REVIEW ARTICLE

Enzymuria-Early Predictors of Acute Kidney Injury in Animals

Maharudra J. Sanap*, Pranesh V. Yeotikar

Abstract

Enzymuria is recently being used as valuable tool for the diagnosis of acute kidney injury (AKI), and urinalysis in clinical cases. The origin of enzymes from different parts of the kidney has diagnostic importance. Enzymes of kidney origin, namely those from brush border of proximal tubuli were recognized for their clinical significance in animals. Now a days, enzymes are more specifically used as tubular markers which determine the location and severity of kidney damage. These enzymes are glutathione S-transferase (GST), N-acetyl-β-D-glucosaminidase (NAG), β-galactosidase (β-GAL), γ-glutamyltranspeptidase and alkaline phosphatase which are used as early diagnostic markers of kidney injury. Normally, enzymes originates from serum (as glomerular filtrate), renal tubular cells, and the urogenital tract (epithelial cells, glandular secretion, and semen). The contribution of serum enzymes is negligible for most urinary enzymes because they are relatively larger (> 80 kDa), due to which those marker enzymes do not sieve through the glomerular membrane and appear in the urine. Urinary enzymes have also been used to determine the presence and location of renal tubular injury. Some bacterial or viral infections can damage kidney tubules, which result in the leakage of some enzymes from the tubular epithelium. Various nephrotoxic drugs used in clinical therapeutics, along with some contrast media may lead to acute kidney injury. Enzymes of kidney origin may be released due to damage to the brush border epithelium. Especially, NAG- a lysosomal enzyme in renal tubular epithelium released into urine in response to tubular damage. These enzymes could be increased or identified in urine of urinary tract infected animals.

Key words: Acute kidney injury, Diagnostic markers, Enzymuria, Nephrotoxic drugs, Urinary enzymes

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Introduction

Enzymuria refers to excretion of enzymes in urine. Enzymuria is recently being used as valuable tool for the diagnosis of acute kidney injury (AKI), and urinalysis in clinical cases. Study on enzymuria shows more interest in animal pathology as an index of nephrotoxicity. Therapeutic treatment involving administration of nephrotoxic drugs such as gentamicin, antifungals as well as NSAIDs results in to huge nephrotoxicity in dogs. Enzymes which are leaked from damaged renal tubular epithelial cells are of diagnostic importance in clinical study (Fig. 1). Enzymes namely Alanine aminopeptidase (AAP; EC: 3.4.11.2) γ-Glutamyl transferase (GGT; EC: 2.3.2.2) and N-acetyl-ß-D-glucosaminidase (NAG; EC: 3.2.1.30) are most commonly measured for the diagnosis of acute kidney injury(AKI). AKI is a multifaceted process resulting from multiple causes such as renal medullary ischemia, insufficient renal perfusion, renal vasoconstriction, and decreased glomerular filtration rate (GFR), renal tubular obstruction, and metabolic changes of renal tubular epithelial cells (Ostermann *et al.*, 2012).

Kidney ischemia/reperfusion (I/R) is identified as a critical problem that is found to be a serious consequence of AKI. There are two vital factors of major concern in acute kidney injury, *i.e.*, i) Vascular factors such as renal vasoconstriction, decreased renal blood flow, and a drop in glomerular blood pressure that causes lateral medullary ischemia, which can lead to tubulo-glomerular feedback aggravation and activation, ischemic tissue damage, or cell necrosis and ii) Renal tubule factors, including obstruction of renal tubules, reabsorption dysfunction, and renal interstitial inflammation (Lameire *et al.*, 2005). In addition, the oxidative stress response activated by inflammatory mediators produced from damaged epithelial cells and the release of various vasoconstrictor substances can all aggravate ischemic damage (Qian *et al.*, 2015), especially of the proximal straight tubule (S3 segment) and medullary thick ascending limb. As a traditional biochemical marker of AKI, serum creatinine is not sensitive enough for early monitoring. Serum creatinine levels rise as a result of loss in glomerular ultrafiltration capacity. On the other hand, several AKI cases occur due to acute renal tubular necrosis caused by ischemia or toxic substances, but they do not directly correlate with glomerular damage.

