

Reno-Protective Effect of Exogenous Glutathione on Experimentally Induced Acute Kidney Injury in Male Rats

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ABSTRACT

Background: Acute kidney injury (AKI) or Acute renal failure (ARF) refers to the sudden damage or failure of the kidney within few hours or days and resulting in acute deterioration of the renal functions. If not properly treated, AKI may lead to chronic renal failure and possibly renal transplantation. The aim of the present study was to evaluate the role of exogenous glutathione (GSH) on ciprofloxacin (GFX)-induced AKI. We also studied the effect of glutathione administration on some genes of interest.

Methods: Forty male Wistar albino rats were equally divided into 4 groups. The control group received intra-peritoneal injection of distilled water for 15 consequent days. The GSH treated group received concomitant intra-peritoneal injection of distilled water and glutathione (200 mg/kg/day) for 15 consequent days. The CFX treated group received concomitant intra-peritoneal injection of distilled water and ciprofloxacin (800 mg/kg/day) for 15 consequent days. The CFX+GSH treated group received concomitant intra-peritoneal injection of CFX and CSH for 15 consequent days. Serum levels of creatinine, urea, cystatin C and GGT were measured. Renal CYP4F1, GPx, GSR gene expression was evaluated.

Results: Exogenous GSH had no significant effect on the kidney functions or the studied genes when compared to the control group. Treatment with CFX resulted in significant increase ($P<0.05$) in creatinine, urea, cystatin C and GGT serum levels when compared to the control group. CFX treatment also significantly ($P<0.05$) down-regulated renal GPx, GSR mRNA levels, while it up-regulated renal CYP4F1, when compared to the corresponding values in the control rats. Serum levels of urea, creatinine and cystatin C were significantly lower ($P<0.05$) in CFX+GSH group when compared to the CFX treated rats. There was significant up-regulation ($P<0.05$) of the renal, GPx, GSR and down-regulation of CYP4F1 mRNA levels in the CFX+GSH group when compared to the corresponding values in the CFX treated group.

Conclusion: Our results suggest a potential prophylactic and possibly therapeutic roles of exogenous GSH administration in the treatment of drug-induced AKI. We also demonstrated that the underlying mechanism could be explained, at least in part, by the antioxidant and gene modifying properties of GSH.

Key words: Acute kidney injury, oxidative stress, glutathione, ciprofloxacin, CYP4F1

INTRODUCTION

Acute kidney injury (AKI) or acute renal failure (ARF) can be defined as the abrupt loss of kidney functions within few days with or without changes in the urine volume. AKI usually exhibit a rise in the serum creatinine as well as other kidney function parameters¹. The incidence of AKI increased both in hospital and community settings. It is estimated that the incidence of AKI could be from 2 to 3 cases per 1000 population². It was reported that around 7% of hospitalized patients and about 66% of patients in intensive care units could develop AKI. If untreated, patients with AKI are at high risk of multiple organ failure and death³⁻⁶.

Ciprofloxacin (CPX), a commonly prescribed antimicrobial fluoroquinolone, can lead to AKI by causing the development of tubulointerstitial nephritis⁷. In experimental animals, CPX was reported to induce crystal nephropathy⁸. Timely intervention proved to be vital for the prevention of AKI-dependent complications⁹. Patients taking

CPX were advised to stay well hydrated and to avoid urine alkalization to prevent crystalluria¹⁰.

The generation of reactive oxygen species (ROS) is typically observed in the pathogenesis of AKI. Furthermore, ROS are considered to be the driving factors in cardiovascular diseases and diabetes mellitus, which either predispose to AKI or present as co-morbidities. Although ROS may play important physiological functions, unregulated accumulation of ROS could result in oxidative damage and perturbed organ functionality. However, enhanced ROS production in different pathophysiological conditions could overwhelm the capacity of the endogenous antioxidant reservoir¹¹. Accumulating data support the idea that tubular and cellular oxidative damage is significantly linked to AKI. Animal models of renal ischemia or nephrotoxicity demonstrated both attenuation of tissue antioxidant status and progressive oxidative tissue damage^{12,13}. Interestingly, critically ill or sepsis patients had increased blood levels of protein and lipid oxidation biomarkers which strongly correlated with inflammatory markers¹⁴.

