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Effective Biomarkers for Miniature Pig in Acute Kidney Injury Using Renal Ischemia-Reperfusion Model

Se-Eun Kim, Kyung-Mi Shim, Seok-Hwa Choi* and Seong-Soo Kang[1]

College of Veterinary Medicine, Chonnam National University, Gwangju 500-757, Korea
**College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Korea*

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Abstract : Acute kidney injury (AKI) is a serious problem associated with high morbidity and mortality. Ischemia-reperfusion is an important cause of acute kidney injury. This study was performed to ascertain clinically useful biomarkers for the diagnosis of AKI. In three miniature pigs, AKI were induced by 60 minutes of bilateral renal ischemia by the clamping renal artery. Blood and urine samples were collected from the pigs prior to clamping (baseline) and 0, 1, 3 and 5 days post-clamping. Serum blood urea nitrogen (BUN), creatinine, sodium and uric acid were measured in serum and urine samples. Fractional excretion of sodium (FE_{Na}) and fractional excretion of uric acid (FE_{UA}) were calculated. Also, interleukin (IL)-6, IL-18, liver type fatty acid binding protein (L-FABP) and glutathione-S-transferase (GST) were detected by Western immunoblotting. Serum BUN and creatinine levels were increased significantly at day 1 post-clamping in all three miniature pigs. However, FE_{Na} and FE_{UA} showed marked individual differences. Western immunoblotting revealed significantly increased levels of IL-6, IL-18, L-FABP and GST in post-ischemic urine, compared to pre-clamping. While more research concerning the variance of FE_{Na} and FE_{UA} is needed, serum BUN, creatinine, IL-6, IL-18, L-FABP and GST may be sensitive urine biomarkers for diagnosis of AKI together with other biomarkers in the porcine ischemia-reperfusion model.

Key words **:** acute kidney injury, biomarkers, renal ischemia and reperfusion, miniature pig.

Introduction

Acute kidney injury (AKI) is commonly defined as a sudden and continuous decrease in renal function. It is induced by the steady accumulation of nitrogenous and non-nitrogenous products and toxins, with rapid development of fluid, electrolyte and acid-base disorders (15). AKI has a high rate of incidence and mortality, which has prompted search for effective treatment (15).

Among the several causes of AKI, ischemia-reperfusion that induces severe tissue damage is a major cause (14,15). Renal ischemia-reperfusion injury (IRI) is often observed in renal artery stenosis, sepsis and surgical operations like cardiac surgery and kidney transplantation. Renal IRI causes renal tubular epithelial cell damage characterized by impaired renal function like tubular reabsorption of sodium and urinary concentration (7,18).

The standard clinical biomarkers for the detection of AKI are serum creatinine, urea and urine output. When AKI occurs, management in the early stage is important. However, standard biomarkers have severe limitations for early detection of AKI because they appear after the progression of renal damage (15,16). Thus, many new biomarkers have been developed recently including enzymes, growth factors, cytokines, adhesion molecules and coagulation factors (1,12). Fractional excretion of sodium (FE_{Na}) is another sensitive diagnostic biomarker for diagnosis of AKI in humans. Fractional excretion of uric acid (FE_{UA}) has been suggested as a good diagnostic biomarker in patients treated with diuretics (8).

In this study, we investigated the clinical usefulness of serum blood urea nitrogen (BUN), creatinine, FE_{Na}, FE_{UA}, interleukin (IL)-6, IL-18, liver type fatty acid binding protein (L-FABP) and glutathione-S-transferase (GST) as diagnostic markers in a porcine ischemia-reperfusion model of induced AKI.

Materials and Methods

Experimental animals

One-year-old, three female miniature pigs were provided from PWG Genetics Korea. The pigs were acclimated and maintained on a standard pig diet with ready access to water. They were housed in an air-conditioned room with a 12 hr light-dark cycle and controlled temperature (23 ± 2ºC) and humidity (55 ± 10%). These experimental and housing protocols were approved by the Chonnam National University (CNU).

