-area and myofibroblast expansion were significantly increase gradually from IR1 to IR12 injury ($p<0.01$). In conclusion, prolonged kidney I/R injury induces the sustainability of hypoxia and inflammatory response, which promotes myofibroblast formation, and decrease the response of vascular remodelling.

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ABSTRAK

Perpanjangan cedera iskemik-reperfusi ginjal (*kidney ischemia-reperfusion injury*/IRI) merupakan fakor risiko penting terjadinya penyakit ginjal kronis (*chronic kidney disease*/CKD). Inflamasi dan hipoksia berkepanjangan diduga merupakan pathogenesis utama cedera kronik, diikuti ekspansi miofibroblas dan kejadian fibrosis. Cedera tubulus, proliferasi sel dan vasokontriksi sebagai respon balik akut terjadi pada fase kronik. Tujuan penelitian ini adalah mengkaji hubungan antara inflamasi, remodelling vaskular dan pembentukan miofibroblas sebagai respon cedera iskemik setelah perpanjangan iskemik/reperfusi (I/R) ginjal. Lima belas mencit Swiss jantan berumus 3-4 bulan digunakan sebagai model cedera setelah dilakukan penjepitan *bilateral pedicle renal.* Tikus dibagi menjadi tiga kelompok dengan 5 ekor setiap kelompok yaitu kelompok *sham operation* (SO), kelompok model IR akut (IR1) dan kelompok model IR kronis (IR12). Pengecatan PAS digunakan untuk menilai cedera tubulus. Terjadinya fibrosis diukur menggunakan pengecatan imunologi merah sirius dan α-SMA untuk ekspansi miofibroblas. Pengecatan imunologi PCNA dan CD68 digunakan untuk mengidentifikasi proliferasi sel dan infiltrasi makrofag. RT-PCR dilakukan untuk mengkaji ekspresi MCP-1, HIF-1α dan ppET-1 yang diukur dengan program ImageJ. Data dianalisis menggunakan ANAVA satu jalan dan uji Kruskal-Wallis dengan tingkat signifikansi 0,05. Kenaikan secara nyata terjadi pada skor cedera tubulus ($p<0,05$) dan sel positif PCNA (0,05) pada kelompok IR1 dibandingkan SO, selain itu terjadi kenaikan HIF-1α pada kelompok IR12. Jumlah makrofag ($p<0,01$) dan ekspresi MCP-1 ($p<0,05$) meningkat secara nyata pada kelompok IR1 dan IR12 dibandingkan kontrol. Ketebalan dinding arteri meningkat (p0,05) diikuti penurunan area lumen vascular ($p<0,05$) dan kenaikan ekspresi ppET-1 ($p<0,01$) pada kelompok IR1 dan pulih secara nyata ($p<0,05$) pada kelompok IR12. Fraksi daerah fibrosis dan ekspansi miofibroblas meningkat nyata secara bertahap dari IR1 ke IR12 ($p<0,01$). Dapat disimpulkan, perpajangan cedera I/R ginjal menginduksi hipoksia dan respon inflamasi berkelanjutan yang menyebabkan pembentukan miofibroblas dan penurunan respon pemodelan kembali vaskular.

Keywords: ischemia-reperfusion injury - kidney – inflammation - vascular remodelling - myofibroblast.

INTRODUCTION

Kidney ischemia-reperfusion injury (IRI) is sudden temporary impairment of blood flow to the kidney, which is characterized by blood supply restriction to kidney and followed by restoration of blood flow and re-oxygenation (perfusion).[1] Kidney IRI is a major cause of acute kidney injury (AKI) and 70% of AKI progress to chronic kidney disease (CKD).[2] CKD is the chronic consequence of ischemia injury and thought to be related to glomerulo-interstitial fibrosis and persistent kidney dysfunction.[3]

The pathophysiology of kidney IRI is complicated. There are 3 stages of tissue response to ischemia injury, that are initiation, extension, and maintenance.[4] In early period or initiation phase, microvascular damage causes hypoxia in corticomedullary junction which is characterized by obstruction, inflammation, and coagulopathy. Then persistent hypoxia and inflammatory responses stimulate extension phase in 24 hours after initiation phase.[4] Loss of tubular brush border, exfoliation, and tubular obstruction are found in this period.[4] On day-3, maintenance phase is began. In this phase, there are repair process, migration, apoptosis, and proliferation to restore and maintain cellular and tubular integrity.[4]

