

Diclofenac-induced acute kidney injury is linked with oxidative stress and pro-inflammatory changes in sprague-dawley rats

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(Submitted: 12 September 2018 – Revised version received: 19 November 2018 – Accepted: 14 December 2018 – Published online: 26 June 2019)

Objectives Diclofenac induces oxidative stress in the body and became the main cause of nephrotoxicity and acute kidney injury (AKI). The traditional markers of AKI are blood urea and serum creatinine which are regarded as low sensitive and low specific in detection the early renal damage. Therefore, the aim of this study is to evaluate oxidative stress and pro-inflammatory biomarkers in diclofenac-induced AKI in rats.

Methods Twenty Sprague-Dawley Male rats were used and randomly divided into two groups. Group 1 ($n = 10$): Rats treated with distilled water plus normal saline for 12 days. Group 2 ($n = 10$): Rats treated with distilled water plus diclofenac 15 mg/kg for 12 days. Rat body weight, body mass index and estimated glomerular filtration rate (eGFR) were evaluated in both groups. Blood urea, serum creatinine, serum malondialdehyde, superoxide dismutase, glutathione reductase, neutrophil gelatinase associated lipocalin, kidney injury molecules (KIM-1) and Cystatin-c were estimated.

Results Diclofenac 15 mg/kg led to significant AKI through elevation of blood urea and serum creatinine with significant reduction of eGFR. KIM-1 serum level was significantly elevated with high sensitivity and specificity compared with the other tested biomarkers.

Conclusion KIM-1 serum level is more sensitive and specific with high accuracy compared with the other renal biomarkers in diclofenac-induced AKI. Estimation of KIM-1 serum levels should be regarded as a cornerstone for early detection of AKI in high risk patients.

Keywords diclofenac, estimated glomerular filtration rate, acute kidney injury, nephrotoxicity

Introduction

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID); belong to the acetic acid group. Diclofenac is used as analgesic, anti-inflammatory and antipyretic agent. Diclofenac sodium acts by inhibiting cyclo-oxygenase-1 (COX-1) and cyclo-oxygenase-2 (COX-2) enzymes, which are responsible for prostaglandin synthesis from arachidonic acid.¹ Diclofenac inhibits COX-1 about 10-fold than COX-2 on contrast to the aspirin which is mainly block COX-1. Indeed, it also inhibits lipoxygenase, phospholipase A2 and other pro-inflammatory autacoids.²

Diclofenac-induced nephrotoxicity is due to the toxic effect of diclofenac on renal glomeruli and tubules. Diclofenac induces formation of glomerular lesions, tubular dilatation and loss of brush border.³ Long-term treatments with diclofenac causes dose-dependent reduction in renal PG lead to disturbances in the glomerular function and reduction of glomerular filtration rate (GFR). In addition, diclofenac induces autolysis that increases renal intracellular osmolarity which is responsible for proximal renal tubules dilatations.⁴

Diclofenac is metabolized in the liver to 4-hydroxydiclofenac, after sulfation and conjugation, which are excreted by the urine (65%) and bile (35%). Diclofenac sodium modifies renal function through inhibition of renal prostaglandins leading to reversible renal ischemia.⁵ Although, NSAIDs related hypertension, salt and water retention, edema and hyperkalemia are highly linked with acute renal failure.⁶

It has been hypothesized that diclofenac has dose-dependent effects on plasma coagulation parameters, oxidative stress, renal damage and adenosine deaminase activity in diclofenac-induced AKI. The toxicity induced by diclofenac sodium result in depletion of ATP, lipid peroxidase, mitochondrial dysfunction and change in renal cells calcium concentration.⁷ Prostaglandin

play an important role in GFR since; renal plasma current is maintained by balanced between the vasoconstrictor impact of the renin-angiotensin system and the vasodilatory effect of prostaglandin. In fluid depleted state, prostacyclin (PGI₂) affect renal homeostatic mechanisms, (PGI₂) and (PGD₂) cause dilatation of renal vascular bed along with the lowering of renal vascular resistance.⁸ Thus, prostaglandins enhances renal perfusion with redistribution of blood flow from the renal cortex to nephrons in the juxta-medullary region. Renal prostaglandins have crucial local function in different parts of renal tissues such as glomeruli and convoluted tubules, thus prostaglandin inhibitors have various deleterious effects.⁹ Diclofenac-induced AKI may be related to the ischemia caused by inhibition of prostaglandins synthesis in renal arterioles.¹⁰ Diclofenac induces oxidative stress in the body and became the main cause of nephrotoxicity and AKI leading to the acceleration of lipid peroxidation and reduction of endogenous antioxidant capacity.¹¹

