

The Effect of Ischemic-Reperfusion on Acute Kidney Injury Pathogenesis to the Glomerular Microscopic Appearance and Cystatin C Level in Wistar White Rat.

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Abstract

Background

Acute kidney injury (AKI) is a sudden decrease of kidney function. The incidence of AKI is increasing every year. One of the causes of AKI is the lack of blood supply to the kidneys (prerenal). At the present time, there are not many studies that report unilateral ischemic-reperfusion (UIR) duration which can cause kidney damage especially glomerulus.

Objective

The aim of this study is to determine the effect of UIR duration to glomerular microscopic appearance, GFR and cystatin C produced in Wistar white rats.

Methods

This study was performed in vivo by using Post-test Only Control Group Design. Unilateral Ischemic-Reperfusion was administered on the rat's left kidney and recovery was done according to specified time. After recovery time, rat's blood was taken, the rat was euthanized, and its kidneys were taken and stained with Picosirius Red coloring. The kidneys were observed by using OptiLAB and the photos were analyzed with ImageJ software. Blood samples were tested by ELISA to measure the cystatin C levels and the levels were converted into Larsson formula to obtain Glomerular Filtration Rate.

Results

The level of cystatin C increased along with the longer duration of UIR and compared inversely proportional to GFR which decreased along with the rise of UIR duration. Cystatin C and GFR had a significant mean difference ($p < 0.05$) with all groups, except for the duration of the UIR group < 60 minutes. The percentage of collagen obtained fluctuated but the whole group which was carried out by UIR had a significantly different collagen amount ($p < 0.05$) with the sham-operated group.

Conclusion

The average glomerular picture showed the addition of collagen, Bowman's capsule thickening and vascular retraction. The longer duration of UIR will worsen the kidney function.

Keywords: Unilateral Ischemic-Reperfusion, Cystatin C, Glomerular Filtration Rate, Collagen Area Fraction, Glomerulus

Background

AKI is a sudden decrease of kidney function (within 48 hours) with specific laboratory and clinical value criteria to help make a diagnosis. The incidence of AKI in hospitalized patients ranges from 2% - 7% with 5% - 10% in patients admitted to the Intensive Care Unit (ICU) due to multiorgan complications and sepsis.¹ The population experiencing Acute Kidney Injury is estimated at 2,000-3,000 patients per million population per year and even continues to increase every year.² AKI still has high morbidity and mortality especially in those who are admitted to the ICU. In addition to high mortality, AKI patients can be at risk of developing Chronic Kidney Disease (CKD) and accelerate the occurrence of End-Stage Renal Disease (ESRD).¹

AKI is most often caused by a disorder of Ischemic-Reperfusion Injury (IRI) which is the result of a lack of oxygen intake, nutrition and disposal of waste products from kidney cells. There is an imbalance between the supply and the need for oxygen in kidney tissue (ischemic) and the accumulation of metabolic waste products.¹ Several studies have suggested that renal vascular fibrosis due to tissue ischemic processes is a regressive process, but glomerular podocytes that are reduced by fibrosis cannot return even after treatment with drugs. This shows that glomerular regeneration depends on the level of damage of the glomerular podocytes.³

Kidney function is measured by using the Glomerular Filtration Rate (GFR). GFR can be calculated from the levels of cystatin C which is converted to the Larrson formula. The use of cystatin C in the measurement of GFR is also more accurate and useful for initial treatment.⁴

Methods

This experimental study was performed with a post test design with a control group in vivo. Wistar white rats aged 10-12 weeks weighing 200-300 grams were obtained from the Eureka Palembang Laboratory, Indonesia with approval from the Research Ethics Committee of the Faculty of Medicine, Sriwijaya University, Palembang, Indonesia. Wistar white rats were placed in different cages by standard feeding and water ad libitum. Rats were acclimatized for 7 days.

This study used 30 wistar white rats which were divided into ten treatment groups with each containing three rats to be treated with Unilateral Ischemic-Reperfusion (UIR) with the clamping of the left renal hilar. The treatment is as follows: Group 1: Sham-Operated, Group 2: 30 minutes UIR + 1 day recovery, Group 3: 60 minutes UIR + 1 day recovery, Group 4: 120 minutes UIR + 1 day recovery, Group 5: 30 minutes UIR + 7 days recovery, Group 6: 60 minutes UIR + 7 days recovery, Group 7: 120 minutes UIR + 7 days recovery, Group 8: 30 minutes UIR + 14 days recovery, Group 9: 60 minutes UIR + 14 days recovery, Group 10: 120 minutes UIR + 14 days recovery.

