Serum and urinary neutrophil gelatinase-associated lipocalin as a predictor of rat kidney histopathology in an early ischemia-reperfusion model

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Abstrak

Latar belakang: *Tingkat keparahan cedera ginjal iskemia-reperfusi (I/R) berhubungan erat dengan tingginya angka kesakitan dan kematian. Hasil penelitian terdahulu pada manusia dan hewan telah mebuktikan bahwa* Neutrophil Gelatinase Associated Lipocalin *(NGAL) dapat mendeteksi dan memprediksi terjadinya cedera ginjal I/R dini. Tujuan penelitian ini adalah untuk membuktikan bahwa peningkatan kadar NGAL serum dan urin berhubungan dengan kerusakan epitel tubuli ginjal pada tikus yang mengalami iskemia reperfusi dini.*

Metode: *Peneltian ini menggunakan 28 ekor tikus* Sprague-Dawley *jantan sebagai hewan model, dikelompokkan dalam 4 kelompok: sham 4 jam (Sham 4), sham 8 jam (Sham 8), iskemia 10 menit reperfusi 4 jam (I/R 4), dan iskemia 10 menit reperfusi 8 jam (I/R 8). Analisis kadar kreatinin serum diperiksa dengan metode Jaffe, sedangkan NGAL serum dan urin menggunakan metode* ELISA Direct Sandwich*. Evaluasi tingkat kerusakan jaringan ginjal dilakukan secara semi kuantitatif pada sediaan histologi dengan pulasan HE. Deskripsi kelainan tingkat seluler ginjal diperjelas melalui evaluasi menggunakan mikroskop elektron dan Imunohistokimia (IHK).*

Hasil: *Kadar NGAL serum berkorelasi bermakna dengan tingkat kerusakan ginjal (ρSpearman NGAL serum = 0,701, p < 0,001), juga kadar NGAL urin berkorelasi bermakna dengan tingkat kerusakan ginjal (ρSpearman = 0,689, p < 0,001). Tingkat ekspresi NGAL lebih tinggi pada kelompok I/R dibanding sham (t-test, t = -26635,046, p < 0,001), juga tingkat kerusakan ginjal tikus (t-test, t = -5,028, p < 0,001), dan kadar NGAL serum dan urin pada kelompok I/R berbeda nyata dibanding sham (Mann-Whitney, U = 0, p < 0,001). Pada* cutoff point *136,95 ng/mL dan 58,69 ng/mL berturut – turut untuk NGAL serum dan urin diperoleh sensitivitas = 1, spesifisitas = 1.*

Kesimpulan: *Peningkatan kadar NGAL serum dan urin berkorelasi dengan kerusakan epitel tubuli ginjal pada tikus yang mengalami cedera ginjal iskemia reperfusi dini.* ***(Med J Indones. 2012;21:208-13)***

Abstract

Background: The severity of ischemia-reperfusion (I/R) kidney injury is highly correlated with mortality and morbidity rate. Research on human and animal prove that NGAL predicts kidney injury at early phase. The objective of this study is to prove that the increase in serum and urinary NGAL are correlated with kidney tubular epithelial damage, and this increase has occurred in initiation phase, indicated by rat kidney histopathology in an early I/R model.

Methods: Twenty eight male Sprague-Dawley rats were divided into 4 groups: 4 hour sham (Sham 4), 8 hour sham (Sham 8), 10 minute ischemia 4 hour reperfusion (I/R 4) and 10 minute ischemia 8 hour reperfusion (I/R 8). Blood, urine and kidney samples were collected. Serum creatinine level was analyzed with Jaffe method, while serum and urinary NGAL level were analyzed with direct sandwich ELISA method. Evaluation of kidney damage were measured semi quantitatively in tissue stained with HE. Further evaluation to confirm cellular changes on kidney was performed by electron microscope and immunohistochemistry.