On contrary, a previous study illustrated that inhibition of COX may not be the main mechanism of diclofenac-induced AKI, which observed that redox signalling and oxidative stress as the main contributing factors in diclofenac-induced AKI.¹²

Normally, reactive oxygen (hydroxyl radical, nitric oxide, peroxynitrite, hydrogen peroxide, superoxide anion and single oxygen) produced in the body are deactivated by non-enzymatic (vitamin C, vitamin D and glutathione) and enzymatic (catalase, glutathione peroxidase and superoxide dismutase) agents.^{13,14}

In general, antioxidants and oxidants are in balance in the body, but oxidative stress happens when the reactive oxygen radicals are produced extremely and/or antioxidants are insufficient, as a result, oxidative stress will be initiated.¹⁵

Furthermore, the toxic dose of diclofenac provokes renal mitochondrial dysfunction through inhibition of mitochondrial

complex causing reduction in ATP generation and apoptosis. Thus, anti-oxidants play a potential role in prevention of diclofenac-induced AKI via attenuation of free radical and oxidative stress effects.¹⁶ Likewise, diclofenac-induced AKI may be related to the induction of pro-inflammatory cytokines including interleukin (IL)-6, IL-1, IL-33 and tumor necrosis factor- α lead to renal nuclear fragmentation and condensation.¹⁷

Biomarkers of Acute Kidney Injury

The traditional markers of acute kidney injury (AKI) are blood urea and serum creatinine which are regarded as low sensitive and low specific in detection the former renal damage. Thus, detection of the initial renal injury required a new biomarkers which are more sensitive and highly specific that give an insight about the site of underlying renal damage.¹⁸

A urinary protein is regarded as a potential marker of acute and chronic renal damage that are induced by nephrotoxic drugs. Normally, the glomeruli restrict the transport and migration of high molecular weight proteins from blood into the lumen of nephron, but during pathological conditions high molecular proteins can be identified and detected in the urine due to nephron dysfunction.¹⁹ High molecular proteins like albumin, transferrin and immunoglobulin G are more sensitive proteins in early detection of glomerular filtration dysfunction and structural glomerular damage.²⁰ On the other hand, low molecular weight proteins are mainly reabsorbed at renal proximal tubules, when there is an excess in the amount of low molecular weight protein concentrations a nephron overload occurred that exceeding the proximal renal tubules reabsorbing capacity causing proteinuria due to failure of the re-absorption capacity.²¹

Low molecular weight proteins such as α 1-microglobulin, β 2-microglobulin, Cystatin-c, retinol binding protein and kidney injury molecule-1 (KIM-1) are obviously recorded as the main proteins that reflect the underlying renal glomerular and/or tubular damage during nephrotoxicity.^{22,23}

Nephrotoxic agents such as cisplatin chemotherapy, NSAIDs and aminoglycoside lead to up-regulation of KIM-1 due to ischemic reperfusion injury thus; serum level of KIM-1 is linked and correlated with the immune-response of renal proximal tubules damage during AKI.²⁴ Additionally, mRNA of KIM-1 is vastly expressed in the injured kidney which is exposed and released into the lumen which finally excreted in the urine. Also, KIM-1 is detected in the blood since; it stable and can be straightforwardly identified.²⁵

Besides, neutrophil gelatinase associated lipocalin (NGAL) which is a 25 kDa protein that attach the granulocytes, it correlated with drug induced AKI since; it is responsible for the renal inflammation during ischemia thus; NGAL serum level be a sign of acute renal injury.²⁶

Additionally, different cytokines such as interleukin, interferon, and colony stimulating factors play an important and integral role in the renal tubular damage and repair so; they are considered as biomarkers of kidney injury during drug induced AKI.²⁷

Cystatin-c is a low molecular weight protein excreted by glomerular filtration and high level of Cystatin-c is linked with reduction of GFR and glomerular filtration. It has been shown that Cystatin-c serum levels predict the stage and progression of renal diseases. Cystatin-c serum levels also increased in cigarette smoking, cancer, neurological and atherosclerosis.^{28,29}

Therefore, novel and new biomarkers may help the determination the site of renal damage and give an important picture about the disease progression and the role of nephro-protecting agents.³⁰

Therefore, the aim of this study was to evaluate oxidative stress and pro-inflammatory biomarkers in diclofenac-induced AKI in rats.