Enzymuria is concerned to the biochemical, patho-biochemical, and morphological essentials of the excretion of urinary enzymes. It is used in clinical diagnostic practice

Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, MAFSU, Parbhani (M. S.) 431402, India.

Corresponding Author: M. J. Sanap, Department of Veterinary Biochemistry, College of Veterinary and Animal Sciences, MAFSU, Parbhani (M. S.) 431402, e-mail: sanap_vet@rediffmail.com

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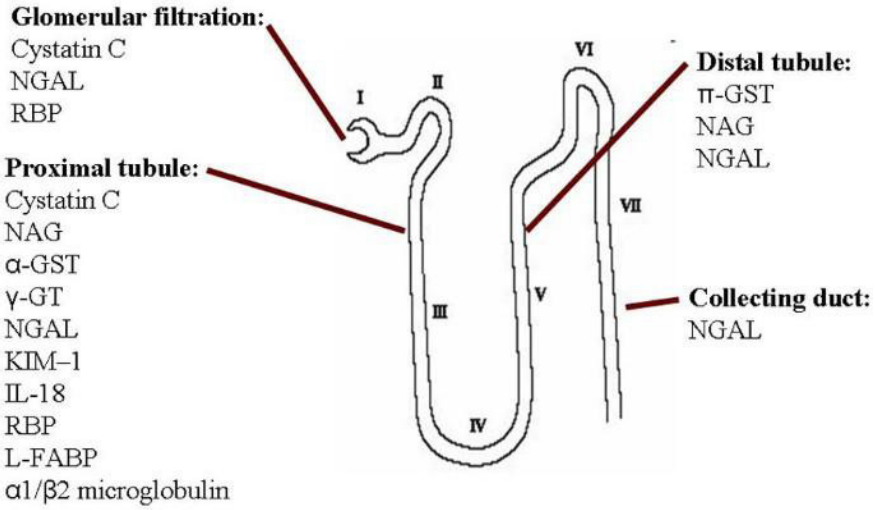

Fig. 1: Origin of acute kidney injury biomarkers within a single nephron. Roman I, II, III, IV, V, VI and VII are subparts of nephron structure. GST, glutathione S-transferase; GT, glutamyltranspeptidase; KIM, kidney injury molecule; L-FABP, liver-type fattyacid-bindingprotein; NAG, N-acetyl-β-D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin; RBP, retinol binding protein.

for renal parenchymal tubular impairment, and assessment of urinary enzymes. One of the most important frequently evaluated urinary enzymes is N-acetyl-β-D-glucosaminidase (NAG) which is a hydrolytic lysosomal enzyme with high molecular weight and low physiological activity. It originates principally in proximal tubules and normally cannot pass through the glomerular membrane. Several urinary enzymes are being used for evaluating impairment in renal functions. Under normal conditions, enzymatic activities of urine may originate from serum (as glomerular filtrate), renal tubular cells, and the urogenital tract (epithelial cells, glandular secretion, and semen). As enzymes molecular weight relatively higher (> 80 kDa), unable to pass through the normal glomerular membrane of kidneys (Clemo, 1998).

Urinary enzymes are extensively being studied as potential biomarkers of injury. As such, enzymes are normally present in proximal tubular cells; after injury, an enzyme may release into the tubular lumen and appear in urine. Several urinary enzymes, including γ-glutamyl transferase (GGT) and N-acetyl-ß-D-glucosaminidase (NAG), have been used to assess nephrotoxicity in dog. The GGT and NAG are ideal enzymes because they are relatively stable at room temperature, and enzyme activity could be maintained by storing urine samples at 4°C. Those enzymes could be measured in urine supernatant without prior removal of enzyme inhibitors (Gossett *et al*.,1987; Matteucci *et al*.,1993 and Reusch *et al*.,1991). Other urinary enzymes, including alkaline phosphatase, leucine aminopeptidase, acid phosphatise, alanine aminopeptidase (AAP), lactate dehydrogenase (LDH), P-glucuronidase, P-galactosidase (PGAL), and lysozyme (muramidase) have also been determined in dog. NAG has been reported to be a very sensitive and reliable marker of renal failure.Therefore, it has been proposed to estimate these biomarkers in various conditions associated with renal injury or dysfunction. Some of the uses of urinary NAG include; nephritic syndrome due to nephrotoxic drugs, urinary tract infection, heavy metal poisoning, kidney transplants, vesico-ureteral reflux, and diabetes mellitus.