After the recovery time, rat's blood was taken, the rat was euthanized, and its kidneys were taken. Then the blood was analyzed by using ELISA to determine the levels of cystatin C and converted into the Larrson formula to obtain the Glomerular Filtration Rate. The kidneys that have been taken are fixed with 10% Neutral Buffer Formalin and then histological preparations are made in the form of slides. Next, the kidney slides were colored using Picosirius Red, reddish color which signifies collagen. The preparation was observed using a microscope and photographed using OptiLED® for analysis using ImageJ®. The collagen area fraction will be expressed in percent.

Data on the difference in mean percentage of collagen, cystatin C and GFR were analyzed by version 24 of the IBM® Statistical Package for the Social Sciences (SPSS®) software. Data analysis was performed using the One Way-ANOVA test because the data were normally distributed and then post-hoc tests were carried out using Bonferroni for the variable of cystatin C and GFR because the data were homogeneous while the percentage variable of collagen was tested with post-hoc Games-Howell because the data were not homogeneous. The significance value was set at $p < 0.05$.

Results

Collagen

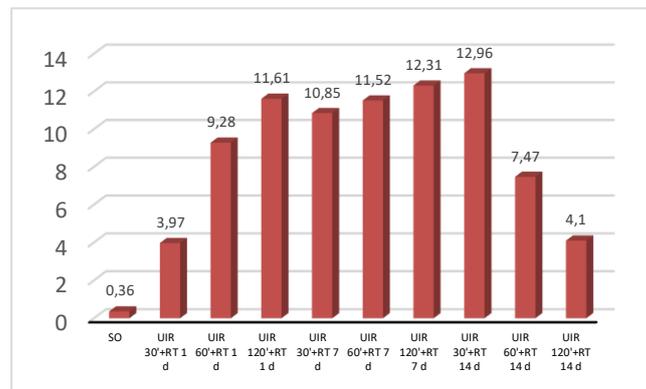


Figure 1. Comparison of the mean percentage of collagen between groups, UIR=Unilateral Ischemic-Reperfusion, RT=Recovery Time, d=day

Figure 1. shows a significant difference between the sham-operated group and other groups ($p=0.000$) using the One-Way ANOVA test. After finding significant differences, the Games-Howell post-hoc test was carried out to ascertain the differences between groups. The results showed a significant mean difference in the ratio of sham-operated with all 1-day-recovery groups, but 7-day-recovery group only differed significantly with the group with 30 minutes of ischemic-reperfusion duration and 14-day-recovery groups at 30 and 120 minutes duration.

Cystatin C

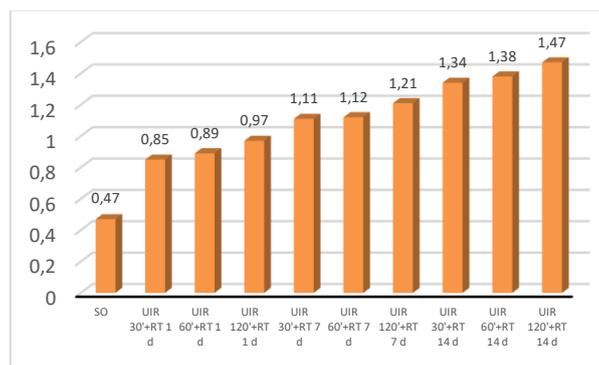


Figure 2. Comparison of mean Cystatin C between groups. UIR=Unilateral Ischemic-Reperfusion, RT=Recovery Time, d=day

Figure 2. shows a significant difference between the sham-operated group and other groups ($p = 0.000$) using the One-Way ANOVA test. After finding significant differences, Bonferroni's post-hoc test was carried out to ascertain the differences

between groups. The results showed significant mean differences in the comparison of all groups except for groups with 30-60 minutes UIR duration and the same duration of recovery.