Inflammation has the important role in early stage. Chemokines are major mediators of the inflammation that regulate pro-inflammatory cytokine, adhesion molecule expression, leukocyte activation and infiltration to the tissue.[1] Inflammatory mediators, reactive oxygen species (ROS), intracellular adhesion molecule (ICAM-1), and P-selectin can promote leukocytes and neutrophil infiltration into post-ischemic tissue.[1] The infiltration of leukocyte, including macrophages, may play an important role in development of kidney injury which is facilitated by chemotactic factors and/or adhesion molecules.[5] Monocyte chemoattractant protein-1 (MCP-1) is a potent chemokine that stimulates migration of monocyte into the intimate layer of arterial walls and organs.[5]

Enhancement of leucocyte-endothelial interaction can cause endothelial injury, then leads to decreasing blood flow to the tissue which aggravate ischemia.[1,6] Hypoxia tissue stimulates hypoxia-inducible factor-1 (HIF-1) expression. Then HIF-1 activates transcription of vascular endothelial growth factor (VEGF), which plays an essential role in angiogenesis.[6] Kidney injury causes tubular system damage, followed by rapid cell proliferation. The proliferation is an acute compensatory mechanism of injury, which is characterized by differentiation of tubular epithelial cell.[7] Those responses are associated with initial phase of injury. Cell proliferation can be represented by the expression of *proliferating cell nuclear antigen* (PCNA). PCNA is a monoclonal antibody which is expressed dominantly on S phase and essential for DNA replication.[8]

Imbalance between vasodilator and vasoconstrictor mediator causes reducing renal perfusion in AKI.[2] Endothelial dysfunction is responsible for reducing renal blood flow by impaired dilator capacity, which is attributed to reduce production of nitric oxide.[9] Endothelial nitric oxide synthase (eNOS) has important role in preservation of medullary blood flow in response to renal vasoconstrictor, such as angiotensin II. However, following renal injury, eNOS function is impaired which is demonstrated by a loss of responses to acetylcholine and bradykinin.[9] Endothelial cells also secrete endothelin-1 (ET-1), a potent vasoconstrictor. Through vasoconstriction effect, ET-1 induces reduction of renal blood flow and glomerular filtration rate,[2] which causes oliguria. In maladaptive response of maintenance phase, ROS can causes interstitial cells expansion and extracellular matrix production by inhibit tubular epithelial cell proliferation.[10] Fibrogenesis process and kidney interstitial fibrosis are shown in interstitial area expansion which is the main characteristic of progressive kidney disease.[11] This study was conducted to elucidate the kidney tissue response to prolonged injury, mainly about tubular injury appearance, inflammatory process, vascular changes, and myofibroblast formation.

MATERIALS AND METHODS

Animal

This was a quasi experimental study with post-test only controlled group design using 15 male Swiss-Webster mice aged 3-4 months old with 30-40 g body weights (BW). Mice were obtained from Animal Model Care Unit, the Integrated Research Testing Laboratory, Universitas Gadjah Mada, Yogyakarta and divided into three groups with five mice in each group i.e. sham operation (SO) as control group, ischemia/reperfusion day-1 (IR1) as AKI model group, and ischemia/reperfusion after 12 days (IR12) as CKD model group. Mice were maintained based on standard laboratory condition and provided diet and

water *ad libitum* before used. Protocol of this study has been approved by the Medical and Health Research Ethic Committee of the Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta.

Kidney ischemia/reperfusion injury model

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

Kidney harvesting

IR1 group was sacrificed in day-1 after operation, while IR12 groups after day 12 after operation. Prior to abdomen and thorax opened, mice were anaesthetized with intraperitoneal injection of pentobarbital sodium (60 mg/kg BW, Somnopentyl®). Perfusion of the organ was done from left ventricle, using 0.9% NaCl solution. Both perfused kidneys were harvested, one kidney was kept into RNA later® for RNA extraction and the rest was fixated into 4% PFA in PBS for 24 hours, and paraffin was used for the embedding tissue process.