Induction of ischemia-reperfusion renal injury

The miniature pigs were fasted for 12 hours prior to sur-

[1]Corresponding author.
E-mail : vetkang@chonnam.ac.kr

gery to prevent any possible adverse effects associated with anesthesia. The animals were premedicated intramuscularly with atropine sulfate (Atropine Sulfate; Huons, Korea, 0.04 mg/kg) and a combination of xylazine hydrochloride (Rompun®; Bayerkorea, Korea, 2.2 mg/kg) and tiletamine/zolazepam (Zoletil®; Virbackorea, Korea, 6 mg/kg). After that, an intravenous line was introduced into an ear vein. Anesthesia was maintained with isoflurane (Forane®; JW Pharmaceutical, Korea) and 100% pure oxygen supply after tracheal intubation. Laparatomy was performed by midline incision and bilateral renal arteries were clamped to induce AKI. After 60 minutes of ischemia, the vessels were unclamped.

Blood sampling and analysis

Blood samples were collected via venipuncture from the jugular vein at pre-clamping (baseline) and post-clamping days 0, 1, 3 and 5. Each sample was centrifuged at 3000 rpm for 10 minutes and pipetted off the top serum layer. Serum BUN levels were measured from serum samples using a VetTest Chemistry analyzer (VetTest Chemistry analyzer®; IDEXX Laboratories, UK). Urine samples were collected by inserting a catheter through the urethra into the bladder at pre-clamping and post-clamping days 0, 1, 3 and 5. Creatinine and uric acid levels were measured from serum and urine samples using the VetTest Chemistry analyzer (VetTest Chemistry analyzer®; IDEXX Laboratories, UK). Likewise, sodium levels were measured from serum and urine samples using an electrolyte analyzer (SPOTCHEM SE-1510; Menarini, Italy) equipped with ion-specific electrodes.

Calculation of FE_{Na} and FE_{UA}

FE_{Na} was calculated as: (urine sodium ÷ serum sodium) ÷ (urine creatinine ÷ serum creatinine). FE_{UA} was calculated as: (urine uric acid ÷ serum uric acid) ÷ (urine creatinine ÷ serum creatinine).

Western immunoblotting analysis

IL-6, IL-18, L-FABP and GST were detected by Western immunoblotting in urine samples for AKI biomarker. IL-6, GST and anti-gout IgG antibodies were purchased from Santa Cruz Biotechnology (USA). Anti-rabbit IgG antibody was purchased from Cell Signaling Technology (USA). IL-18 and L-FABP antibodies were prepared in our laboratory by protein injection in rabbits. Each urine was added four times the sample volume of cold (−20°C) acetone and incubated for 60 minutes at −20°C. Precipitated protein was isolated by centrifugation at 13,000 × g and the pellet was washed in acetone and air dried pellet was dissolved in 1 ml of sodium dodecyl sulfate (SDS) sample buffer. Twenty microliters of sample were loaded in different lanes, and analyzed by 14% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Twenty microliter aliquots of the urine samples were also separated by 14% SDS-PAGE. The resolved proteins were transferred to enhanced nitrocellulose membranes. Each blot were incubated in 5% skim milk for 1 h at room temperature, then washed with TBS-T (10 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% Tween-20) and incubated with the primary antibody at 4°C. Each membrane was washed and the particular primary antibody was added and incubated overnight at 4°C with secondary antibody conjugated to horseradish peroxidase. The bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech, UK) on X-ray film (Amersham Pharmacia Biotech, UK).

Statistical analysis

All values are expressed as mean ± SD. Data were analyzed using analysis of paired samples *t*-test and a *p*-value of < 0.05 was considered as significant. All statistics were performed using SPSS 12.0 for Windows.

Results

The serum BUN, creatinine, sodium and uric acid levels measured at pre-clamping and post-clamping days 0, 1, 3 and 5 are summarized in Table 1 and Fig 1. The serum BUN, creatinine and uric acid levels were increased at day 0 and were highest at day 1, and thereafter they decreased gradually. Serum BUN was increased significantly compared with base-

Table 1. Serum biochemistry, FE_{Na} and FE_{UA} data at pre-clamping (baseline) and post-clamping days 0, 1, 3 and 5 in miniature pigs