Causes of Acute Kidney Injury

Ashalatha *et al*. (2015) studied the effect of contrast radiographic administration on urinary enzymes and microalbuminuria as biomarkers of renal dysfunctions. It revealed that the nephrotoxicity of radiographic contrast agents is serious clinical problem. The use of radiographic technique utilizing contrast media is increasing for both diagnostic and interventional procedures. Contrast-induced nephropathy (CIN) has been identified as the third leading cause of hospital-acquired AKI. Contrast-induced nephropathy increases serum creatinine concentration of greater or equal to 0.5 mg/dL or by a relative increase of 25% or more from the baseline value within 48 h of radiographic medium administration (Thomsen and Morcos, 2003). The current definition of CIN is based on the changes in serum creatinine levels after administration of contrast medium. However, serum creatinine is a good marker of glomerular function rather than kidney injury. Nicloe *et al*. (2011) determined the effect of storage time and temperature on canine urine enzymes such as NAG and GGT. Prolonged therapy, malnutrition, volume depletion, liver diseases, pre-existing renal disease, potassium and magnesium depletion, and concomitant exposure to other nephrotoxic drugs (amphotericin B, cyclosporin, vancomycin, and NSAIDs) that pose a risk to the kidneys.

Tubular Marker Enzymes

In recent years, several studies have revealed that some proteins, especially enzymes released from the damaged proximal or distal renal tubular cells, can predict early tubular

damage, and enzyme activities were positively correlated with the degree of damage (Westhuyzen *et al*., 2003). Currently, diagnostic tubular marker enzymes are primarily derived from two parts of renal tubular epithelial cells; brush border membrane and cytolysosome. Therefore the presence of enzymes in urine including GST, NAG, γ -glutamyl transpeptidase and alkaline phosphates can specify damage position as well as severity of kidney problems.

Glutathione S-transferase (GST), (EC:2.5.1.18)

Glutathione S-transferase **(**GST) is a soluble cytosolic enzyme, possessing two subtypes: α and π, which is expressed in the proximal convoluted tubule and distal convoluted tubule. The possible application of this enzyme is to identify the α-GST isoenzyme (proximal renal tubule specific) and π-GST isoenzyme (distal renal tubule specific) for localising the tubular defects (Shu *et al*.,2016). It can be detected in urine before an increase in serum creatinine reflecting kidney damage.

N-acetyl-β-D-glucosaminidase (NAG), (EC: 3.2.1.30)

N-acetyl- β-D-glucosaminidase (NAG) is a lysosomal enzyme which is predominantly located in the renal tubules. It cannot be filtered through glomerular membrane due to its high molecular weight (140 kDa). Urinary NAG activity is increased in a variety of tubule-interstitial diseases; thus, its activity detected in urine reflects tubular dysfunctions (Kazumi *et al*.,1999; Nielsen *et al*.,2012). Urine NAG still has several advantages besides being sensitive and quantitative, which is positively correlated with the proximal renal tubular degree of damage. However, some extra renal factors, such as industrial solvents, heavy metals, and certain disease states (including rheumatoid arthritis, abnormal glucose tolerance, and hyperthyroidism) may lead to elevated level of urinary NAG. Hence, all these factors must be ruled out while predicting the cause of acute kidney injury (Vaidya *et al*.,2006; Fujita *et al*.,2020). The NAG has been shown to be a sensitive and reliable marker of AKI. Increased NAG levels correlated with nephrotoxicants exposure (Emeigh Hart, 2005), delayed renal allograft function (Mukhopadhyay *et al*.,2004), chronic glomerular disease (Bazzi *et al*., 2002) and diabetic nephropathy (Ikenaga *et al*.,1993). Bonventre (2010) estimated NAG, a lysosomal enzyme at proximal tubular brush border which is released into the urine after renal proximal tubule injury. There are two main NAG isoenzymes in human kidneys; isoenzyme A is the soluble part of the intralysosomal compartment and is normally secreted in urine by exocytosis, and isoenzyme B, a component of the lysosomal membrane is excreted in the urine during tubular injury.

