

Original Paper

Evaluation of KIM-1 and NGAL as Early Indicators for Assessment of Gentamycin-Induced Nephrotoxicity *In Vivo* and *In Vitro*

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Key Words

Gentamycin • Nephrotoxicity • KIM-1 • NGAL • HK-2 cells

Abstract

Background/Aims: The aminoglycoside Gentamicin is a widely used antibiotic, applied in equine medicine. Despite its clinical use, concerns remain regarding the potential toxic side-effects, such as nephrotoxicity. Early detection of renal damage is critical in preclinical drug development. This study was aimed to determine whether kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) may be early indicators in the assessment of Gentamycin-induced nephrotoxicity. **Methods:** In our study, a model of Gentamicin-induced nephrotoxicity in male Sprague Dawley rats treated for up to 7 days at 50 or 100mg/kg/day was used to monitor the expressions of novel biomarkers of renal toxicity during the progression of acute kidney injury (AKI). Additionally, biomarkers were assessed in human kidney proximal epithelial cells (HK-2) treated with Gentamicin for 2, 6, 12, 24, 36 or 48h *in vitro*. **Results:** Repeated administration of Gentamicin to rats for 1, 3, or 7 days resulted in a dose- and time-dependent increase in the expression of KIM-1 and NGAL. The expressions of the two biomarkers changed prior to renal tubule damage and increases in serum creatinine (SCr) and blood urea nitrogen (BUN) levels, suggesting their usefulness for predicting Gentamicin-induced acute kidney injury (AKI) *in vivo*. **Conclusions:** In contrast, no significant increase in the expression of the biomarker genes and proteins were evident in HK-2 cells after treated by Gentamycin for up to 48h, suggesting that they may not be suitable endpoints for sensitive detection of nephrotoxic effects *in vitro*.

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Introduction

More than 50 years since their introduction, aminoglycosides have represented highly effective antimicrobial agents especially in Gram-negative infections despite nephrotoxicity and ototoxicity. An understanding of the antibiotic's nephrotoxicity therefore has substantial clinical significance [1]. But early detection of renal damage is difficult. The monitoring of patients in clinical trials was primarily based on serum creatinine (SCr), blood urea nitrogen (BUN), and the detection of urinary components like electrolytes, enzymes, and other waste products [2]. Owing to the poor sensitivity and specificity of these traditional markers of renal injury, new indicators for earlier and more accurate detection are needed, and to aid in predictive safety assessment during drug development [2, 3].

Many protein products might serve as novel biomarkers for the initiation phase of AKI have been studied, including kidney injury molecule-1 (KIM-1), Neutrophil gelatinase-associated lipocalin (NGAL) and others [4-6]. KIM-1 is a type I transmembrane glycoprotein which is undetectable in healthy kidneys. The increased expression of this protein was found at very high levels on the apical membrane of proximal tubule cells after ischemic and nephrotoxic injury [7]. Urinary KIM-1 outperforms SCr, BUN and urinary NAG in multiple rat models of kidney injury and may facilitate sensitive, specific and accurate prediction of human nephrotoxicity in preclinical drug Screens [8]. NGAL, also known as lipocalin-2, was found in activated neutrophils, in accordance with its role as an innate antibacterial factor [9]. NGAL may eventually have prognostic value in predicting not only acute, but also chronic, worsening in renal function in patients already affected by chronic nephropathies [10].

However, the ability of the two putative biomarkers of nephrotoxicity to indicate or even predict mild histopathological changes following exposure to aminoglycosides still needs to be evaluated. In this study, we investigated the expressions of KIM-1 and NGAL in rats treated with Gentamicin for up to 7 days. Although the majority of toxicological investigations have been performed in vivo experiments using animals, the use of non-animal in vitro test methods which have become invaluable tools can provide important information on the mechanism of toxicity [11]. We therefore investigated the effects of Gentamicin on novel kidney biomarkers in stable HK-2 cells for up to 48h. The principal goal was to determine if changes in the expression of the newly identified biomarkers correlate with the progressive damage to the kidney as evidenced by histopathology and might even precede traditional endpoints of nephrotoxicity.