Results: Serum NGAL was found significantly correlated with degree of kidney tissue damage (ρSpearman NGAL serum = 0.701, p < 0.001), also urinary NGAL (ρSpearman = 0.689, p < 0.001). NGAL expression differs significantly between I/R group and sham (t-test, t = -26635.056, p < 0.001), also kidney damage (t-test, t = -5.028, p < 0.001), and serum and urinary NGAL levels (Mann-Whitney, U = 0, p < 0.001). With cutoff points of 136.95 ng/mL and 58.69 ng/mL subsequently for serum and urinary NGAL , it is found that sensitivity = 1, specificity = 1.

Conclusion: Elevation of serum and urinary NGAL are significantly correlated with epithelial tubular kidney damage on rat undergoing early ischaemia reperfusion. ***(Med J Indones. 2012;21:208-13)***

Keywords: *Early I/R kidney injury, kidney histopathology, NGAL*

Acute Kidney Injury (AKI) represents very important and potentially devastating disorders in clinical practice. Its prevalence varies from 5% of all patients admitted to hospital to 30 – 50% of those in intensive-care units. Despite substantial technical improvements in treatments, mortality and morbidity associated with AKI remain dismally high, more than 50% in patients admitted to intensive-care units. Overall incidence of AKI was 24 cases per 1000 discharge, with rate increasing by 11% yearly.[1] Dialysis-requiring AKI was

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independently associated with a 28-fold increase in the risk of developing stage 4 or 5 chronic kidney disease (CKD), and more than 20% become dialysis dependent in 3 years.[2]

Hospitalized AKI patients with minimal increase of serum creatinine of 0.3 mg/dL associated with 7 fold of risk of death.[3] The current concept and diagnosis of AKI are mainly based on increase in serum creatinine indicating loss of excretory renal function. Increase of serum creatinine is used in RIFLE classification of AKI and by Acute Kidney Injury Network consensus criteria. Unfortunately serum creatinine is a delayed and unreliable indicator of AKI for a variety reasons. First, even normal serum creatinine is influenced by many non-renal factors such as gender, age, body weight, medications, hydration status, nutrition status and kidney tubular secretion. Second, Due to the concept of renal reserve, it is estimated that over 50% of kidney function has been lost when serum creatinine rises. Third, several hour or days must elapse before study state production and decreased excretion of creatinine is established. Fourth, alterations in serum creatinine represents and may lag behind a functional change in glomerular filtration rate (GFR), and this in turn lags behind important structural changes that occur in the kidney during the early damage of AKI.[4]

Animal studies identified some interventions can prevent and treat AKI if given at early stage before the serum creatinine begins to rise. Lack of early biomarkers has hindered our ability to translate these promising therapy to human. Detecting this early stage of damage requires emerging structural biomarkers. Since AKI is largely asymptomatic, this early biomarkers allow early detection of AKI, which allow timely therapeutic interventions and in turn to allow better prognosis.

A biomarker that is released into the blood or urine by the injured kidney is analogous to the troponin release by injured by myocardial cells, that is more sensitive and specific marker of early AKI than serum creatinine is urgently needed. Earlier detection of AKI with a specific biomarker may result in earlier nephrology consultation, more optimal dosing of antibiotic, avoidance of nephrotoxic agents and even earlier specific therapy to repair the damage kidney. An ideal biomarker of AKI would allow the earlier detection of kidney injury before an increase in serum creatinine; would differentiate the etiology of AKI; would be able to monitor the effects of an intervention or treatment; and would predict the need for dialysis, mortality and long-term kidney outcome.

The criteria for an ideal protein biomarker for AKI would include: (1). the protein must originate from the injured organ; (2). the amount of the protein in the biofluid must be proportional to its expression in the injured organ; (3). the biomarker should be temporarily related to the inciting stimulus, so as to alert the clinician to a potentially reversible of the illness; (4). the expression of biomarker should rapidly decrease in amount when the acute phase of injury has terminated; (5); the biomarker should be critical component of organ pathophysiology. Several promising biomarkers have been studied in animal models of AKI and in human, such as: NGAL, IL-18, KIM-1, Cystatin C, and L-FABP.[5]

Ischemia is known as the most frequent cause of AKI in human. In 20 children undergoing cardiopulmonary bypass[6] and developed ischemic kidney injury, but diagnosis with serum creatinine was only possible 1–3 days after. Serum and urinary NGAL increased significantly form baseline 2 hour after the procedure and begin to decrease after 4 hour. Experiment on Swiss-Webster mice with 10 ischemia shows urinary NGAL has increased after 4 hour reperfusion and peaked at 12 hour reperfusion. Sprague-Dawley rats with 30 minute ischemia shows urinary NGAL has increased after 3 hour reperfusion.[7] These results shows that ischemia and reperfusion periods has a role on NGAL levels increase.