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Even though the exact mechanism of AKI-induced overproduction of ROS is not yet fully elucidated, reducing ROS generation, and thereby oxidative damage, remains a potential therapeutic target. Antioxidants could interfere in the early stages of AKI pathogenesis, possibly by direct elimination of ROS or even the oxidant source. Reno-protective agents with antioxidant activities has been studied in various settings of renal cells, kidney tubules, and whole animal models of AKI^{11,15}. Glutathione (GSH) plays important roles in various physiological processes^{16,17}. GSH is principally considered as an antioxidant, an immunity booster, and a detoxifier¹⁸. Due to its low molecular weight and the presence of specific cell membrane transporters, GSH can rapidly cross the cell membrane. This could enable exogenous GSH to act as an intracellular antioxidant¹⁹.

Due to the complexity of AKI as a clinical disorder associated with severe morbidity and mortality, all possible preventive measures should be sought to avoid the deleterious consequences of the condition. In the present study, we explored the therapeutic potential of exogenous GSH in CPX-induced AKI rat model.

METHODS

All experiments and animal care and use were approved by the Faculty of Medicine Menoufia University Ethics Committee. Experiments were conducted in adherence to the Guiding Principles in the Use and Care of Animals published by the National Institutes of Health (NIH Publication No 85–23, Revised 1996).

Forty male Wistar rats weighing 150–200 g were recruited for the present study. Rats were purchased from a local providing facility. Rats were left to acclimatized for 10 days and were given free access to normal diet and water in an air-conditioned room with a 12-hour light-dark cycles.

Following acclimatization, rats were randomly divided (10 rats per group) into: 1- Control group: rats received intra peritoneal injection of distilled water for 15 consecutive days, 2- GSH-treated group: rats received intra-peritoneal injection of 200 mg/kg/day GSH (Advanced Glutathione, Rex Pharma Group, Budapest, Hungary) for 15 consecutive days, 3- CFX-treated group: rats received intra-peritoneal injection of 800 mg/kg/day CFX (Ciproxin solution for infusion, Bayer plc, Reading Berkshire, United Kingdom) for 15 consecutive days, and 4- CFX+GSH-treated group: rats received concomitantly intra-peritoneal injection of CFX and GSH.

Collection of Blood Samples: At the end of the experiment (after 15 days of treatment), rats were fasted overnight. The next morning rats were anaesthetised using intraperitoneal injection of sodium thiopental (STP, 60 mg/kg). Blood was collected then via cardiac puncture. Blood samples were left to coagulate for almost 30 minutes at room temperature. Samples were then centrifuged (3000 rpm for 15 min), and the serum was separated. Samples were stored at -20 °C for further biochemical analysis. At the end of the study, all rats were scarified by cervical dislocation.

Biochemical Analysis: ELISA kits (MyBioSource Inc, San Diego, CA, USA) were used to measure the serum level of creatinine, urea, cystatin C and gamma-glutamyl transferase (GGT) enzyme activities. An automatic optical reader (SUNRISE Touchscreen, TECHAN, Salzburg, Austria) was used to perform the tests.

Quantitative RT-PCR (qRT-PCR): Immediately after scarifying the animal, the kidneys were removed and stored at -80 or freshly used for real time quantitative reverse transcription-polymerase chain reaction (RT-PCR) experiments. Gene specific primers were designed using Primer Express Software version 2.0 (Applied Biosystems, USA).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the housekeeping control gene (Table 1). RT-PCR assays were performed in duplicate for all target and housekeeping genes using Applied Biosystems 7500 FAST 96-well PCR machine (USA). To evaluate the effects of GSH on CFX-induced AKI, the mRNA expression levels of glutathione peroxidase (GPx), glutathione reductase (GSR) and cytochrome P450-family 4- subfamily F1 (CYP4F1) genes were evaluated by RT-PCR assay as described previously 20-22. Briefly, total RNA was extracted following homogenizing frozen or fresh kidney specimens using TRI reagent (Sigma-Aldrich, UK). Reverse transcription of renal RNA was performed using high-capacity RNA-to-cDNA kit (Applied Biosystems, CA, USA). Afterwards, the generated cDNA was used to measure mRNA expression for the target genes. With GAPDH serving as the endogenous housekeeping gene control, relative quantity of mRNA expression of the target gene of interest was calculated using the comparative Ct (2^{-ΔCt}) method. Data were expressed as a ratio (target gene/GAPDH%) and were shown as the mean ± standard error of mean of at least three independent experiments.