Materials And Methods

Animals and Cells

Male Sprague-Dawley rats (180-220g) were housed under conditions of $23 \pm 2^\circ\text{C}$ and 40–70% relative humidity at all times in an alternating 12 h light-dark cycle. All experiments were conducted according to the Guidelines for Animal Experiments. Gentamicin sulfate (Sichuan Changzheng pharmaceutical co., Ltd, Chengdu, China) was intravenously administered at 0, 50 or 100 mg/kg/d ($n = 5$ rats/dose group/time point) and the animals were necropsied on days 1, 3 or 7 for toxicity evaluation.

HK-2 cells were cultured in DMEM medium with 10% fetal bovine serum, stored in a humidified incubator (95% air with 5% CO_2) at 37°C . Gentamicin was diluted with cell culture medium to reach the desired concentrations (100 $\mu\text{g}/\text{ml}$). Gene expressions were detected at various time points (2hr, 6hr, 12hr, 24hr, 36hr and 48hr).

Blood chemistry

Rats were fasted overnight before necropsy and bled from the abdominal aorta with 3 ml collected into serum separator tubes and centrifuged 1,500g for 10 min at 4°C . Isolated plasma and serum samples were stored at -80°C until use. SCr and BUN were measured using a standard clinical chemistry analyzer.

Table 1. Sequence of Forward and Reverse Primers for Real-Time PCR

Gene title	Accession no.	Forward primer 5'-3'	Reverse primer 5'-3'
KIM-1 (Rat)	AF035963	AACGCAGCGATTGTGCATCC	GTACACTCACCATGGTAACC
NGAL (Rat)	NM_130741	GATGAACTGAAGGAGCGATTTC	TCGGTGGGAACAGAGAAAAC
GAPDH (Rat)	BC059110	CCTGGAGAAACCTGCCAAGTAT	AGCCCAGGATGCCCTTTAGT
KIM-1 (Human)	AW604297	TCACCTCAGCCAGCAGAAACCC	CACCCAAAAGAGCAAGAAGCAC
NGAL (Human)	BC033089	TTCTCGGCCCTGAAATCATG	TCAGCCGTGCATACACTGGTC
GAPDH (Human)	BE893087	CAGCCTCAAGATCATCAGCA	GTCTTCTGGGTGGCAGTGAT

Histopathology and Immunohistochemistry

After fixation in 4% paraformaldehyde for 48h, the kidneys were rinsed in tap water, dehydrated in a graded alcohol series, embedded in paraffin, and cut into 5µm thick sections. The paraffin sections were processed for hematoxylin–eosin staining and immunohistochemistry. Detections of biomarkers were used rabbit anti-KIM-1/Tim-1 (Abcam, USA) and rabbit anti-NGAL/LCN2 (Millipore, USA). Sections were incubated with biotinylated secondary antibody (mouse anti-rabbit, Invitrogen, USA) and following incubation with Streptavidin–Peroxidase conjugate (Invitrogen, USA), peroxidase activity was visualized using AEC/DAB substrate-chromogen mixture (Invitrogen, USA).

Quantitative Realtime PCR

RNA was isolated from kidney tissue or cell samples using the Total RNA Extractor Kit (Sangon Biotech Co.,Ltd. Shanghai, China) and further purified using the RNeasy Kit (Takara, Dalian, China) including DNase treatment. RNA integrity was confirmed by 1.2% agarose gel electrophoresis and the concentration was measured by spectrophotometry measuring the absorbance at 260 nm and 280nm. Complimentary DNA (cDNA) was synthesized from 1µg RNA using the Prime SScript RT reagent Kit (Takara, Dalian, China). Quantitative real-time PCR was performed using the Bio-rad CFX96 Sequence Detection System in 25-µl reactions containing 2×mastermix with SYBR Green I, 2µl of cDNA and 0.5µl of each primer. Amplification was carried out using the following temperature profile: 3 min enzyme activation at 95°C, followed by 40 cycles of 95°C for 10s, 60°C for 30s, and 72°C for 10s. Primer sequences are shown in Table 1. PCR product formation was determined by measuring the fluorescence signal emitted by the incorporation of SYBR Green I. Gene expression changes relative to untreated controls were determined by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed by ANOVA and Dunnett's test using the SPSS 19.0 software (USA). Values significantly different from control are indicated as *p < 0.05, **p < 0.01, and ***p < 0.001.