Glomerular Filtration Rate (GFR)

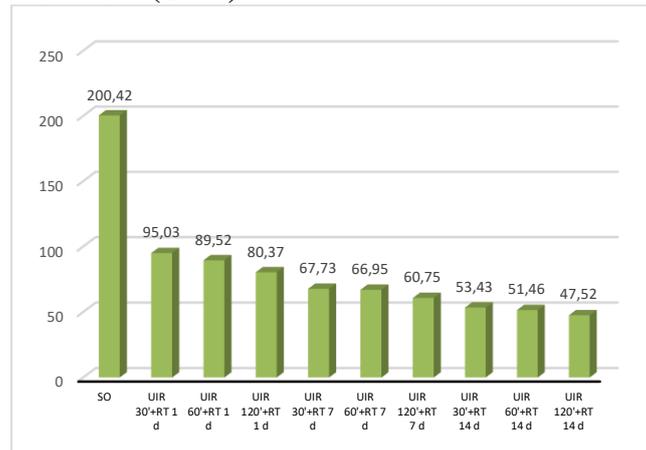


Figure 3. Comparison of mean GFR between groups, UIR=Unilateral Ischemic-Reperfusion, RT=Recovery Time, d=day

Figure 3. shows a significant difference between the sham-operated group and other groups ($p = 0.000$) using the One-Way ANOVA test. After finding significant differences, Bonferroni's post-hoc test was carried out to ascertain the differences between groups. The results showed significant mean differences in the comparison of all groups except the group with 30-60 minutes UIR duration and 1-day-recovery and 7-day-recovery duration and 120 minute UIR group + 14 day-recovery did not differ significantly from 30 and 60 minute UIR with the same recovery.

Glomerulus Microscopic Appearance

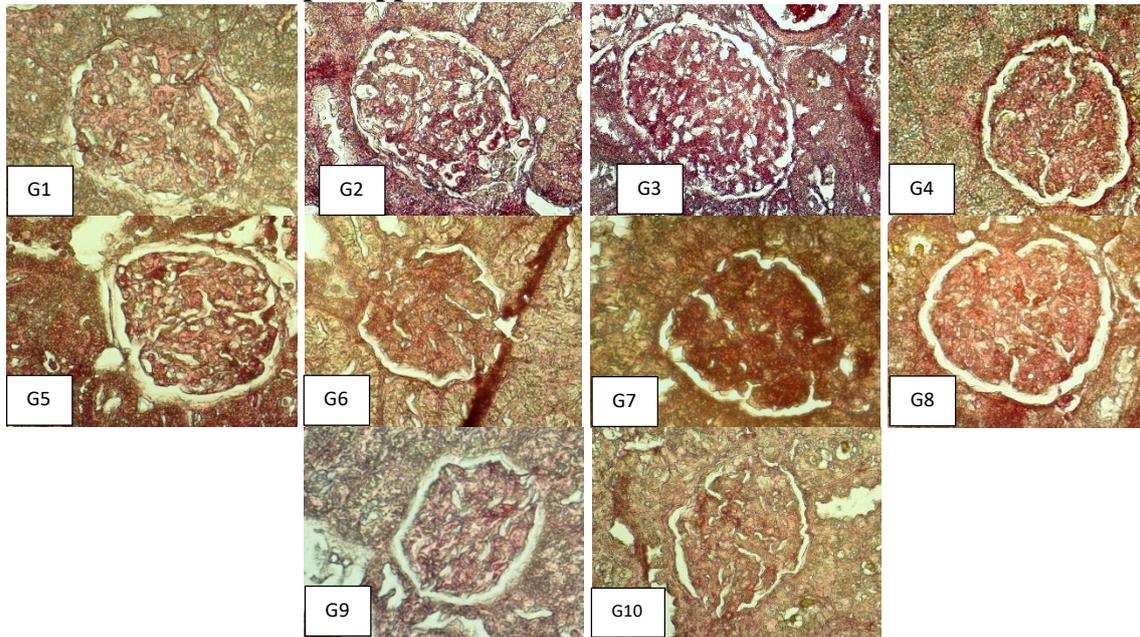


Figure 4. Glomerulus Microscopic Appearance

Figure 4. shows that in the group that was performed a unilateral ischemic-reperfusion procedure, there is a thickening in the basal membrane, increasing amount of collagen, vascular retraction and capsular adhesion when compared to the sham-operated group.

Discussion

Ischemic damage in the human kidney has implications for about 50% of AKI cases with a variety of causes, including hypovolemic conditions, sepsis, cardiopulmonary bypass, and aortic and renal surgery. The physiological healing response of mesenchymal cells to injuries requires the formation of scar tissue. If the injury persists, the wound healing response will lead to fibrogenesis and accumulation of the extracellular matrix.⁵ Although there are specific organ differences in the fibrogenic pathway, certain major elements are characteristic of the fibrogenic response in almost every tissues. Epithelial or endothelial damage will trigger the release of inflammatory mediators and initiate fibrogenic responses.