Histological analysis and immunohistostaining

The kidney was embedded in paraffin block with 4 µm sections. Paraffin sections were deparaffinized and rehydrated using xylene and alcohol serial. Specimens were then stained with sirius red (SR) for measuring fibrosis interstitial fraction area and periodic acid-schiff (PAS) to determine tubular injury. For immunohistochemical staining, after deparaffinized and rehydrated, followed antigen retrieval, blocking peroxidase using H_2O_2 3% in PBS solution, and then blocking non-specific antigen using background sniper. The slides were incubated with α-SMA (1:400, Sigma, A2547), CD68 (1:400, Abcam, ab125212), and PCNA (1:200, Abcam, ab29) as 1st antibodies, TrekAvidin-HRP, 2nd antibody anti rabbit Trekkie Universal Link (Biocare Medical®), then diaminobenzidine tetrahydrochloride (DAB). α-SMA antibody immunostaining was used for measuring myofibroblast expansion, CD68 antibody for counting macrophage cells, and PCNA for assessing cell proliferation in kidney injury. Quantification was measured from 15 fields for each sample with 400x magnification, using ImageJ software.

Tubular Injury and Interstitial Fibrosis Fraction-area quantification

Tubular injury score was assessed by PAS staining, which was determined using semi quantitative scoring system in 15 fields for each specimen with 200x magnifications. The variables of scoring are tubular atrophy and dilatation, loss of brush border, accumulation of inflammatory cells, and intraluminal cast. Scale of the lesion are from 0 to 4: 0, normal; 1, mild, injury <25%; 2, moderate, injury 25-50%; 3, severe, injury 50-75%; 4, extensive damage, injury >75%. Fibrosis fraction-area was stained using Sirius red, which was quantified using ImageJ software on 15 non-overlapping fields and expressed in percentage (%).

Vascular remodeling

Lumen area and wall thickness were measured on sirius red staining of intra renal arteries. 10-15 arteries were randomly chosen,

and then assessed using ImageJ software. The arteries were measured vessel area (diameter of outer layer), lumen area (diameter of inner layer), vessel perimeter, lumen perimeter, wall area (the difference between vessel area and lumen area), and central perimeter (the average of vessel and lumen perimeter). Wall thickness calculation is from the ratio between wall area and central perimeter.

Reverse transcriptase PCR (RT-PCR)

RNA was extracted using Genezol solution (Genezol®, Cat. No. GZR100), followed by RNA concentration quantification using sphectophotometry. cDNA was synthesized using Rever Tra Ace® (Toyobo, Japan, Cat. No. TRT-101) and random primer (Toyobo, Japan, Cat. No. 3801). Reverse transcriptase PCR was done for assessing the expression of following genes: HIF-1α forward AGCTTCTGTTATGAGGCTCACCATC3', reverse AATGTCAAGATCACCAGCAC-3'), MCP-1 (forward 5'-CTTCTGGGCCTG CTGTTCA-3', reverse 5'- CTTCTGG GCCTGCTG TTCA-3'), ppET-1 (forward 5'-GCCACAGACCAGGCAGTTAGA-3', reverse 5'-ACCAGCTGCTGATA GATACACTTC-3'), GAPDH (forward 5'-TTGCTGTTGAAGTCGCAGGAG-3', reverse 5'-TGTGTCCGTCGTGGAT CTGA-3') were used as reference. The gene expressions were quantified using densitometry analysis (ImageJ software) and GAPDH gene was used to normalized the gene expressions (housekeeping gene).

Statistical analysis

Data were presented as mean ± standard error of mean (SEM) for PCNA, HIF-1α, MCP-1 levels, fibrosis fraction-area, myofibroblast expansion, vascular remodelling (wall thickness and lumen area), and ppET-1 expression. For tubular injury score and macrophage cell counting were presented as median (min-max) data. Median data were analyzed using non-parametric test, Kruskal-Wallis, and then each group was compared using post-hoc Mann-Whitney. While the rest data, which have normal data distribution, were analyzed using one-way ANOVA test, followed by post-hoc LSD test. The level of statistical significance was $p<0.05$.

RESULTS

Kidney IRI induced tubular injury, cell proliferation, and inflammation

Tubular injury in kidney IR1 model is a response to acute injury. It was shown in tubular injury score of IR1 group was increased significantly ($p<0.001$), compared to control group and IR12 (FIGURE 1.B). This result was parallel to PCNA immunostaining. PCNA positive cell was extremely increased in IR1 group, and it was decline in IR12 ($p< 0.001$, FIGURE 1.C). Paradoxically, HIF-1α was more expressed significantly in the CKD model (IR12 group) as shown in FIGURE 1.E. It was due to HIF-1α expression is associated with the maintenance phase.