Results

In Vivo Study

After the single intravenous administration of Gentamicin at 50mg/kg and 100mg/kg, traditional biomarkers for nephrotoxicity, SCr and BUN levels, and kidney histopathology were examined on Days 1, 3 and 7. No noteworthy findings were recorded in the control group at any time point. SCr and BUN levels were significantly increased on Day 7 (Fig.1), which was consistent with the progression of histological damage (Fig.2a). Mild tubular cell vacuolar degenerations were seen treated by 50mg/kg bw Gentamicin (no shown). Tubular cell degenerations, necrosis, tubular dilatation, hyaline cast tubules and inflammation were observed in the distal and proximal tubules in the Gentamicin-treated rats on Day7 (Fig.2a). As previously reported [12, 13], Gentamicin treatment resulted in histopathological changes, depending on dose and treatment time.

Consistent with progression of renal damage, KIM-1 and NGAL were undetectable in renal tubules of control kidneys, but they were detected as early as Day1 in kidneys of both low-dose (no shown) and high-dose animals (Fig.2). In the treated animals, an increase

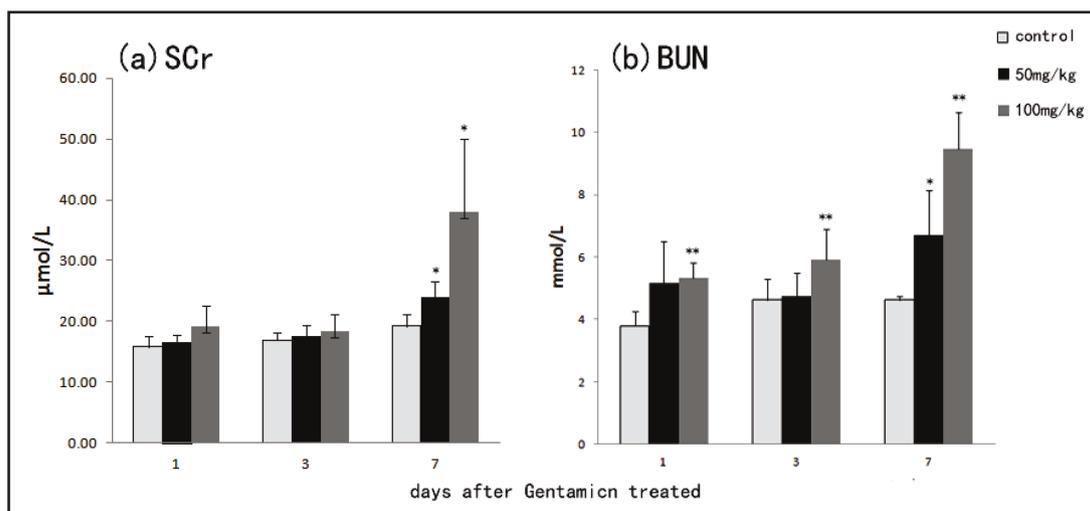
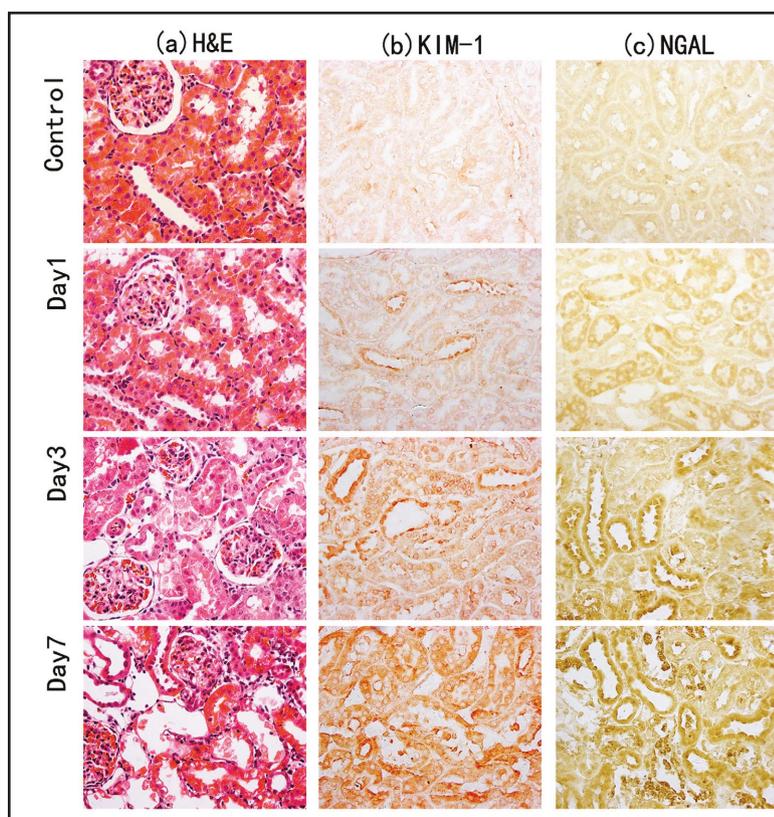