Ischemic is thought to be the first of many fibrogenic signals that trigger a cascade of cellular and molecular responses that give rise to extracellular matrix formation. Inadequate blood supply to the kidneys causes kidney size to decrease, another

consequence is the hardening of the arteries involving thickening of the intima. The earliest consequences of inadequate renal perfusion include increased production of renin by the juxtaglomerular apparatus with angiotensin II, endothelin-1, transforming growth factor- β (TGF- β) and platelet-derived growth factor-B (PDGF-B) related fibrosis causes. Ang II promotes endothelin-1 secretion in mesangial cells and endothelin-1 induced renal vasoconstriction. Ang II increases the expression of TGF- β gene and mRNA B-chain interstitial PDGF, thus contributing to the formation of extracellular matrix, and especially collagen IV in the renal interstitium.⁶ Loss of vascular mass causes collapse of glomerular tuft. The ischemic in collapsed glomerular tuft can shows disorder on the viability of podocytes and attaches to the glomerular basal membrane. Renal ischemia causes vasoconstriction of the glomerular efferent arterioles by widening the tuft, which exerts physical traction on the podocytes. Ischemia also causes a high increase in angiotensin II which causes changes in podocytes.⁶

Decreased remnants of the nephron, including the glomeruli, can be completely removed and replaced by fibrous tissue. Thus, with nephron degeneration, the interstitial damage score will increase, while the glomerular damage score will remain constant or even decrease due to the loss of the total sclerotic glomeruli. More recent studies clearly show that decreased kidney function in chronic kidney disease correlates best with the number of nephrons remaining. If there is a glomerular injury, loss of podocytes will increase the loss of other podocytes.⁸

The structure of the glomerular matrix consists of varying proportions of type IV and V collagen, laminin, fibronectin, entactin, and glycosaminoglycan sulfate among other components. Type I, II and III collagen are interstitial collagens or fibrillary collagens which are the highest amount. Types IV, V and VI are non-fibrillary forms and are found in interstitial tissue and basal membrane. Kidney fibroblasts secrete type IV collagen, which increases when fibroblasts are stimulated with TGF- β .⁹ The functional properties of the matrix can be altered by changes in composition without variation. Expansion of the extracellular matrix compartment is a key feature of the prolonged glomerular inflammatory process, often leading to sclerosis and obliteration of glomeruli.¹⁰ The frequency of cells expressing collagen does not provide information about the amount of collagen produced by certain cell types. Resident cells

(fibroblast/pericytes) occur early in collagen I production, while hematopoietic cells follow later, most likely because they need time to migrate to the kidneys. Kidney function also increases when collagen I is specifically lost in hematopoietic cells. There are several interactions between fibroblasts, collagen-producing hematopoietic cells (fibrocytes), and inflammatory cells that promote or inhibit fibrosis.¹¹

In this study, the amount of collagen increased with the addition of ischemic-reperfusion duration but decreased in the 14-day-recovery group. The absolute quantity of extracellular matrix formation represents the dynamic balance between synthesis and degradation. In mesangial cells of the kidneys, autophagy will improve fibrosis by reducing collagen. In renal fibrosis, autophagy induction during kidney damage protects mesangial cells and increases their presence. The disruption of the essential Atg7 autophagy gene with specific RNA has been associated with a decrease in type I collagen levels. Autophagy has potential during kidney injury as an inducer of synthesis and degradation of collagen type 1.¹²

Autophagy shows an important role for the presence of fibroblasts and high autophagy levels have been reported in fibrogenic cells in almost all body tissues such as stellate cells, cardiac fibroblasts, mesangial cells, skin fibroblasts and synovial fibroblasts. Autophagy is a catabolic pathway that is important for cellular energy homeostasis which involves self-degradation of the intracellular component in lysosomes. The fibrogenic response by blocking autophagy in fibrogenic cells of the kidneys and lungs shows that the autophagy consists of core fibrogenesis pathways and antifibrotic candidates can be targeted. Integrin represents the main communication method among the extracellular matrix, inflammatory cells, fibroblasts and parenchymal cells involved in the process of initiation, maintenance and resolution of tissue fibrosis. Integrin is the transmembrane protein and main receptor for cell adhesion to extracellular matrix proteins and cell-to-cell adhesions. This molecule can mediate the translation of extracellular signals into various cell forms including cell adhesion, migration, proliferation, differentiation and apoptosis. The expression of integrin $\alpha\beta6$ is low in the normal kidney, but this induction of integrin causes kidney disease associated with chronic inflammation and fibrosis.¹³