NGAL as an AKI biomarker has successfully passed the pre-clinical, assay development and clinical testing, as a promising AKI early biomarker. It is now in the screening stage, facilitated by the commercial tool for measurement of NGAL on large population in a point of care setting. NGAl has shown to be good in predicting AKI, as a functional biomarker; however there is a need to show the correlation of the NGAL level increase in serum and urine with rat kidney histopathology in an early ischemia-reperfusion model.

METHODS

The study is experimental type with animal model of 28 male Sprague-Dawley rats, weight 250-300 grams. The rats divided into 4 groups, each of 7, those groups are: sham 4 hours (Sham 4), sham 8 hours (Sham 8), ischemia 10 for minutes and reperfusion for 4 hours (I/R 4), and ischemia 10 for minute and reperfusion 8 for hours. Reperfusion time of 4 hours were chosen to ensure that reperfusion has increased NGAL levels and 8 hour reperfusion time were chosen as an estimation time before NGAL reach its peak level.

To induce ischemia in I/R 4 and I/R 8 the bilateral renal arteries were clammed for 10 minutes, and then

clams were released, the incisions were sutured, and after 4 hour and 8 hour reperfusion time, for I/R 4 and I/R 8 respectively, urine were collected via urinary space puncture, blood were collected via cardiac puncture, and afterwards the rats were re-anesthetized and then euthanasia were performed, the kidneys were harvested. For the Sham 4 and Sham 8 groups, clamping of renal arteries were not performed, they were only incised on the front abdomen, renal arteries identified, and the incision were closed by sutures, after 4 and 8 hours, urine and blood were collected the same way as in I/Rs groups, re-anesthetized and then euthanasia were performed, the kidneys were harvested.

Serum creatinine, serum NGAL and urinary NGAL were measured. Kidney tissue was stained with HE, kidney NGAL expression was stained with immunohistochemistry method. This study was performed in Centre of Primate Study (*Pusat Studi Satwa Primata* / PSSP), Bogor Agriculture Institute and Department of Anatomical Pathology, Faculty of Medicine University of Indonesia, Jakarta, in October 2010 to May 2011.

Serum and urinary NGAL were assayed with ELISA, serum creatinine with colorimetric photometry Jaffe method. NGAL expression in kidney tissue were calculated with semi quantitative method based on the expression intensity, range from (-) to (++++). The amount of NGAL expression observed and calculated in 6 fields with 40 x magnification for each specimen. Six indicator of kidney damage according to histopathological changes were identified and scored semi-quantitatively based on the severity of its presence: thinning or loss of brush border, score (+), denuded area of tubular membrane base (++), tubular cast (++), tubular dilatation (++), interstitial edema (+++), and tubular necrosis (++++). The amount of the six histopathological changes calculated in 6 fields with 40 x magnification for each specimen.

The correlation between serum and urinary NGAL and kidney damage were calculated with Spearman's rho. To find significant differences between the four groups on levels of creatinine, serum NGAL, urinary NGAL, and kidney damage, Kruskal-Wallis tests were performed. To study the effects of ischemia-reperfusion on NGAL levels raise and kidney damage Sham 4 and Sham 8 were joined to constitute Sham group, and I/R 4 and I/R 8 were joined to constitute I/R group. Mann-Whitney U test were performed to find significant difference on serum and urinary NGAL levels between Sham and I/R, and *t*-test were performed on kidney damage levels. Sensitivity analysis also performed to evaluate the performance of serum and urinary NGAL as diagnostic test in predicting early kidney I/R injury.