Parameter	Baseline	Day after unclamping			
		Day 0	Day 1	Day 2	Day 3
BUN (mg/dl)	8.58 ± 1.26	10.04 ± 0.57	27.81 ± 3.67[a]	23.28 ± 7.65	19.00 ± 3.03
Cr (mg/dl)	0.97 ± 0.1	1.2 ± 0.14	2.01 ± 0.46	1.37 ± 0.12	1.02 ± 0.24
Na (mEq/L)	142.67 ± 0.58	140.67 ± 0.58[b]	145.67 ± 4.51	143.67 ± 2.08	141.67 ± 2.08
UA (mg/dl)	0.08 ± 0.05	0.12 ± 0.07	0.26 ± 0.25	0.22 ± 0.05	0.21 ± 0.15
FE_{Na} (%)	1.15 ± 0.88	1.58 ± 0.78	1.22 ± 0.59	0.45 ± 0.31	0.15 ± 0.1
FE_{UA} (%)	198.96 ± 124.2	110.2 ± 20.99	133.61 ± 99.17	51.49 ± 19.32	82.90 ± 105.79

Data are expressed as mean ± SD.
[a]$p < 0.05$ as compared with post-clamping in serum BUN.
[b]$p < 0.05$ as compared with pre-clamping in serum Na.
BUN, blood urea nitrogen; Cr, creatinine; Na, sodium; UA, uric acid; FE_{Na}, fractional excretion of sodium; FE_{UA}, fractional excretion of uric acid.

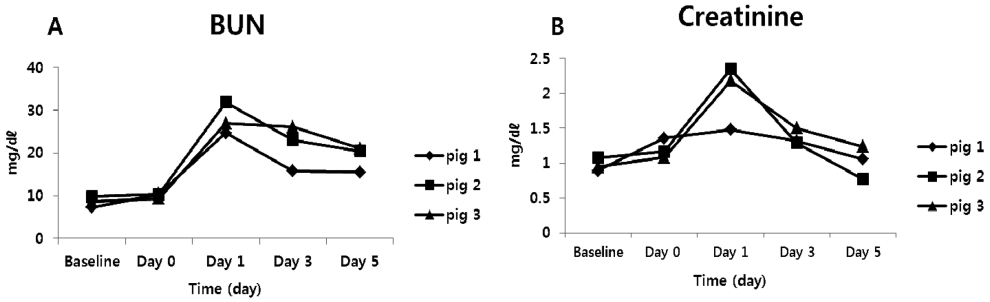

Fig 1. Changes of blood urea nitrogen (BUN) and creatinine at pre-clamping (baseline) and post-clamping days 0, 1, 3 and 5 in miniature pigs.

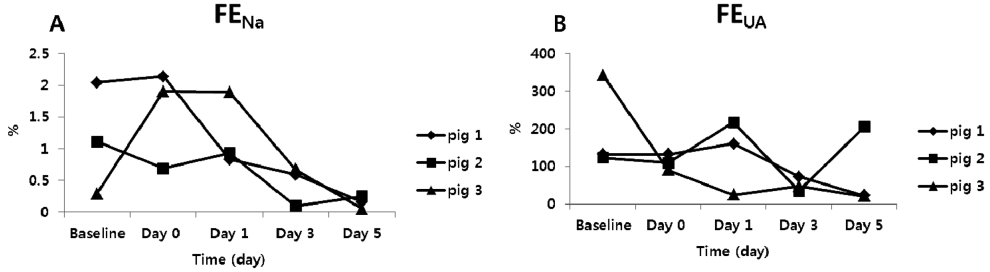

Fig 2. Changes of FE_{Na} (A) and FE_{UA} (B) at pre-clamping (baseline) and post-clamping days 0, 1, 3 and 5 in miniature pigs.

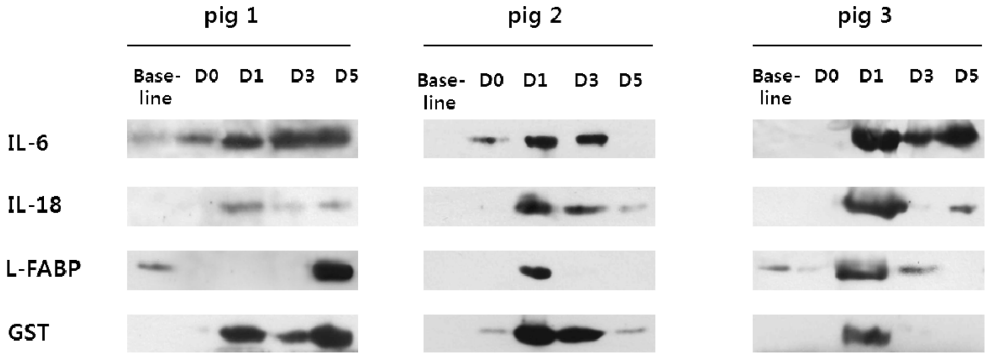

Fig 3. IL-6, IL-18, L-FABP and GST expression in urine sample at pre-clamping (baseline) and post-clamping days 0, 1, 3 and 5 in miniature pigs.