Thickening of the glomerular basal membrane can occur due to spasm of capillary clots or ischemic due to atrophy. Although changes are distributed in the arterial supply area, more severe changes occur in the outer cortex. The glomerulus tends to gather in the subcapsular area of the kidney. Capillary clots in the glomerulus become dense due to the collapse of capillaries. The glomerular basal membrane appears to constrict around the collapsing capillaries. Ischemia due to inadequate blood flow causes overall glomerular collapse. Glomerular mesangial cells react strongly with the anti-actomyosin smooth muscle and cells, which are considered mesangial cells, growing in tissue culture and having prominent intracellular fibrils containing actomyosin. The axial distribution of mesangial cells in the glomerulus and their anatomic relations close to the capillaries will allow these cells to have a considerable effect on glomerular blood flow. Mesangial contractions can also cause glomerular capillaries collapse, capillary closure of peripheral fibers by mesangial contractions can result in shunting of blood through anastomosis within and between the glomerular lobules. The mesangial area appears to be widened in part due to condensation of the capillary basement membrane in glomerular tuft.¹⁴

As the basement membrane thickens around the collapsing capillary, glomerular tuft is converted to a protein mass. Initially, the Bowman capsule contracted around the shrinking glomerular bundle so the urinary space did not increase too much and the ratio of the beam area or total glomerular area remained very constant. Bowman's capsule basal membrane thickened. In some glomeruli, collagen is deposited internally into the capsular basal membrane. Initially it appeared around the hilum but then spread to fill Bowman's cavity. Some cells, which originate from the capsule, are trapped in fibrous tissue that proliferates and the basal membrane clump is replaced by collagen.¹⁴

Overall, the measurement of kidney function is using GFR.⁷ Research shows that cystatin C serum increases when GFR is 88 ml / minute where creatinine changes can be detected when the GFR decreases to 75 ml / minute so that the variance of GFR is more reflected by cystatin C.¹⁵ Cystatin C is a protein produced by all nucleated cells at a constant rate that is used to be a marker of kidney function because cystatin C is a low-weight molecular protease inhibitor that is freely filtered in the glomerular membrane and then reabsorbed and metabolized in the proximal tubule of the kidney so it does not return to blood.¹⁶ Thus the Cystatin C level in blood represents GFR. When the GFR decreases

due to catabolism in the proximal renal tubules, cystatin C begins to increase.^{4,15} A previous study shows a strong correlation of calculated GFR from cystatin C, suggesting that cystatin C better describes a decrease in GFR.⁴

A severe decrease in glomerular filtration rate (GFR) associated with ischemic kidney injury that occurs is due to the combined effect of changes in intrarenal hemodynamics and tubular injury. Hemodynamic changes associated with AKI include afferent arteriolar narrowing and mesangial contractions, both of which directly reduce GFR. Afferent arteriolar vasoconstriction will reduce glomerular plasma flow, while mesangial contraction will reduce the availability of glomerular filtration surface area for filtering and decreasing glomerular ultrafiltration coefficient (Kf).¹⁷ Mesangium regulates a single glomerular nephron filtration rate (SNGFR) by changing glomerular ultrafiltration coefficient (Kf). Mesangium is surrounded by capillary loops. Mesangial cells are special pericytes with contractile elements that can respond to vasoactive hormones. Mesangium contractions can close and prevent the perfusion of glomerular capillary loops that are anatomically related. This reduces the available surface area for glomerular filtration and reduces glomerular ultrafiltration coefficients.¹⁷

Conclusion

The longer the duration of unilateral ischemic-reperfusion, the lower kidney function was characterized by an increase in cystatin C levels and a decrease in GFR and an increase in glomerular collagen except in the 14-day-recovery group where collagen decreased in 60 and 120 minutes UIR compared to 30 minutes. In addition, glomerular microscopic images showed basal membrane thickening, vascular retraction and capsular adhesion in the UIR treatment group when compared to the sham-operated group.

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