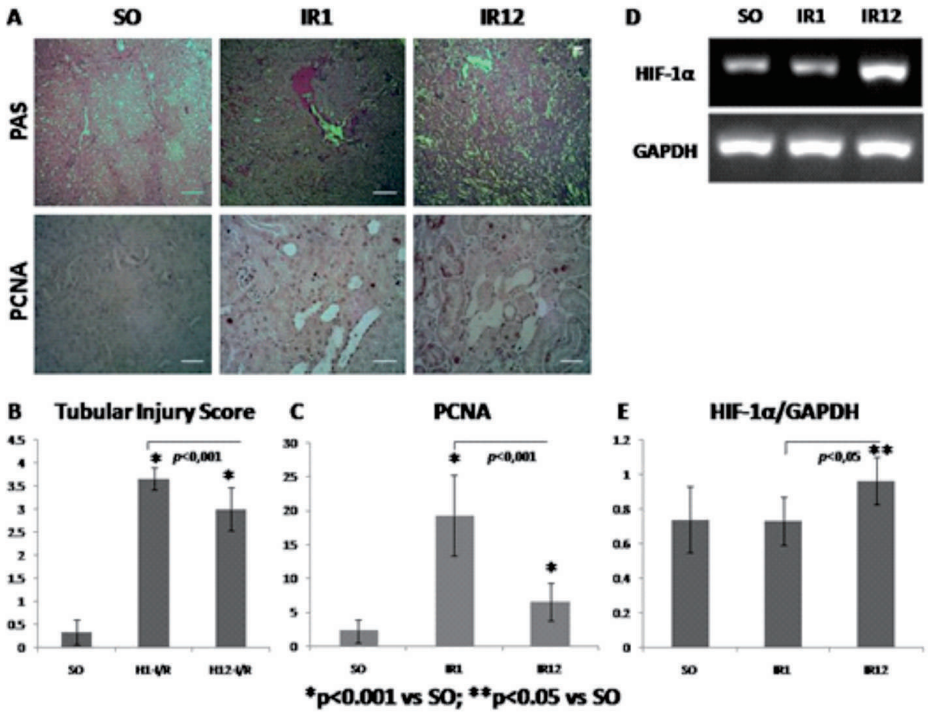

FIGURE 1. A. Microscopic picture of PAS and PCNA staining to show tubular injury and cell proliferation; B-C Quantitative analysis of tubular injury score and PCNA positive cell count; D-E Electrophoresis band and RT-PCR measurement of HIF-1α.

This study used MCP-1 and macrophage as representation of inflammation process. MCP-1 is a regulator of macrophage migration and infiltration. Therefore, increase of MCP-1expression in IR1 group was followed by increasing macrophage cell number, using CD68 immunostaining (FIGURE 2 C-D). This enhancement was persisted until chronic phase. It can be observed in IR12 group, which was still increased.