RESULTS

For serum creatinine levels, there is no significant difference between the four groups (Kruskal-Wallis; $\chi^2 = 2.446$, p = 0,485). It is concluded that I/R treatment did not increase the serum creatinine levels. However, for serum NGAL levels (Kruskal-Wallis, $\chi^2 = 23.369$, $p < 0.001$), urinary NGAL levels (Kruskal-Wallis, $\chi^2 = 23.825$, p<0,001), and NGAL tissue expression (ANOVA, F = 10,530.046, $p < 0.001$), there are significant differences were found between the 4 groups. And also for kidney damage (ANOVA, F = 9,101, p < 0,001)there is significant differences were found between the 4 groups (Table 1).

Significant correlations were found between serum NGAL levels and kidney damage ($\rho_{Spearman} = 0.701$, p < 0,001), and between urinary NGAL levels and kidney damage ($\rho_{Spearman} = 0.689$, $p < 0.001$).

Increased of serum and urinary NGAL levels have occurred in I/R group (Mann-Whitney U = 0, $p < 0.001$). In this I/R group, the kidney damage shows significant difference with Sham (*t*-test, $t = -5.028$, $p < 0.001$).

Reperfusion has different effect between serum NGAL and urinary NGAL levels In I/R group. Serum NGAL levels unexpectedly lower in I/R 8 group, while urinary NGAL levels were higher in I/R 8 group than in I/R 4 group (Figure 1).

Table 1. Levels of serum creatinine, serum and urinary NGAL, NGAL expression, and kidney damage on each group

	Sham 4	Sham 8	I / R 4	I / R 8
Serum Creatinine (mg/dL)	0.74 ± 0.10	0.74 ± 0.11	0.71 ± 0.04	0.79 ± 0.11
Serum NGAL (ng/mL)	28.80 ± 24.89	84.20 ± 10.32	325.12 ± 61.41	274.15 ± 63.10
Urinary NGAL (ng/mL)	11.54 ± 42.62	41.90 ± 15.56	128.29 ± 61.95	235.14 ± 97.66
NGAL expression (%)	1.30 ± 0.28	1.096 ± 0.33	73.53 ± 1.58	94.74 ± 2.80
Kidney damage (%)	0.64 ± 0.39	0.75 ± 0.58	1.67 ± 0.79	2.07 ± 0.67

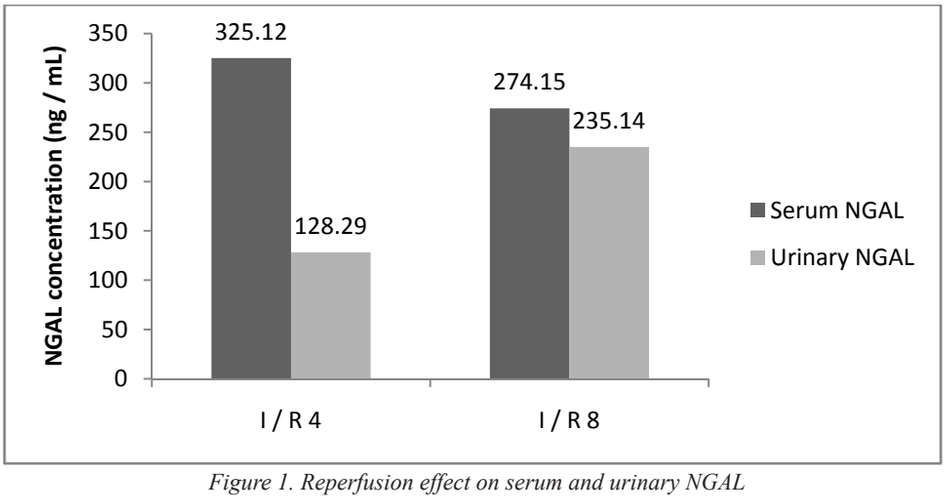

Figure 1. Reperfusion effect on serum and urinary NGAL

On doing senitivity analysis serum and urinary NGAL were set as test values and kidney damage in Sham and I/R groups were set as gold standard on performing sensitivity and specificity analysis. With cut off points of 136.95 ng/mL and 58.69 ng/mL for serum and urinary NGAL respectively, it is found that sensitivity = 1, specificity = 1. The AUC = 1 were found for both biomarkers.