Materials and Methods

About 20 Sprague-Dawley Male rats were used in this study. These animals were gained from the National Center for Drug Control and Research. Rats' age ranged from 3 to 4 months and their body weight ranged from 200 to 400 g. The animals were isolated as three rats in each sterilized cage and placed with suitable temperature (22–25°C) with artificial 12/12 light cycle. They left for 1 week for adaptation without any intervention with free access to normal chow pellets and water. Human care for animals was according to the guide to the care and use of laboratory animal. After acclimatization period, weights of rats were taken and rat with wound infection were excluded. Then rats were randomly divided into two groups, 10 rats in each group. The study protocol and method for induction of AKI was according to Singh et al's,³¹ method.

Control group (n = 10): Rats treated with distilled water (5 ml/kg, p.o.) for 12 days, on days 6–12 received an intraperitoneal injection of normal saline (5 ml/kg) daily.

AKI group (n = 10): Rats treated with distilled water (5 ml/kg, p.o.) for 12 days and on days 6–12 received diclofenac 15 mg/kg, i.p.

Anthropometric Measurements

Length was measured by graduated tape measure from nose to the anus (naso-anal length in cm). Rat body weight was done by specific digital balance in gram. Body mass index (BMI) equal body weight in grams over the square of length in cm, $BMI = BW (g)/length (cm)^2$. Estimated glomerular filtration rate (eGFR) was measured according to Schwartz formula, $eGFR = k \times height (cm)/serum creatinine (mg/dl)$, $k = 0.55$.³²

Sample Collection

On day 12, chloroform was used to anesthetize the rats and sharp scissors were used to exudate the rats. The blood sample was allowed to drain in sterile gel tube, and then centrifuged for 10 min at 5000 rpm at room temperature, after that the formed supernatant layer was isolated as serum sample and kept in freezer at –20°C to be assessed later.

Assessment of Biochemical Variables

Blood urea and serum creatinine were estimated by using an auto-analyzer (ILab-300-Biomerieux Diagnostic, Milano, Italy) they are expressed as mg/dl. Serum malondialdehyde (MDA), superoxide dismutase (SOD), glutathione reductase (GSH), NGAL, KIM-1 and Cystatin-c were measured by ELISA kit methods according to the instruction of the kit manufacture (Myo-bio source, USA).

Statistical Analysis

Statistical Package for the Social Sciences software (SPSS, Inc., Chicago, IL, USA) was used for data analysis. Data of the this study presented as mean \pm SD. Unpaired student *t*-test between

control and treated groups was used for detecting the significance of differences. The levels of significance was regarded when $P < 0.05$.

Results

During diclofenac-induced AKI, rat body weight was increased to 286.87 ± 27.24 g compared with the control group 268.00 ± 25.01 g but; not reached to the significant level ($P = 0.20$). The BMI was increased significantly in AKI group compared with the control group ($P = 0.01$). Blood urea was raised significantly in AKI group up to 70.5 ± 12.53 mg/dl compared with the control group (41.83 ± 7.46 mg/dl, $P = 0.0003$). Besides, serum creatinine in AKI group was increased significantly (1.52 ± 0.49 mg/dl) compared with the control group (0.7 ± 0.14 mg/dl, $P = 0.0019$). The estimated GFR reduced significantly in AKI group (7.59 ± 1.7 ml/min/1.37) compared with the control group (16.89 ± 4.21 ml/min/1.37, $P = 0.0001$). Regarding the oxidative stress and endogenous anti-oxidant capacity, there were insignificant increase in the MDA serum levels in AKI group (427.65 ± 210.39 ng/ml) compared with the control group (289.85 ± 44.18 ng/ml, $P = 0.14$) while; both SOD and GSH serum level were reduced in AKI group compared with the control group ($P = 0.2$ and 0.4929 respectively). KIM-1 was significantly raised in AKI group (269.03 ± 29.61 pg/ml) compared with the control group (73.78 ± 16.29 , $P = 0.0001$) (Table 1).

Indeed, Cystatin-c serum level was insignificantly increased during induction of AKI by diclofenac from 0.024 ± 0.0005 ng/ml in the control group to 0.0277 ± 0.009 ng/ml in the AKI group ($P = 0.33$) (Fig. 1).

Blood urea was significantly correlated with serum creatinine ($r = 0.99$, $P = 0.01$), MDA ($r = 0.99$, $P = 0.008$), KIM-1 ($r = 0.89$, $P = 0.02$) and Cys-c ($r = 0.98$, $P = 0.01$) but negatively correlated with SOD ($r = -0.99$, $P = 0.001$) in AKI group (Table 2).