Fig. 1. Levels of SCr and BUN in rats treated with Gentamicin: Data are expressed as 0, 50 or 100 mg/kg bw Gentamicin for 1, 3, and 7 days. Data are presented as mean fold change \pm SD compared with control animals (n=5). Statistical analysis was performed by ANOVA and Dunnett's test (*p < 0.05, **p < 0.01).

Fig. 2. (a) Hematoxylin and eosin-stained kidney sections of rats treated with 100mg/kg bw gentamicin for up to 7 days, showing normal renal architecture in control group, slight tubular cell vacuolar degenerations in the proximal tubules 3 days after Gentamicin treatment and tubular cell degeneration, necrosis, tubular dilatation and inflammation observed in the distal and proximal tubules in Gentamicin-treated rats at 7 days. (b) Marked KIM-1 induction in tubules with cellular degeneration in kidneys of rats treated with 100mg/kg bw Gentamicin for up to 7 days. (c) Treatment with 100mg/kg bw Gentamicin for up to 7 days resulted in NGAL observed at the apical side of distal tubules in the cortex and in the deep OSOM. Original magnification 400 \times .



of KIM-1 staining was seen first in the OSOM and cortex at day 1, occurred in epithelial cells of S3 tubules that were affected by Gentamicin toxicity as seen by cell degeneration and regeneration. Its intensity and extent increased with the treatment duration, in close

Table 2. Changes in Gene Expression of novel Biomarkers of Nephrotoxicity in HK-2 cells after Treatment with Gentamicin for 2, 6, 12, 24, 36 or 48h

Gene title	Fold deregulation - Interval (h)					
	2	6	12	24	36	48
KIM-1	2.07±0.23	0.73±0.18	0.71±0.08	0.60±0.18	0.66±0.15	0.66±0.06
NGAL	0.22±0.01	0.54±0.22	0.45±0.08	0.38±0.15	0.40±0.19	1.18±0.69

Note. Data are presented as mean ± SD compared with controls (3 independent experiments)