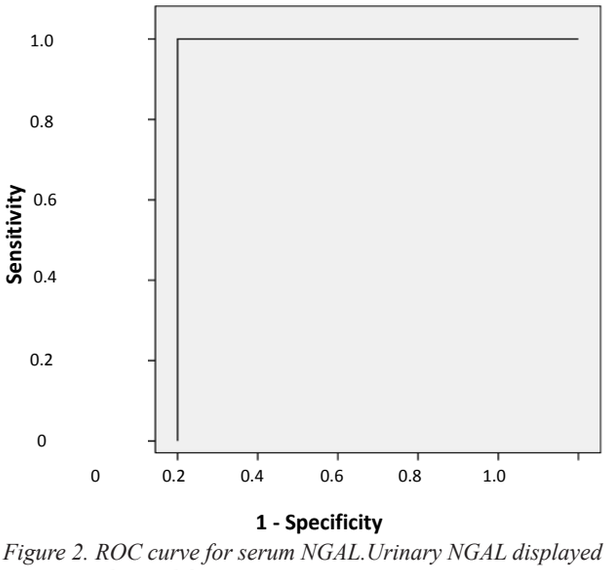

Figure 2. ROC curve for serum NGAL.Urinary NGAL displayed identical form

DISCUSSION

Animal model of I/R kidney injury

The I/R treatment has created early I/R kidney injury model, in which kidney histopathology change has occurred while serum creatinine levels not yet increased. Ten minute ischemia followed by 4 and 8 hours reperfusion was determined after pilot study was done. In the pilot study, 15 and 20 minute ischemia resulted in increased serum creatinine levels.

Serum and urinary NGAL correlate strongly with the degree of rat kidney tissue injury

Increase of serum and urinary NGAL have a good correlation with the degree of kidney injury. This shows that the increase of serum and urinary NGAL concentration has a role as a good predictor of kidney tissue injury. In a clinical study by Haase et al[8] in hospitalized patients with increased serum NGAL in the absence of increase in serum creatinine, NGAL detects patients with likely subclinical AKI who have an increased risk of adverse outcomes, implying important biochemistry and molecular morphology might be present before the filtration function disturbance. The author proposed that the concept and definition of AKI might need re-assessment and also proposed "*Future prospective studies should confirm histopathological agreement of NGAL with tubular injury.....*".

Serum and urinary NGAL levels increased in the early phase of kidney tissue injury

This study showed that the difference in serum and urinary NGAL level between injured and normal kidney tissue, those between Sham and I/R group of rats. Serum and urinary NGAL levels increased 6 and 7 fold respectively in I/R group of rats, compared to Sham, while the degree of kidney tissue injury is almost 3 fold compared to Sham. Urinary NGAL level in I/R 8 group significantly higher than in I/R 4 group, meaning that longer reperfusion time increases production NGAL in kidney tubular tissue. This shows NGAL has a role in the process of recovery in kidney tissue damage.

The lower serum NGAL levels in I/R 8 group than in I/R 4 were unexpected. One possible explanation for this result is the diminishing role of interleukin-1β due to ATP depletion.

This study has successfully identified a phase of early kidney tissue injury in which NGAL levels have been increased compared to that of normal kidney. This phase is important to identify in taking preventive measures on high risk patients to AKI, such as diabetic patients, hypertensive patients, elderly patients, post-kidney transplantation patients, post-CPB patients, and in ICU patients. This is a phase of early kidney injury proposed by Jo et al[9] *as a narrow therapeutic window,* a short phase that should be sought and recognized in which preventive intervention showed beneficial effects in animal model of AKI, and could be a prospective phase for therapeutic clinical trial in translational research.

Kidney tissue damage in I/R group

Light microscopy with HE staining shows tubular kidney damage less than 5% in I/R group but no significant differrent found between I/R 4 and I/R 8. The damage dominated by loss of brush border, which is the mildest structural changes found in kidney tubular injury. This shows that the damage were still in initiation phase. This study has identified an early phase of AKI in which serum and urinary NGAL increased, tubular damage ocured, while serum creatinine level is in normal range. This early phase of AKI is important to identifed in order to be able to modify the risk factor for AKI, such as in diabetics, hypertensives, and critically ill patients. Jo et al[9] called this phase as narrow therapeutic window. They showed in experimental study, interventional therapy given in this early phase had a good result.