Kidney injury molecule-serum level was highly sensitive and specific biomarker in diclofenac-induced AKI while; GSH was the least sensitive biomarker (Table 3).

Table 1. Effect of diclofenac on the anthropometric variables, biochemical and inflammatory biomarkers in diclofenac induced AKI

Biomarkers	Control (n = 10)	AKI (n = 10)	P
Weight (g)	268.00 ± 25.01	286.87 ± 27.24	0.20
Height (cm)	21.50 ± 0.83	21.00 ± 0.53	0.19
BMI (g/cm ²)	1.02 ± 0.02	1.64 ± 0.03	0.01*
Blood urea (mg/dl)	41.83 ± 7.46	70.50 ± 12.53	0.0003**
Serum creatinine (mg/dl)	0.70 ± 0.14	1.52 ± 0.49	0.0019**
Estimated GFR (ml/min/1.37)	16.89 ± 4.21	7.59 ± 1.7	0.0001**
MDA (ng/ml)	289.85 ± 44.18	427.65 ± 210.39	0.14
SOD (pg/ml)	48.12 ± 32.92	30.62 ± 15.54	0.20
GSH (µg/ml)	15.94 ± 2.39	14.88 ± 3.02	0.4
KIM-1 (pg/ml)	73.78 ± 16.29	269.03 ± 29.61	0.0001**
NGAL (pg/ml)	15.78 ± 3.07	18.76 ± 4.13	0.16

* $P < 0.05$. ** $P < 0.01$ unpaired *t*-test. BMI: body mass index; GFR: glomerular filtration rate; MDA: malondialdehyde; SOD: superoxide dismutase; GSH: glutathione reductase; IL-18: interleukin-18; KIM-1: kidney injury molecule-1; NGAL: neutrophil gelatinase associated lipocalin.

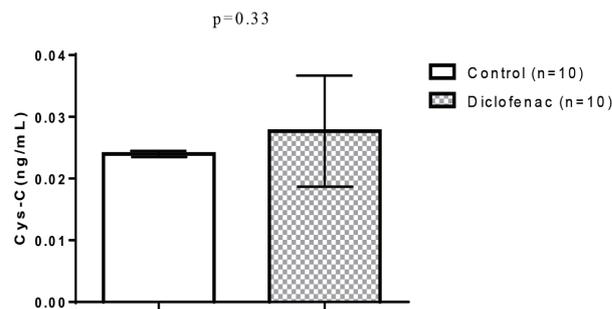


Fig. 1 Cystatin-c serum levels in diclofenac-induced AKI.

Table 2. Correlation of blood urea with biochemical parameters and renal biomarkers in diclofenac-induced AKI group compared with the control group

Variables	Group I		Group II	
	r	P	r	P
Serum creatinine (mg/dl)	0.62	0.33	0.99	0.01**
GFR (ml/min/1.37)	0.33	0.50	-0.91	0.08
MDA (ng/ml)	0.57	0.23	0.99	0.008*
SOD (pg/ml)	0.72	0.09	-0.99	0.001*
GSH (µg/ml)	0.45	0.43	-0.89	0.09
KIM-1 (pg/ml)	0.86	0.06	0.89	0.02**
Cys-c (ng/ml)	0.66	0.15	0.98	0.01**
NGAL (pg/ml)	0.50	0.29	0.87	0.05

* $P < 0.01$. ** $P < 0.05$ unpaired *t*-test. GFR: glomerular filtration rate; MDA: malondialdehyde; SOD: superoxide dismutase; GSH: glutathione reductase; KIM-1: kidney injury molecule-1; Cys-c: Cystatin-c; NGAL: neutrophil gelatinase associated lipocalin.

Table 3. Sensitivity and specificity of biomarkers in diclofenac-induced AKI

Biomarkers	Sensitivity	Specificity	Accuracy	PLR	NLR
Blood urea (mg/dl)	70	58.33	63.64	1.6	0.5
Serum creatinine (mg/dl)	72.73	63.64	68.18	2.0	0.4
MDA (ng/ml)	60.00	33.33	50.00	0.9	1.2
SOD (pg/ml)	55.56	40.00	50.00	0.93	1.1
GSH (µg/ml)	50.00	40.00	46.67	0.83	1.25
KIM-1 (pg/ml)	90.00	90.00	90.00	9.00	0.11
NGAL (pg/ml)	57.14	57.14	57.14	1.33	0.75
Cys-c (ng/ml)	81.82	50.00	70.59	1.64	0.36

MDA: malondialdehyde; SOD: superoxide dismutase; GSH: glutathione reductase; KIM-1: kidney injury molecule-1; Cys-c: cystatin-c; NGAL: neutrophil gelatinase associated lipocalin, PLR: positive likelihood ratio; NLR: negative likelihood ratio.

