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ORIGINAL ARTICLE

Neutrophil gelatinase-associated lipocalin (NGAL) as a potential biomarker for early detection of acute renal failure

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Abstract Neutrophil gelatinase-associated lipocalin (NGAL) is a biomarker that can potentially be used for the detection of acute renal failure earlier and more accurately than current standard examinations, such as serum levels of creatinine and urea which are less sensitive and less specific. The objective of this research was to assess the ability of NGAL as a biomarker of acute renal failure by comparing the levels of serum urea and creatinine in rats with induced acute renal failure. Twelve male Wistar rats (*Rattus norvegicus*), 2 months of age with a body weight (BW) of 150–200 g, were allocated into two groups. Group I (n=2), as the control group, were injected with sterile distilled water 10 mL/kg BW intramuscularly and group II (n=10) were injected with 50 % glycerol 10 mL/kg BW intramuscular, the rats in all group had been fasting 12 h before injection. In group II, two rats were selected at each time point (0, 6, 12, 24 and 48 h after injection) bloods collected for analysis of urea and creatinine concentration and identification of NGAL and they were necropsied and the kidney removed for histopathological analysis and NGAL identification. The levels of creatinine and urea were analysed by independent t test. Identification of NGAL was conducted by one-step reverse transcriptase polymerase chain reaction and sequencing. Results showed that the glycerol injection significantly elevated the levels of urea at 6, 12 and 24 h compared to the control group (p<0.05). Creatinine levels were significantly increased at 24 h compared to the control group (p<0.05). The expression of NGAL in kidney and blood samples could be detected at 6, 12 and 24 h after induction. In conclusion, the blood NGAL could be detected before increasing of creatinine levels and could be used as an early detection of acute renal failure.

Keywords Neutrophil gelatinase-associated lipocalin (NGAL) · Biomarker · Glycerol · Acute renal failure

Introduction

Acute renal failure is a condition with a high risk of mortality in both humans and animals. The incidence of renal failure in humans is approximately 20–30 % classified as critical, and eventually about 6 % of all patients require kidney transplantation therapy (Ferguson et al. 2008; Ronco et al. 2010). Pathophysiological mechanisms associated with acute renal damage have lately gained more attention in the medical field (Ronco et al. 2010). Despite advances in diagnosis and treatment, acute renal failure is a major risk factor related to the emergence of a broad range of serious complications in patients (Coca et al. 2007; Coca et al. 2009).

In current clinical practice, acute renal failure is usually diagnosed by measuring the levels of creatinine and blood urea nitrogen (BUN) in serum. However, creatinine is an unreliable indicator during acute changes in renal function (Nguyen and Devarajan 2008; Clerico et al. 2012). Measurement of BUN levels is known to be non-sensitive, non-specific and do not represent the actual state of the progressive nature of the renal failure (Nguyen and Devarajan 2008).

Many biomarkers have been developed for the early and accurate detection of acute kidney disease (Ronco et al. 2010; Cruz et al. 2011). Among these biomarkers Cystatin C and

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neutrophil gelatinase-associated lipocalin (NGAL) have been identified as being capable of providing results that are both sensitive and reliable (Cruz et al. 2011). NGAL messenger RNA (mRNA) expression dramatically increased in renal tubular secretion regions in renal failure with acute ischemia, sepsis and post-transplantation (Stejskal et al. 2008; Huynh et al. 2009). Efforts to develop early detection of acute renal failure with appropriate and sensitive biosensors are needed to overcome the problems of diagnosing acute kidney failure. This study will examine and assess NGAL as a biomarker for acute renal failure, through the detection of the presence of NGAL in rats with acute renal failure induced using 50 % glycerol.