NGAL kidney tissue expression increases strongly in early kidney injury

NGAL kidney tissue expression in I/R group of rats increases 66 fold compared to Sham group. And the intensity of NGAL expression in proximal and distal tubule, is proportionately to the severity of injury. Increase of NGAL in kidney injury come about in serum, urine and kidney tissue, implying that NGAL could be an ideal biomarker with an excellent technique on reporter mouse with I/R AKI, showed in real time the expression of NGAL in serum, kidney tissue and urine simultaneously. The criteria for an ideal protein biomarker for AKI would include: (1). the protein must originate from the injured organ; (2). the amount of the protein in the biofluid must be proportional to its expression in the injured organ; (3). the biomarker should be temporarily related to the inciting stimulus, so as to alert the clinician to a potentially reversible if the illness; (4). the expression of biomarker should rapidly decrease in amount when the acute phase of injury has terminated; (5); the biomarker should be critical component of organ pathophysiology.[10]

Serum and urinary NGAL as a powerful diagnostic test

Using degree of injury in Sham and I/Rgroups as gold standard, in ROC analysis, perfect value of AUC = 1 were found for serum NGAL and also for urinary NGAL, and both sensitivity and specificity = 1. The very strong association of serum and urinary NGAL levels and the degrees of injury between Sham and I/Rgroup should be an explanation to overlapping of the sensitivity and the specificity. It is concluded that as a diagnostic test, serum NGAL and urinary NGAL

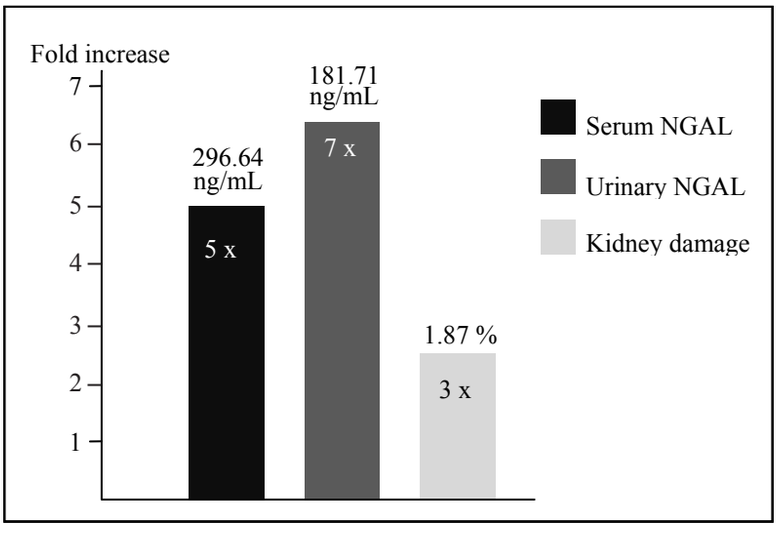

Figure 3. NGAL and kidney damage in I/R group fold increase over Sham

performance are equal, both are powerful. A study by Mishra et al found at 2 hours after cardiac surgery serum NGAL AUC was 0.906 and for urinary NGAL AUC was 0.998, sensitivity was 1, and specificity was 0.98. [6]

In, conclusion, serum and urinary NGAL levels increased in initiation phase of I/R kidney injury, and in agreement with degree of kidney tissue damage. NGAL serves as an ideal structural biomarker and possess powerful performance as a diagnostic test for early kidney injury: increased while kidney function is still normal, sensitive and specific, expressed in the injured kidney tissue, proportionally with the severity of the injury. Urinary NGAL level increase seems to be more consistent than of serum NGAL in its general performance as a biomarker for early kidney injury.

Serum and urinary NGAL should be serially examined in patients with high risk factor for kidney injury. Clinical platform for NGAL examination with *Point of Care Testing,* quick and practical method should be available in emergency departments and intensive care units.Consideration should be given to include NGAL levels in formulating AKI criteria.

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