line ($p < 0.05$) at day 1 (Table 1). FE_{Na} and FE_{UA} varied individually. In pigs 1 and 3, FE_{Na} was increased at day 0 and then decreased. In contrast, in pig 2, FE_{Na} was decreased at day 0 and increased at day 1, and then decreased gradually (Fig 2). FE_{UA} in pig 1 was increased, with the peak observed on day 1. Thereafter, FE_{UA} decreased markedly. In pig 2, FE_{UA} was decreased at day 0 and on day 3. In pig 3, FE_{UA} was decreased on day 1, increased on day 3 and again decreased on day 5 (Fig 2).

IL-6, IL-18, L-FABP and GST were detected in urine samples in Western immunoblotting analysis (Fig 3). Their levels were significantly increased compared to pre- and post-clamping. In pigs 1 and 2, the level of IL-6 was increased at day 0. In pig 3, the level of IL-6 was increased on day 1. The levels of IL-18 were significantly increased on day 1 in all three miniature pigs. L-FABP varied with individuals. In pig 1, the level of L-FABP increased on day 5. In pig 2, L-FABP was markedly present on day 1. In pig 3, L-FABP was evident on days 1 and 3. The level of GST was increased on days 1, 3 and 5 compared to baseline in pigs 1 and 2. In pig 3, GST only appeared on day 1.

Discussion

As the major cause of AKI, renal IRI results in sudden reduction of glomerular filtration rate and renal blood flow, and impaired urine concentrating ability. In addition, renal IRI induces an immune response, leading to both local and systemic inflammation (7,15,18), and it leads to delayed graft function and reduces long-term organ survival in transplantation (4).

In this study, serum BUN and creatinine changed little

compared to baseline and day 0. However, on day 1, serum BUN increased significantly compared to baseline and day 0, and serum creatinine did not show a significant difference, although it exhibited an increasing trend similar to serum BUN. In our previous study, serum BUN and creatinine did not show significant difference at 1 and 3 h after reperfusion compared with pre- and post- clamping (12). Thus, serum BUN and creatinine will be effective biomarkers at least 1 day post-clamping.

Also, we investigated the diagnostic usefulness of FE_{Na} and FE_{UA} in this study. When AKI occurs, renal tubule sodium and water reabsorption increase by compensatory mechanisms, such as glomerulotubular balance to decrease of body fluid, activation of renin aldosterone system and increase of antidiuretic hormone release (20). Thus, sodium excretion and FE_{Na} decrease in urine. Generally, in human medicine, a FE_{Na} value below 1% is indicative of prerenal AKI or transient AKI, and a FE_{Na} value more than 1% indicates ATN or permanent AKI (5,13,20). FE_{UA} is not affected by diuretics that affect the distal tubule, because uric acid is mainly controlled in the proximal tubule. Thus, FE_{UA} has been suggested to be a good diagnostic biomarker in patients treated with diuretics (8). In this study, FE_{Na} showed a downward tendency and was reduced to less than 1%. However, it did not have statistical significance. The change of FE_{UA} varied among the three pigs. Therefore, early diagnosis of AKI in miniature pigs may be difficult using FE_{Na} and FE_{UA}.

Apart from FE_{Na} and FE_{UA}, the present results indicate that IL-6, IL-18, L-FABP and GST are clinically useful for the diagnosis of AKI. IL-6 is a pleiotropic cytokine that has both pro- and anti-inflammatory properties (3). Copious amounts of IL-6 are produced by endothelial cells in response to pro-inflammatory signals including tumor necrosis factor-alpha and hypoxia. IL-6 production is also a common reaction to tissue injury and organ failure. The main target cells of IL-6 include hepatocytes and some leukocyte subpopulations, including monocytes and neutrophils, and some T and B cells (9,17). In this study, the level of IL-6 was markedly increased compared to pre- and post-clamping in all three miniature pigs.