β-Galactosidase , (EC : 3.2.1.23)

β-Galactosidase **(**β-GAL); lysosomal enzyme derived from renal tubular epithelial cells which has increased in pyelonephritis and glomerulonephritis. Significantly increase in β-GAL activity may occur in acute tubular necrosis due to lysosomal damage. Elizabeth *et al*. (2016) expressed that the urinary β-galactosidase enzyme in mice stimulate the calcium transport by stabilizing transient receptor potential vanilloid type 5 (TRPV5) channel at plasma membrane. The authors used *in vitro* Ca^{2+} transport assays including immune-blot analysis, immune-histochemistry, patch clamp electrophysiology and total internal reflection fluorescence microscopy by which they revealed that glycosidase β-galactosidase (β-gal), an enzyme that hydrolyzes galactose. They also detected β-gal expression in the apical membrane of the proximal tubules and identified β-galactosidase (β-gal) protein in urine.

γ –glutamyl transpeptidase,(EC: 2.3.2.2)

γ -glutamyltranspeptidase (GGT) is found in proximal tubular brush border cells. Urinary GGT activity can be determined by using commercial testing kits (Uechi *et al*., 1998; Brunker *et al*.,2009). The urinary GGT index is affected by the pH of the urine. In alkaline urine samples, enzyme activities were elevated and GGT is commonly used for the detection of acute kidney injury (Brunker *et al*.,2009).

Alkaline phosphatase, (EC: 3.1.3.1)

Alkaline phosphatase (ALP) is a most important renal brush-border enzyme.This is glycoprotein in nature exist in four isoenzymic forms and it is stable at alkaline pH (pH 8.0). Theses isoenzymes found mainly in liver, bone, intestine, kidney, placenta and germ cells. Normally, serum alkaline phosphatase activity ranges from 24-147 U/L in dog (Mcatee and Lidbury, 2017). All ALP isoenzymes act as homodimers which is attached to the outer cell membrane by a glycosylphosphatidylinositol (GPI) anchor. The release of soluble (anchor-free) ALP to the circulation is catalysed through cleavage by GPI-specific phospholipase D (Anh *et al*., 1998, 2001; Magnusson *et al*., 2002). Its increased activity in urine has been associated with proximal tubular damage in dogs.

Heiene *et al*. (1991) identified urinary ALP as indicator of acute kidney injury. These activities were measured in dogs with chronic renal failure as well as acute renal failure where the average values for urinary ALP were 5.8 IU/litre, 6.7 IU/litre respectively. Raekallio *et al*. (2006) determined the adverse effect of long-term administration of carprofen through estimation of concentration of total protein, albumin, urea, and creatinine and serum activities of ALP and alanine aminotransferase (ALT). Further, they also evaluated the urinary ratios of ALP-to-creatinine, γ-glutamyltransferase (GGT)-to-creatinine, and protein-to-creatinine for confirmation of adverse effect of carprofen in dogs.