Materials and methods

Experiment

Twelve, 2 months old, Wistar rats (Rattus norvegicus) were allocated into two treatment groups: Group I (n=2), the control group, had an intramuscular injection of sterile distilled water at 10 mL/kg BW after being fasted for 12 h and were euthanized at the end of research; Group II (n=10) had, after being fasted for 12 h, an intramuscular injection of 50 % glycerol at 10 mL/kg BW to induce acute renal failure (Singh et al. 2012). At 0, 6, 12, 24 and 48 h, after induction, two rats were randomly selected from group 2 and blood was taken via the retro-orbital plexus for analysis of urea, creatinine and NGAL. After blood sampling, the rats were euthanized and renal tissue collected. Half a kidney was stored in 10 % formalin for histopathological examination with haematoxylin and eosin staining (H&E) and half renal stored in the freezer for RNA NGAL isolation. Identification of NGAL was performed using reverse transcriptase polymerase chain reaction (RT-PCR) and then analysed by electrophoresis. This study complies with the ethical clearance with the registration number no. 122/KEC-LPPT/X/2013.

Determination of urea and creatinine

In both groups, renal function was evaluated using the Jaffe colorimetric method (Creatinine and Urea kit DiaSys Diagnostic Systems GmbH, Germany) to calculate the serum levels of urea and creatinine. This was done by measuring changes in absorbance values after 1 min in the sample compared with the standard solution (Thomas 1998).

RNA extraction and RT-PCR

RNA from the blood and kidneys were extracted with the High Pure RNA Isolation Kit, Roche. A total of 20–25 mg of tissue was added with 400 μL of lysis buffer and 4 μL beta-mercaptoethanol then mixed in a 1.5 mL tube; the samples were homogenized with a homogenizer. After all RNA had been extracted, it was stored in the freezer −80 °C (Schettler et al. 2003).

NGAL gene was amplified using one-step RT-PCR Kits (Roche), and the procedure was performed according to the method of Rolfs et al. (1992). NGAL gene primer pair was used to produce 245 bp amplicons. Primers used for amplification of genes encoding NGAL consist of the forward primer 5′ ACAGCTACCCTCAGATACAGAGCTA 3′ and reverse 5′ TCAGTTGTCAATGCATTGGTCGGTG 3′. The following thermocycler programme was used: RT (temperature of 50 °C for 30 min); pre denaturation (94 °C for 5 min); denaturation (94 °C for 1 min); annealing (55 °C for 30 s); elongation (68 °C for 30 s); Post-elongation (68 °C for 5 min)—30 cycles. RT-PCR product then underwent electrophoresis on 1 % agarose gel, visualized with UV transilluminator and compared with the control and marker 100 bp DNA Ladder. Sequencing was performed on the kidneys and blood samples, and sequencing results were analysed using MEGA 6.0 software.

Data analysis

NGAL gene amplification results were compared with the control and marker 100 bp DNA Ladder. The levels of urea and creatinine were analysed by SPSS 17 statistical programme, test data normality using the Kolmogorov-Smirnov test and data analysed using the independent t test with 95 % confidence level.

Results and discussion

Urea level

Mean levels of urea in the control rats ranged from 26.75 to 35.05 mg/dL in the first 24 h which are still within normal limits (10–58 mg/dL) according to Mitruka and Rawnsley (1981). Group II (treated) showed significantly increased levels of urea at 6, 12 and 24 h after induction, compared with control rats (p<0.05; Table 1).

According to Yang et al. (2012), glycerol can cause renal damage characterized by elevated urea levels. Increased levels of urea in the treated animals at 6 h are probably due to hypovolemia and dehydration as the early post-rhabdomyolysis response. Rhabdomyolysis can lead to a decrease in the body fluid volume (Xavier et al. 2009).

Creatinine level

Creatinine levels in the control rats were within the normal range—0.20–0.80 mg/dL according to Mitruka and Rawnsley

Table 1 Serum urea (mg/dL) levels

Calculation	Treatment	Time (h)			
		0	6	12	24
Mean±SD urea (mg/dL)	Control	35.05±1.77	26.75±7.14	31.75±3.88	29.90±7.35
	Glycerol	33.65±2.87	62.34±13.98*	104.70±33.44*	213.27±65.26*

*t test significant ($p<0.05$)

(1981). Mean creatinine levels in Group II gradually increased and peaked at 24 h with an average serum creatinine of 2.23 mg/dL, which is a significant increase when compared with the controls ($p<0.05$; Table 2).