Discussion

This study illustrated significant diclofenac-induced AKI which revealed through elevation of blood urea and serum creatinine compared with the control group in the experimental rats as supported by Alabi et al's,³³ study that illustrated diclofenac leads to significant renal tubular necrosis and

raising in the renal biomarkers. The induced AKI in this study as well showed significant reduction in the estimated GFR which was due to the development of acute tubular necrosis and glomerular damage since; Tomohiro et al.,³⁴ exposed that short-term administration of diclofenac leads to proximal renal tubular damage and significant glomerular damage due to potential inhibition of renal prostaglandins which *per se* reduce renal blood flow and causing renal ischemia. BMI of the experimental rats was higher compared with the control group this might due to daily body weight gain or fluid retention which caused by renal damage. Moreover, reduced GFR also take part in raised BMI via reduction of urine output and development of peripheral oedema as a number of rats developed ascites. Observational study suggested that fluid retention is the main element of clinical syndrome of kidney disease since; there are similarities in the fluid retention and component in the patients with either heart failure or advanced renal disease.³⁵ Oxidative stress, free radical generations, lipid peroxidations and reduction of endogenous anti-oxidant capacity are the main mechanisms of diclofenac-induced AKI as described by Hickey et al.,³⁶ study that demonstrated a high dose of diclofenac leads to significant lipid peroxidation through elevation of MDA serum levels and reduction of SOD (marker of anti-oxidant). But in this there were insignificant elevation in MDA serum level and insignificant reduction in the anti-oxidant capacity (SOD, GSH) respectively which might due to insufficient diclofenac dose, small sample size or short duration of the experimental study. Indeed, many cytokines are elevated during diclofenac-induced AKI that reflects the inflammatory process in the renal tissues. Bunel et al.'s,³⁷ study showed that IL-18 and NGAL sera levels were significantly increased within short period of renal injury induction (within hours) and return to the normal levels within few days due to partial regeneration of damaged renal tubules. This may explain the insignificant elevation of these biomarkers in this study. In additional, renal biomarkers are subjected to different variability including dynamic of their excretion, diurnal variations and baseline assessment as supported by Harrill et al.,³⁸ study that confirmed the variable results of this study. Likewise, diclofenac in this study failed to increase serum levels of NGAL significantly.

Furthermore, KIM-1 was significantly elevated in this study compared with the control. Lan et al.'s,³⁹ study confirmed significant elevation of KIM-1 in Cadmium-induced nephrotoxicity.

KIM-1 serum levels are elevated during initiation of acute nephrotoxicity as it increased in both serum and urine since; it shed from renal proximal tubules. KIM-1 serum is significantly correlated with different grades of renal damage in acute nephrotoxicity and renal injury because; it is highly sensitive and specific for renal tubular toxicity⁴⁰ as revealed in our study.

On the other hand, Luo et al.'s,⁴¹ *in vitro* and *in vivo* study showed that repeated and consecutive administration of nephrotoxic agents in rats for 7 days result in time dependent increase in both KIM-1 and NGAL serum levels before the initiation of renal proximal tubules damage suggesting that both KIM-1 and NGAL are insensitive in detection of early renal damage and these biomarkers may not be appropriate in evaluation and detection of nephrotoxic effect of certain agents.

Cystatin-c is a sensitive biomarker for detection of acute renal damage seeing as; it more sensitive than blood urea and serum creatinine.⁴² Harman et al.'s,⁴³ study confirmed that Cystatin-c is a surrogate biomarker for acute renal injury and should be incorporated in the estimation of GFR as it is more sensitive than creatinine but; Cystatin-c serum levels in diclofenac-induced AKI of this study were elevated insignificantly compared with the control which might be due to small sample size, biomarker variability and short duration of the study, these factors may contribute to the insignificant elevation of Cystatin-c in diclofenac-induced AKI in rats model of the current study.

Conclusion

Kidney injury molecule-1 serum level is more sensitive and specific with high accuracy compared with the other renal biomarkers in diclofenac-induced AKI. Therefore, estimation of KIM-1 serum levels should be regarded as a cornerstone for early detection of AKI in high-risk patients.

Acknowledgment

The authors would like to thank the Research committee in College of Medicine, Al-Mustansiriyia University for the great supports.

Conflicts of Interest

None. ■

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