Advantages of Enzymuria

The advantages of employing urinary enzymes over urine protein for assessment of tubular injury are increased sensitivity that could predict renal malfunction and its onset in urinary system affected animals and dose response (the amount of enzyme activity in the urine accurately reflects the degree of tubular injury present) (Price,1982; Westhuyzen *et al*.,2003). An additional advantage of using urinary enzymes

is its ability to localize the injury at specific nephron site, and the subcellular location of the enzyme that can act as an index to the severity of the underlying injury (as it is well known that the brush border enzymes specify less severe damage than cytosolic, mitochondrial, or microsomal enzymes) (Price, 1998; Dubach *et al*., 1988)

Precautions in Enzymuria

Some precaution must be taken while employing method of enzymuria in assessing acute kidney injury; sample collection and handling are very critical to analyse urinary enzyme activity more accurately.

Contamination of collected specimens must be avoided, as enzymes present in feces, food, or bacteria can contribute some more activities to the enzyme activity present in the urine. Enzyme activity may also result from the presence of increased numbers of erythrocytes, leukocytes, or epithelial cells in the urine.

Technique of centrifugation can be employed for removal of contaminating cells which resolves some sorts of errors. Examining the resulting sediment will allow the investigator to discard the heavily contaminated urine samples.

However, normal urine frequently contains a variety of low-molecular-weight substances (urea is a notable example) that can act as inhibitors of the enzymes of interest. These substances can be removed by dialysis, dilution, Sephadex filtration, ultrafiltration, or gel filtration but these methodologies have not been validated for enzymes in all species.

Moreover, enzymes are generally less stable in urine than in serum or plasma, urinary enzyme assessment needs to be performed quickly (within a few hours of sample collection) or a stabilizing substrate needs to be added to the sample if storage is anticipated. This is a particular problem with γ-glutamyl transferase in most of the species, even with added stabilizers. The addition of albumin, ethylene glycol, glycerol, or erythritol preserves the urinary activity of enzymes when the samples are stored at –20°C (Jung and Grutzmann, 1988).

Diagnosis of AKI

Several factors, including glomerular filtration rate (GFR), rate of tubular secretion, rate of generation, and volume of distribution affect the rise in serum creatinine levels after the onset of AKI. Hence, it seems to indicate that the large changes in GFR are associated with a relatively small variation in serum creatinine over the first 24-48 hours of AKI. This leads to not only delay in diagnosis and intervention but also in estimating the degree of injury (Moran and Myers, 1985). Certain novel biomarkers have been identified for early detection of kidney injury, like neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), interleukin-18 (IL18), and cystatin C, which have been proved to be specific and sensitive in identifying acute kidney injury. However, these markers are costly and cannot be applied in routine clinical practice. Tubular injury may lead to the excretion of lysosomal and brush border enzymes into the ultrafiltrate and thus increase enzyme activity in the urine. These studies have demonstrated that urinary N-acetyl-β-D-glucosaminidase (NAG) can be used to predict AKI. Nicloe *et al*. (2011) determined the effect of storage time and temperature on canine urine enzymes. They measured the urine NAG and GGT activities in the fresh urine supernatant, aliquots of urine supernatants refrigerated at 4°C for 5 days and urine sample frozen at -20°C for 5 and 30 days, at these days urine enzyme activities were compared with day 0 values. Additionally, they stored the aliquots of urine at 4°C for 30 days and -70° C for 30 days. They observed the GGT concentrations in >50% of samples had effect of storage time and temperature on urine enzymes. The urine GGT/creatinine ratios in samples frozen under -70°C for 30 days carried >0.10 FPR, whereas those refrigerated at 4°C for 5 days or 30 days had <0.01 FPR (false positive rate). They concluded that the time and temperature storage conditions affected urine GGT activity and urine GGT activities were most severely affected by freezing at -20° C.