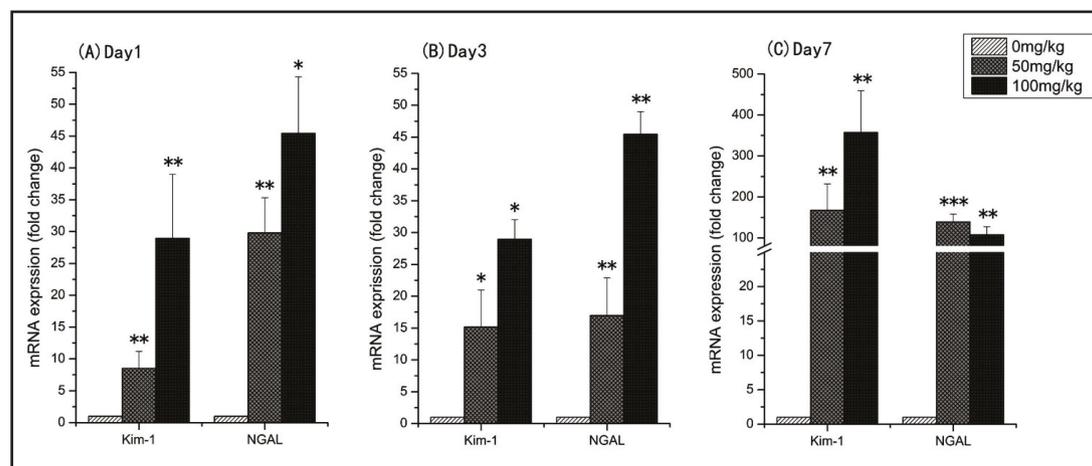


Fig. 3. Changes in mRNA expression of KIM-1 and NGAL biomarkers of nephrotoxicity in kidneys of SD rats repeatedly dosed with 0, 50 or 100 mg/kg bw Gentamicin for 1, 3, and 7 days. Data are presented as mean fold change ± SD compared with control animals (n=5). Statistical analysis was performed by ANOVA and Dunnett's test (*p < 0.05, **p < 0.01, ***p < 0.001).

correlation with the histopathological changes (Fig.2b). NGAL was exclusively detected at the apical side of some distal tubules in the cortex and in the deep OSOM (Fig.2c). Time and dose-related effects on the two biomarkers expressions were observed at the target site of Gentamicin toxicity.

Repeated administration of 50 or 100 mg/kg bw Gentamicin to male SD rats resulted in a dose- and time-dependent increase in the expression of genes suggested as candidate biomarkers of renal injury (Fig. 3). Changes in the expression of KIM-1 and NGAL were found to correlate with the progressive histopathological alterations observed in kidneys of Gentamicin-treated animals and preceded changes of clinical parameters indicative of impaired kidney function. Induction of KIM-1 and NGAL gene expressions were the earliest responses of the kidney following Gentamicin treatment, evident as early as Day1 in both low-dose and high-dose animals, where only subtle histopathological changes were observed. Furthermore, the degree of KIM-1 mRNA increase in the individual animals was more upregulated in high-dose animals after Gentamicin treated for 7 days (Fig.3C).

In Vitro Study

In contrast to the significance of biomarkers expression in vivo, treatment of HK-2 cells with 100µg/ml Gentamicin for up to 48h, compared with controls, did not result in marked changes in the mRNA expression of KIM-1 and NGAL (Table 2). Both of the two biomarkers were expressed at low levels in vitro, and no increase in mRNA was observed after treatment with Gentamicin.

Discussion

The kidney is one of the routinely assessed organs during preclinical safety evaluations. The present study was designed to assess the value of novel tissue biomarkers for improved detection of kidney injury in rats using the aminoglycoside antibiotic Gentamicin as a model compound [14-16]. Gentamicin may accumulate in epithelial tubular cells causing a range of effects starting with loss of the brush border in epithelial cells and ending in overt tubular necrosis, activation of apoptosis and massive proteolysis which was confirmed in our study.