According to Ayvaz et al. (2012), creatinine levels in rats can increase up to 7.56 mg/dL 48 h after injection with 50 % glycerol. The increasing of creatinine levels in this study was likely due to rhabdomyolysis and a decreasing of renal function due to glycerol's toxic effects. Histopathological changes showed decreasing renal function evidenced by necrosis and dilatation of tubular renal epithelium at 24 h.

Current standard tests to determine the status of kidney function is by measuring serum creatinine and urine production. However, the initial measurement of serum creatinine may not reflect the extent of the injury as the accumulation of creatinine always lags behind injury events (Lameire and Hoste 2004). The increase in serum creatinine can indicate damage to the kidneys but has limited ability to determine the damage in the early stages (Devarajan 2007). Acute renal failure can be detected with a blood chemistry test and via NGAL gene expression. Blood chemistry test results showed an increase in urea starting at 6 h, and creatinine at 24 h, whereas the NGAL gene expression detection began at 6 h.

Rats in group II showed increased urea levels at 6, 12 and 24 h after glycerol injection whilst increased creatinine was observed at 24 h. According to Stockham and Scott (2002), in most mammals, increased levels of creatinine and urea generally occur simultaneously but creatinine was the preferred indicator over urea in determining the status of renal function because creatinine levels were more constant and not reabsorb by the tubules. Increase in levels of urea in the blood does not always mean there is a kidney damage, because increasing urea can be influenced by several other factors such as hypovolemia, high protein diet, shock, dehydration, congestive heart failure and bleeding in the gastrointestinal tract (Stockham and Scott 2002).

The identification of genes encoding NGAL in this study showed NGAL mRNA expression was detected at 6, 12, 24 and 48 h after injection of 50 % glycerol. NGAL expression at 6 h emerged as an indication of the beginning of kidney damage. This study suggests that NGAL biomarkers provide a faster response than serum creatinine.

Kidney histopathology

Microscopically, the kidneys from the control group did not exhibit pathological changes whilst the treated group showed histopathological changes in the kidney from 6 to 48 h. In rats, injection of 50 % glycerol can cause acute tubular necrosis (ATN) with morphological characteristics such as tubular cell necrosis, tubular lumen dilation and swelling of the proximal tubular cells with brush border loss (De Jesus et al. 2007; Ibrahim et al. 2011).

In this study, ATN was characterized mostly by necrosis of tubular epithelial lumen; some tubular lumen were dilated and some contained homogeneous eosinophilic (or yellowish red) masses, and there was infiltration of lymphocytes into the interstitial tissue (Fig. 1). The acute tubular necrosis can be caused by ischaemia and/or toxic agents in kidney. These agents include aminoglycoside antibiotics, cisplatin chemotherapy agents, heme pigment due to haemolysis and rhabdomyolysis (Rennke and Denker 2007).

Table 2 Serum creatinine (mg/dL) levels

Calculation	Treatment	Time (h)			
		0	6	12	24
Mean±SD creatinine (mg/dL)	Control	0.35±0.07	0.30±0.00	0.20±0.00	0.20±0.00
	Glycerol	0.34±0.07	0.40±0.12	0.71±0.30	2.23±0.96*

*t test significant ($p<0.05$)