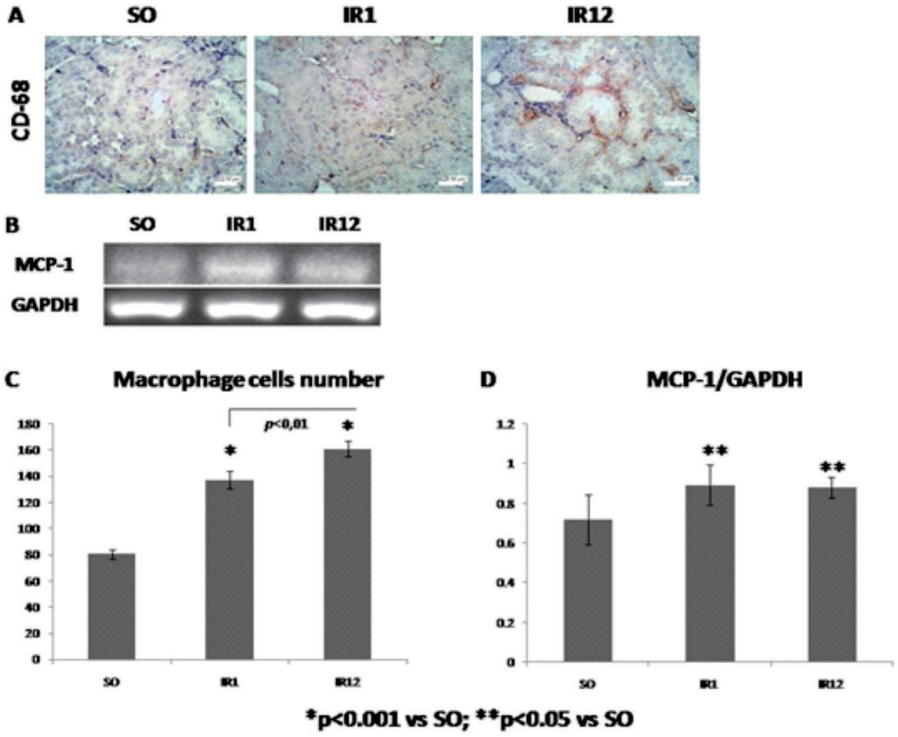

FIGURE 2. A. Representative picture of CD-68 immunostaining; B. Electrophoresis band of MCP-1 and GAPDH; C-D Quantitative analysis of macrophage cell count and RT-PCR measurement of MCP-1

Vascular remodelling of intrarenal artery

Vasoconstriction response in acute phase is related to the expression of ppET-1 gene. It was found that there were histological vascular changes of intrarenal artery (FIGURE 3.A). One day post-exposure, the vessel was constricted. It was proved by increase of wall thickness as well as decrease of vascular lumen area in IR1 group (FIGURE 3 D-E). And after 12 day post I/R, that condition was returned, showed by no significant difference between IR12 group and control group (FIGURE 3.D-E). Consistently, this condition was followed by increase of ppET-1 gene expression one day after IRI, then decline in IR12 group ($p < 0.05$, FIGURE 3.C).

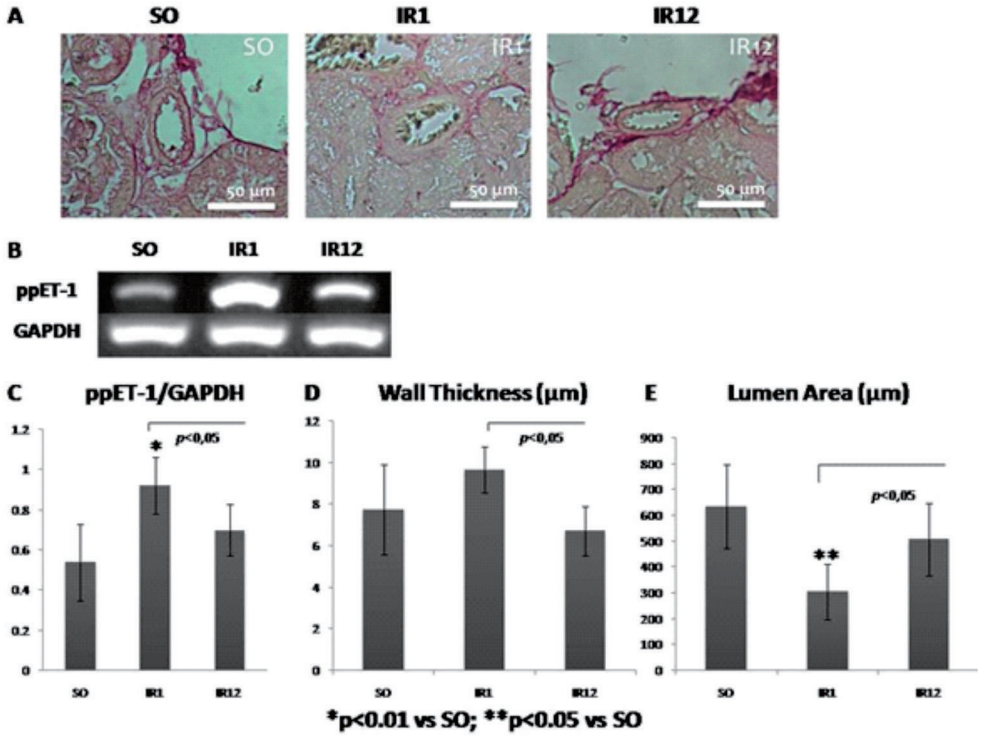

FIGURE 3. A. Histological changes of intrarenal artery are evaluated using sirius red staining; B-C. Electrophoresis band and RT-PCR measurement of ppET-1; D-E. Quantitative analysis of intrarenal artery on wall thickness and lumen area.