Our data clearly reveal that Gentamicin induced renal injury in all study animals, as reflected by increased tissue KIM-1 and NGAL. We observed upregulation of mRNA expression of KIM-1 and NGAL in kidney of rats following treatment with Gentamicin in the absence of effects on traditional clinical chemistry markers of nephrotoxicity. These increases were evident 1 day after treated by Gentamicin and lasted for 3 and 7 days, respectively. Both the time-course and the degree of the response correlated well with the severity of tissue injury. Changes in marker gene expression were subsequently confirmed by immunohistochemistry. Our observations that expression of KIM-1 and NGAL occurs within 1 day after treated by Gentamicin accept that these two biomarkers reflected renal injury faster and more accurately than SCr and BUN [6, 8].

Changes in the expression of NGAL were one of the earliest effects observed in kidneys of animals treated with 50 or 100 mg/kg bw Gentamicin, occurring at time-points at which had few histopathological changes in epithelial tubular cells were injured evident. As compared with NGAL, the mRNA level of KIM-1 was more prominent after treated by Gentamicin for 7 days. Upregulation of the two genes were thought to be associated with proliferation/regeneration and repair in response to toxicity or disease [8, 10], consistent with the histopathological alterations observed following treatment with Gentamicin.

An ancillary aim of this study was to determine if the two novel kidney biomarkers might also serve as sensitive endpoints for in vitro toxicity testing. Development of reliable in vitro test systems may contribute to reduction of animal experiments required for drug and chemical safety assessment [11]. It is known that Gentamicin inhibits the activity of lysosomal phospholipases in proximal tubular epithelial cells. Both in vivo and in vitro studies showed that Gentamicin has enhanced the generation of reactive oxygen metabolites (ROS) [17]. Therefore, mRNA expressions of KIM-1 and NGAL were studied in HK-2 cells, which may allow rapid screening of large numbers of compounds. However, treatment resulted in only minor alterations in gene expression following exposure to Gentamicin. KIM-1 and NGAL, which were sensitive markers for toxicity in vivo, were not significantly affected by treatment. Similar effects were also observed in Ochratoxin A-treated NRK-52E cells, which was explanted that, in contrast to the kidney, some of the marker genes were already expressed at relatively high levels in vitro [18]. This is supported by the fact that expression of NGAL reported to increase during isolation and cultivation of proximal tubular cells [19]. Both KIM-1 and NGAL were thought to be associated with proliferation/regeneration which may not occur in monolayers of cultured kidney cells in response to a continuous nephrotoxic insult. Thus, it is not feasible to complete replacement of animal studies with Gentamicin-induced nephrotoxicity. However, in vitro assays may still be valuable tools for evaluation some drug's potential nephrotoxicity [20]. This may serve to reduce the need for animal testing, particularly within pharmaceutical industries, because potent nephrotoxins may be excluded from further development and only those candidates showing no effects in vitro may need to be tested in vivo.

Measuring these novel biomarkers may enable clinicians to make an early diagnosis of aminoglycosides-induced AKI, which does not evolve into acute renal failure. Support for this notion comes from numerous clinical studies in which plasma KIM-1 and NGAL or urinary excretion of these biomarkers increased a few hours after the induction of AKI of various etiologies, including kidney transplant rejection [21, 22], nephron sparing surgery [23] and nephrotoxic drugs induced nephropathy [24].

The goal of reducing or protecting against aminoglycosides' nephrotoxicity has attracted much effort and attention during the last decade [25-27]. As this research was designed to evaluate novel biomarkers used in Gentamicin-induced early renal injury, we did not study how these markers express after exposure to Gentamicin for a long time. Future investigations are required to not only confirm a potential application of KIM-1 and NGAL as useful biomarkers for such a diagnosis but also as parameters to prevent the development and progression of chronic kidney disease.

Conclusion

Alterations in the excretion of KIM-1 and NGAL were detected before marked changes in clinical chemistry parameters were evident, providing further support for the use of the two biomarkers as sensitive, mechanistically anchored indicators of Gentamicin-induced nephrotoxicity *in vivo*. In contrast, results from this study do not support their use as reliable endpoints of toxicity *in vitro*.

Disclosure Statement

The authors declare no conflicts of interest.

Acknowledgement

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