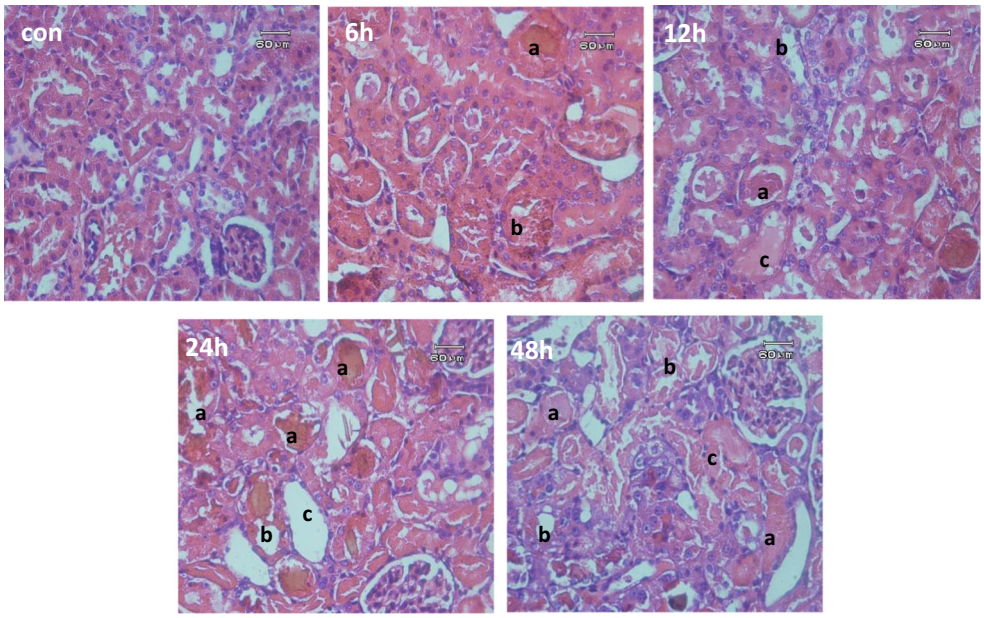

Fig. 1 Renal histopathology of controls and 6, 12, 24 and 48 h after glycerol injection; (*a*) tubular lumen containing homogeneous eosinophilic mass, (*b*) tubular epithelial cell necrosis, (*c*) dilated tubular lumen (H&E staining, 10×40)

Acute renal failure caused by glycerol injection can lead to rhabdomyolysis (Bagley et al. 2007; Yang et al. 2012; Gu et al. 2014). Rhabdomyolysis can induce renal failure as a result of reduced blood supply to glomeruli, decreased glomerular filtration, tubular epithelial damage and tubular obstruction by residual myoglobin (Boutaud and Roberts 2011). Iron in myoglobin and haemoglobin released into the bloodstream as a result of myolysis and haemolysis induces the formation of free radicals and lipid peroxidation, therefore, plays a critical role in the pathogenesis of acute renal failure (Baliga et al. 1997; Vanholder et al. 2000; Aydogdu et al. 2006).

Identification genes encoding NGAL

In this study, amplification of genes encoding NGAL showed the amplicon size was 245 bp; PCR product was confirmed on electrophoresis gel with a 1 % agarose concentration. Using gene amplification, the mRNA expression of NGAL in kidney samples was detected at 6, 12, 24 and 48 h. The negative controls and time point 0 showed no NGAL gene expression in kidney tissue (Fig. 2a). The same pattern of expression was seen in the blood samples (Fig. 2b).

NGAL is also known as lipocalin 2 (Lcn2), and according to Aigner et al. (2007), Lcn2 mRNA in normal rats showed higher gene expression in bone marrow, much weaker expression in spleen, lungs and granulocytes and no gene expression in liver, heart, kidney, small intestine or thymus.

Identification of NGAL gene expression in this study was performed using RT-PCR. Studies on NGAL expression in acute renal failure due to glycerol induced rhabdomyolysis have not been reported. Our studies show that NGAL expression was detected during the early stages of acute tubular necrosis. We found that NGAL can be a useful biomarker for the diagnosis of renal epithelial injury. Human NGAL was originally identified in the neutrophil as a 25 kDa protein covalently bound to the matrix metalloproteinase-9 (MMP9) (Mishra et al. 2004). Homological NGAL in the rats is known as lipocalin 2 (Lcn2); Gene Lcn2 has six exons and encodes for two functional transcripts (Chakraborty et al. 2012). NGAL is synthesized during maturation of granulocytes in the bone marrow and expressed on epithelial cells that undergo inflammation or malignancy. NGAL protein accumulates