Fibrosis and myofibroblast expansion

Fibrosis area was stained in purplish-red by sirius red staining (FIGURE 4.A). The widest fibrosis fraction-area was shown from IR12 group (4.927%), then followed by IR1 group (3.260%), and SO group (1.021%) (FIGURE 4.B). These finding was similar to myofibroblast expansion. There were abundant of myofibroblast in IR12 group, compared to IR1 and control group. Statistical analysis showed significant difference between groups of fibrosis fraction-area and myofibroblast expansion ($p<0.01$, FIGURE 4.B-C).

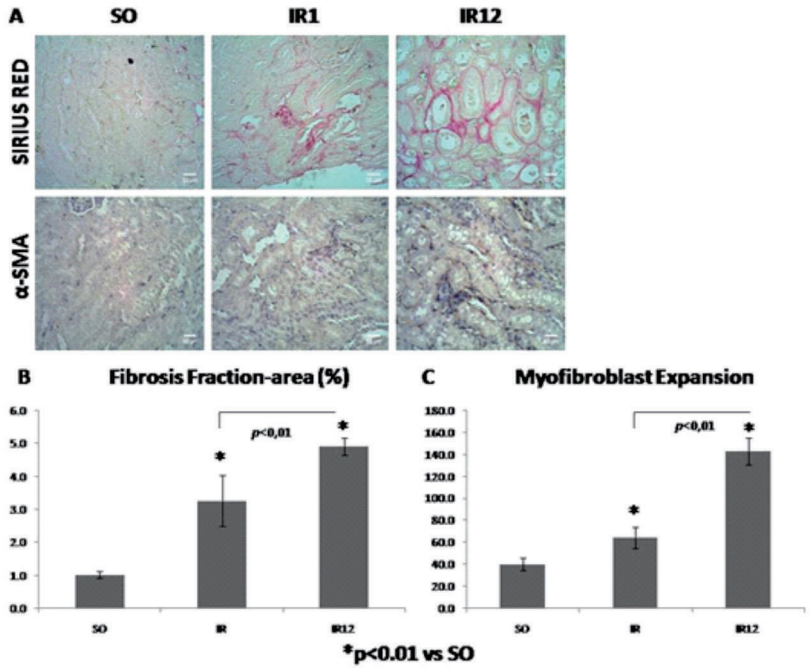

FIGURE 4. A. Sirius red and α-SMA IHC staining to show fibrosis and myofibroblas expansion; B-C. Quantitative analysis of fibrosis fraction-area and myofibroblast expansion

DISCUSSION

Kidney IRI effect on cell proliferation and inflammation response to tubular injury

The principle of kidney IRI is sudden temporary impairment of blood flow to the kidney.[1] There is injury/regeneration process in kidney injury which is characterized by impairment of cell polarity, cytoskeleton integrity, and loss of renal tubular brush border which can induce apoptosis and necrotic process.[12,13] Brush border and cell debris can cause intraluminal obstruction which form an intraluminal cast in the distal tubules. These obstructions can promote the dilatation and atrophy of proximal tubules in acute kidney injury.[14] Therefore, those characteristics were used for assessing tubular injury score. After 7 days post injury, there is a repairing process by the differentiation tubular cell and restoration of renal function and structure.[7] In this study, IR1 group, as an acute response, was impaired significantly compared to SO and IR12 group (FIGURE 1.A-B). It was correspond with study that was done by Basile *et al.*[9] reduction of tubular injury score in IR12 group was affected by repairing mechanism of tubular cells which was restored incompletely (FIGURE 1.B).