Athanasiou, *et al*. (2021) evaluated the impact of BTV (bluetongue virus) serotype 4 infection on kidneys using common renal biomarkers. They performed complete urine analysis for determination of urine protein to creatinine ratio (UP:C) and urine γ -glutamyl transferase to creatinine (UGGT:C) ratio. They also estimated numerous parameters including blood urea nitrogen (BUN), creatinine, total proteins, albumin (ALB), and inorganic phosphate (P) in serum samples. It was found that UP:C and UGGT:C were significantly higher ($p < 0.05$) in BTV sheep compared to normal, whereas urine specific gravity was significantly lower ($p < 0.05$). Further, they observed the absence of azotaemia in BTV infected sheep as compared to normal and concluded that there is renal tubular injury or renal dysfunction in blue tongue affected sheep. Biljana *et al*. (2010) determined renal tubular enzyme changes on induction of gentamicin, aminoglycoside antibiotic doses resulting into nephrotoxicity in neonates. They found that the urinary activities of enzymes namely alanine aminopeptidase (AAP), γ-glutamyl transferase (GGT) were increased in contrast to control when treated with gentamicin@ dose rate of 5.0 mg/kg b. wt. for 10 consecutive days. They concluded that both aminopeptidase (AAP), γ-glutamyl transferase were sensitive indicators of nephrotoxicity.

Saraswathi and Shoba (2015) analysed the levels of tubular enzymes and albumin in urine of Gentamicin induced renotoxic rats. They found that the urinary levels of NAG, GGT, ALP, LDH and albumin were increased as compared to control group. They concluded that these markers could be used to determine the extent of renal damage. Whereas, Ali Mohammadi-Karakan *et al*. (2007) reported that the increase in urinary N-acetyl-β-D-glucosaminidase indicated tubular dysfunction whereas other markers like microalbuminuria and a decreased creatinine clearance were observed. It was concluded that the routine screening for diabetic patients could be possible with measurement of urinary NAG level that reflect the kidney failure in diabetic patients. Geor (2003)

revealed that the acute renal failure was more evident in horses susceptible to hypovolemia or endotoxemia (eg, colic, colitis and sepsis) as well as in horses those had history of treatment with nephrotoxic drugs such as aminoglycosides and non-steroidal anti-inflammatory (NSAIDS) drugs.

Kumari Pallavi *et al*. (2020) proved that the urinary enzymes such as GGT/ALP/LDH would be used as reliable biomarkers of tubular damage for early diagnosis of diabetic nephropathy. Rosemary *et al*. (2019) determined the NAG activity in horse urine and found that this lysosomal enzyme was released in urine from renal tubular epithelium in response to kidney injury. Increased activities of certain urinary biomarkers namely GGT, NAG, P-GLU, and muraminidase were found superior to blood urea nitrogen (BUN), serum creatinine, creatinine clearance, urine protein excretion, and urine specific gravity for detecting Gentamicin induced renotoxicty as well as acute tubular necrosis (Adelman *et al*., 1979a; 1979b, Conzelman *et al*., 1979; Greco *et al*., 1985 and Rivers *et al*., 1996). Salvage (2008) revealed that the measurement of glomerular filtration rate and urinary biomarkers in horses would improve diagnostics in kidney problems. Currently, methods to assess acute kidney injury in equine include measurement of plasma creatinine and urine specific gravity, examination of urine sediment, calculation of urine fractional excretion of electrolytes and urine gamma-glutamyl transferase (GGT) index (Sato *et al*.,2002).

Conclusions

The increased level of blood creatinine indicates kidney diseases which has occurred after 60 to 75% glomerular dysfunctions. This is considered as a poor biomarker in detecting renal disorders. The enymuria has become a reliable tool for the diagnosis of acute kidney injury in animals. Enzymuria can assess the presence of tubular markers originating from brush border of proximal tubuli such as alanine amino peptidase, γ-glutamyltranspeptidase, alkaline phosphatase and lysosomal enzymes (β glucuronidase and N-acetyl- β -D-glucosaminidase) in urine samples in response to acute kidney injury. Severity of kidney damage could be assessed by analysing the urinary enzymes such as glutathione S-transferase, N-acetyl- β -D-glucosaminidase and β-galactosidase, gamma-glutamyltranspeptidase and alkaline phosphatase. In addition, urinary estimation of Cystatin C and Podocin and their correlation with glomerular filtration rate would be useful in the early detection of acute kidney injury.

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