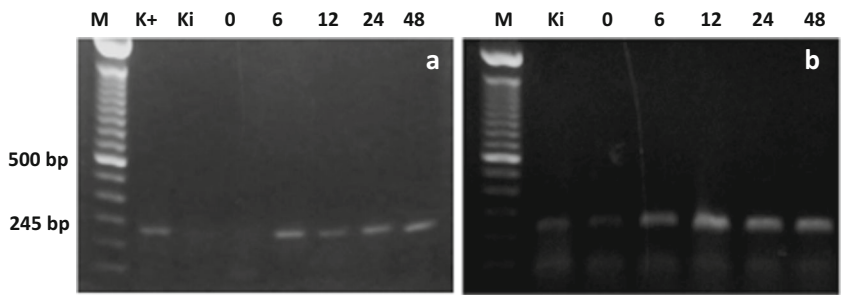

Fig. 2 Electrophoresis for mRNA NGAL in kidneys (**a**) and blood (**b**)

in the blood, urine, proximal and distal renal tubules following damage (Mori et al. 2005).

Mishra et al. (2003) reported that NGAL mRNA expression appeared at 3, 12 and 24 h post-renal ischemic injury induced by cisplatin compounds in rats, and increased expression of NGAL mRNA was highest at 12 h, assessed by looking at the amount of NGAL mRNA folding by using quantitative RT-PCR. Increased expression of NGAL mRNA in rats can be caused by upregulated NGAL genes as an increase in gene expression that occurs in the cell is triggered by internal and external cell signalling pathways, and the result is the release of the proteins encoded by these genes (Davidson and Levin 2005; Schmidt-Ott.K.M 2011). mRNA NGAL may rise as a result of an increase in the expression level or stimulation or interaction of inflammatory cells in renal epithelial cells (Nielsen et al., 1996).

Devarajan (2007) and Nickolas et al. (2008) indicate that the detection of NGAL protein in serum and in urine is a sensitive and specific biomarker in predicting the incidence of acute renal failure. Due to the role of NGAL, NGAL protein increase can limit injury to the renal tubules by modulating a variety of cellular responses, such as proliferation, apoptosis and differentiation, but the specific mechanisms for these actions are not yet known (Schmidt-Ott et al. 2007).

Clinical studies by Nickolas et al. (2008) showed that kidney injury can result in 10 times higher concentrations of NGAL in the blood plasma and more than 100 times higher in the urine. NGAL protein can be detected quickly in a few hours in animal models after a kidney injury. NGAL fulfils many characteristics making it suitable as a biomarker for early detection of kidney injury, following renal transplantation, for example (Mishra et al. 2006; Bataille et al. 2011). Furthermore, the NGAL gene is also significantly regulated in the kidney following ischemic insult (Mishra et al. 2004) and has proved a highly predictive biomarker of acute and chronic kidney injury (Devarajan 2010; Atzori et al. 2011).

NGAL was able to bind several ligands, including siderophores—strong iron-binding agents which play a role in cellular respiration and DNA synthesis. Interaction between NGAL and siderophores can modulate most of the biological effects (Borregaard and cowland 2006; Clifton et al. 2009; Devarajan 2010).

DNA sequencing was performed to ensure that the DNA fragments amplified by RT-PCR were genes that encode NGAL. The results of bioinformatics analysis of the NGAL gene sequences carried out on the kidneys and blood samples showed NGAL mRNA amplification (245 bp). The results of sequence similarity have 100 % homology with the GenBank database NGAL gene sequences (forward and reverse). Results alignment with MEGA 6.0 software also show a percentage of similarity of 100 % with the gene accession number NM_130741.1 NGAL and BC089053.1.

Conclusion

Neutrophil gelatinase-associated lipocalin could be used as a biomarker for acute renal failure that is more accurate and able to detect results earlier than the current urea and creatinine biomarkers.

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