Bonventre & Duffield[15] study showed that injury in renal tubules was increased in 24 hours after IRI, followed by cell proliferation and optimized in 48 hours post IRI induction. This study used the expression of PCNA antibody for describing cell proliferation in acute and chronic phase after IRI. In normal physiologic condition, tubular epithelial cells have less ability to proliferate, it is shown by slowness of cell turnover. After injury, proliferation rate is increased significantly to

replace the necrotic/apoptotic cells.[16] PCNA positive cell of IR1 group was more expressed than in chronic period (IR12) ($p< 0.001$, FIGURE 1.C). It meant that the proliferation rate in chronic injury was reduced. Decreasing of cell proliferation is caused by repair process which is began from day-7 after IRI.[7] Furthermore, there is a maladaptive repair process that decrease cell proliferation by discontinuing G2/M phase, then PCNA, which is dominantly expressed in S phase, will be decreased.[16]

Persistent hypoxia in I/R injury can causes sustainable injury and increase of HIF-1α expression.[17] HIF is activated by low oxygen condition and induces widespread changes in gene expression.[17] Many of genes whose expression is increased by HIF are expected to improve the cellular capacity when oxygen supply is reduced.[17] Therefore, activation of HIF may improve the survival of ischemic cell and also promote adaptive changes, such as increased angiogenesis.[18] HIF appear in 10 minutes after ischemia, the most optimal period is 2 hour post-ischemia, and will be decreased in 8-24 hours after the optimum phase.[18] HIF-1α expression of IR1 group was not increased, it was estimated that the level was decline after 24 hours post-exposure, due to HIF-1α is degraded rapidly (FIGURE 1.E). Several study revealed that HIF has protective effect in acute phase of ischemia injury. Paradoxically, in prolonged period of hypoxia, HIF-1α will be increased. Higgins *et al*.[19] found that the ablation of HIF-1α gene can prevent fibrosis tubulointerstitial expansion, through mesenchyme-epithelial cell transition. Moreover, Haase[20] showed that the prolonged HIF signal activation can stimulate fibrosis and persistent destruction of tissue. When HIF is stable and not degraded, it can lead to enhancement of pro-fibrotic gene transcription, connective tissue growth factor (CTGF).[20] Hence, the highest level of HIF-1α was expressed in IR12 group significantly ($p<0.05$) (FIGURE 1.E). It is due to maladaptive response of repair process, so that in certain condition the level of HIF is associated with chronic injury.

Inflammatory response is the main role in pathogenesis of kidney injury. It can affect on acute and chronic (maladaptive amelioration) phase.[21] When kidney is exposed to ischemia injury, the epithelial cells will be change, the barrier and endothelial integrity can be damaged.[9] This process produce proinflammatory cytokine and chemotactic, such as TNF-α, MCP-1, IL-8, IL-6, TGF-β, RANTES and ephitelial neutrophil-activating protein 78 (ENA-78), which activate inflammatory cells, including macrophage.[21] Enhancement of MCP-1 is associated with the presence of macrophage.[5] Sutton *et al*.[4] reported that in initial phase (less than 24hour) cytokine and chemokine are increased, including MCP-1. MCP-1 can be produced by vascular smooth muscle cells.[22] Ischemia condition will stimulate endothelial dysfunction, followed by inflammatory cells infiltration. As a compensated mechanism, smooth muscle tone will be increased, then induces the activation of vascular smooth muscle to produce MCP-1.[22] Therefore, MCP-1 level will be increased and macrophage infiltration will be stimulated. Increase of MCP-1 in exposed group (IR1 and IR12) is significant ($p<0.05$, FIGURE 2.D). This enhancement was followed by increase of macrophage cell significantly ($p<0.05$), which is observed by CD68 immunostaining (FIGURE 2.C).

Repair phase of kidney injury consist of two conditions, complete and incomplete restoration. When injured tissue is restored completely, tubular cell will differentiate and proliferate to replacing the dead cells.[21]

Contrarily, when the repair response is incomplete, it will induce maladaptive process, such as fibroblast proliferation, excessive extracellular matrix deposition, and inflammatory response will be persistent,[21] so that proinflammatory chemokine still produced. It can be found in MCP-1 expression and macrophage level of IR12 group, which still increased (FIGURE 2.C-D).