IL-18 is an 18 kDa proinflammatory cytokine. It is up-regulated during endogenous inflammatory processes. Urinary IL-18 originates from proximal tubular epithelial cells. Intracellular IL-18 is responsible for kidney injury in the course of AKI, given its property as a neutrophilic attractant (6,15, 16). Thus, it is powerful mediator of ischemia-induced AKI in the animal model. Presently, IL-18 was markedly increased on day 1.

L-FABP is expressed in various organs including liver, heart muscle and kidney. It functions in the cellular uptake of fatty acids (FAs) from plasma and promotion of intracellular FA metabolism. L-FABP may inhibit the accumulation of intracellular FAs, thus, it may be an important cellular antioxidant during oxidative stress (10,11,16). In preclinical studies, renal L-FABP expression protected against tubulointerstitial damage because of its antioxidant function in models of proximal tubule protein overload, as well as against unilateral ureteral obstruction (2,10). L-FABP can be filtered through the glomerulus and reabsorbed in the proximal tubule epithelial cells. However, because of its small size, L-FABP excretion increases in urine when proximal tubule cells are injured (16). In this study, L-FABP expression was up-regulated at post-clamping. In spite of its individual differences in the pigs, the level of L-FABP was changed significantly compared to pre- and post-clamping.

GST represents a family of cytosolic, microsomal and membrane-bound enzymes. GST-α is produced in the proximal tubule epithelial cells and GST-π in the distal tubule epithelial cells. Increase in urine excretion of GST may indicate that renal tubule gets damaged. Also, comparing to GST-α and GST-π may indicate the location of damaged lesion (15,19,21). The change of the level of GST was evident in this study. And the peak of GST was observed on day 1 in all miniature pigs.

As for this study's results, the early diagnosis of AKI was difficult by use of FE_{Na} and FE_{UA} although these factors are used for diagnosis of AKI in human medicine. However, serum BUN, creatinine and IL-6, IL-18, L-FABP, GST in urine were increased significantly compared to pre- and post-clamping. Therefore, detection of serum BUN, creatinine and IL-6, IL-18, L-FABP, GST in urine can be useful diagnostic biomarkers for diagnosis of AKI. Also, it suggested that more investigation is needed for clinical diagnosis of AKI by these biomarkers in other AKI models in miniature pigs.

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미니돼지의 신허혈-재관류에 의한 급성신손상 모델에서의 유용한 바이오마커

김세은 · 심경미 · 최석화* · 강성수[1]

전남대학교 수의과대학, *충북대학교 수의과대학

요 약 : 급성신손상은 높은 이환율과 치사율을 나타내는 심각한 질환이며, 허혈-재관류에 의한 신손상은 급성신손상의 중요한 원인이 된다. 본 연구는 미니돼지에서 급성신손상을 진단하는데 임상적으로 유용한 바이오마커를 찾아내기 위해 실시되었다. 세 마리의 미니돼지에서 60분간 신동맥을 결찰하여 양측성 신허혈을 유도하였다. 각 미니돼지에서 결찰 전, 결찰 후 0, 1, 3, 5일에 혈액 및 뇨 검체를 채취하였다. 혈청 및 뇨 검체에서 BUN, creatinine, 나트륨 및 요산을 측정한 후 나트륨 및 요산의 분획배설율(FE_{Na}, FE_{UA})을 산출하였다. 또한 IL-6, IL-18, L-FABA 및 GST를 Western immunoblotting을 실시하여 측정하였다. 결과에서 세 마리 미니돼지 모두 혈청 BUN과 creatinine 농도는 결찰 후 1일째에 유의적으로 증가하였다. 그러나 FE_{Na}와 FE_{UA}는 현저한 개체차를 보였다. 수술 전과 후를 비교했을 때 허혈 이후의 뇨 검체에서는 IL-6, IL-18, L-FABP 및 GST의 농도가 유의적으로 증가하였다. 결론적으로, FE_{Na}와 FE_{UA}에 대해서는 추가적인 연구가 필요하다고 생각되며, 혈청 BUN, creatinine과 뇨 IL-6, IL-18, L-FABP 및 GST는 돼지의 허혈-재관류 모델에서 다른 바이오마커와 함께 급성신손상을 진단하는 민감한 바이오마커가 될 수 있을 것이라 생각된다.

주요어 : 급성신손상, 바이오마커, 신허혈 및 재관류, 미니돼지