Kidney IRI effect on vascular remodelling

Renal ischemia affects the renal vascular and tubules. There is morphological and structural changing of renal tubules post-exposure, while auto regulation disturbance and vasoconstriction are the vascular response to ischemia. Furthermore endothelial dysfunction, stimulated by ROS, also play role in vascular maladaptive response.[14] One of endothelial reaction to any type of injury, including ischemia injury, is remodelling of vascular wall.[14] This mechanism involves cell growth, cell death, cell migration and degradation or cellular matrix production.[9] These changes eventually result in intimal accumulation of smooth muscle-like cells and extracellular matrix, medial smooth muscle degeneration, and adventitial fibrosis.[23] The histopathological changes can be observed by thickening of vascular wall and narrowing of lumen area, which increase vascular resistance. Based on the result, there were enhancement of vascular thickness and narrowing of lumen area after one day exposure, then restored after day12 (FIGURE 3.A). Those alteration between IR1 and IR12 group was significant statistically ($p<0.05$, FIGURE 3.D-E). The changes that occurred in IR12 group are considered as restoration mechanism in maintenance phase of ischemia injury.[4]

Vasoconstriction response in ischemia injury is influenced by the presence of ET-1, mediated by ET_AR.[2] Endothelial cells secrete ET-1 as a response to endothelial injury, caused by ischemia.[1] ET-1 can stimulate hypertrophy, migration, and proliferation of vascular smooth muscle cell by transduction signal.[24] Therefore, ET-1 can promote the thickening and narrowing of intrarenal vessel, caused by smooth muscle cell proliferation. ET-1 expression is proportional to the vascular remodelling mechanism. It is consistent with this study, ET-1 expression was increased significantly ($p<0.05$) in IR1 group as well as enhancement of wall thickness and narrowing of lumen area (FIGURE 3.C-E). Those expressions then decrease in chronic group, it signified the presence of repairing process. It was correspond to the previous study conducted by Arfian *et al*.[2] that reduction of the remodelling level is associated with ET-1 deletion in IRI model.

Kidney IRI effect on myofibroblast formation

It has been explained that there is amelioration mechanism which is characterized by cell differentiation and restoration of kidney function. When the injury process is extended and the restoration mechanism is incomplete, it will progress to chronic injury which is observed by the presence of fibrosis in tubulointerstitial area.[12] In acute periode, tubular injury can be compensated by adaptive amelioration process, through inconsiderable fibrosis formation and tubular cell proliferation as compensation to maintain the kidney structurally.[4] Kidney tissue is intact, but the function is reduced. Reduction of kidney function is caused by replacement of fibrotic tissue which is loss of elasticity, proliferation capacity, and differentiation ability.[25] However, adaptive mechanism often continues to be maladaptive response,

supported by persistent inflammatory process.[4] This mechanism is stimulated by hypoxia environment which activate proinflamatory cytokines, profibrotic, growth factor.[13]

Tubulointerstitial fibrosis was illustrated on IR1 and IR12 group significantly ($p<0.05$, FIGURE 4.B). Purplish-red colored area in sirius red staining (FIGURE 4.A) indicates the presence of type I and II collagen.[25] The widest fibrosis area was found in IR12 group (4.927%), represented chronic injury in IRI model (FIGURE 4.B). It was correspond to study conducted by Skrypnyk *et al*.[26] and Varrier *et al*.[27] that reported the highest level of fibrosis was presence in chronic condition and there is TGF-β1 expansion which stimulate myobibroblast activation. The activated myofibroblast will produce and degrade a matrix, then stimulate connective tissue formation in tubulointerstitial area. α-SMA expression, as spesific marker, is used for indicating the presence of myofibroblast.[28] It was found enhancement of α-SMA expression in IR1 and IR12 ($p<0.05$, FIGURE 4.C). Significantly, IR12 group was the most expressed between goups ($p<0.05$) (FIGURE 4.C).

Fibrosis fraction-area and myofibroblast expansion are more progressive on IR12, it is related to ROS which is originated from inflammatory metabolism.[26] ROS can promotes death cell of tubular system and stimulates proliferation factor in interstitial tubular cell.[4] Kim *et al*.[10] showed that enhancement of interstitial cell proliferation parallel to ROS level in kidney tissue. It is proved by increasing of α-SMA, FSP1, dan protein NADPH oksidase-2 expression.[10] The excessive proliferation and expansion of extracellular matrix, followed by apoptotic tubular cell without regeneration mechanism, will accelerate the progressivity of injury.[9,10]

CONCLUSION

Prolonged IRI leads to chronic injury via persistent hypoxia and inflammatory response, signified by myofibroblast formation in tubulointerstitial area. Vascular remodelling and cell proliferation response are reduced in long-term period of injury.

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