Statistical Analysis: Origin® software was used for the statistical analysis of the data. Analyses of Variances (ANOVA) with Tukey's post hoc tests were applied following checking of data normality using Kolmogorov–Smirnov test. Results were expressed as mean ± standard error (SE), and p values < 0.05 were considered significant.

Table 1: Primers used for measuring the expression of GPx, GSH AND CYP4F1 genes

GPx	Sense	AGTTCGGACATCAGGAGAATGGCA
	Anti-sense	TCACCATTACCTCGCACTTCTCA
GSH	Sense	GGAAGTCAACGGGAAGAAGTTCCTG
	Anti-sense	CAATGTAACCGGCACCCACAATAAC
CYP4F1	Sense	AAACGGTTGATTTTCAGAAGGCAGTT
	Anti-sense	ACAGCAGTCCATGAACAGSAAAGG
GAPDH	Sense	TGCACCACCAACTGCTTAGC
	Anti-sense	GGCATGGACTGTGGTCATGAG

RESULTS

There was no significant difference ($P>0.05$) between the serum levels of creatinine, urea, cystatin C and GGT in the GSH treated group (0.65±0.06 mg/dl, 4.13±1.84 mg/dl, 1.87±0.09 mg/l, and 1.91±0.25 mg/dl respectively), when compared to the corresponding values in the control group (0.62±0.06 mg/dl, 3.22±0.55 mg/dl, 1.89±0.13 mg/l, and 1.87±0.19 mg/dl respectively). There was no significant difference ($P>0.05$) in the relative quantities (RQ%) of GPx, GSR and CYP4F1 mRNA in the GSH treated group (113.27±16.51, 110.41±12.89 and 104.83±12.37 % respectively), when compared to the corresponding values in the control group (100%) (Figures 1 and 2).

The serum levels of creatinine, urea, cystatin C and GGT in the CFX treated group (1.86±0.17 mg/dl, 26.63±2.65 mg/dl, 3.23±0.22 mg/l, and 89.77±13.57 mg/dl respectively) were significantly higher ($P<0.05$) when compared to the corresponding values in the control or the GSH treated groups. There was significant decrease ($P<0.05$) in the serum levels of creatinine, urea, cystatin C and GGT (0.85±0.07 mg/dl, 5.04±1.96 mg/dl, 1.95±0.26 mg/l, and 2.14±0.34 mg/dl respectively) in the CFX+GSH treated group when compared to the corresponding values in the CFX treated group. Although serum creatinine was still significantly higher, there was no significant difference ($P>0.05$) in serum urea, cystatin C and GGT when comparing the CFX+GSH with the control and GSH treated groups (Figure 1).

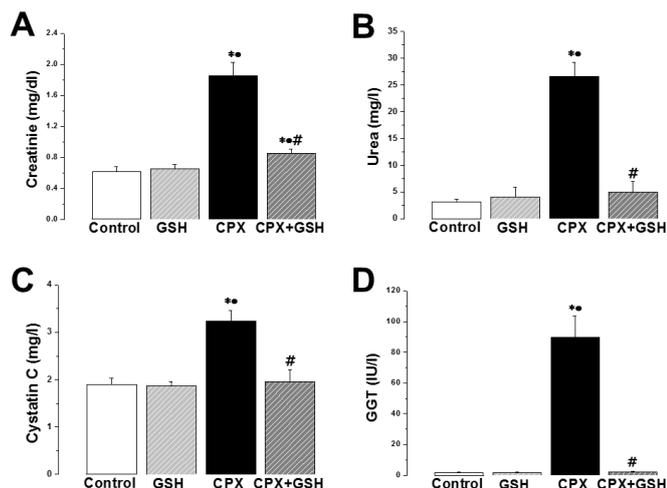


Figure 1: Exogenous GSH counters CFX-induced deterioration of kidney function. (A) Serum creatinine level in control, GFX treated, GSH treated and CFX+GSH treated groups. (B) Serum urea level in control, GFX treated, GSH treated and CFX+GSH treated groups. (C) Serum cystatin C level in control, GFX treated, GSH treated and CFX+GSH treated groups. (D) Serum GGT level in control, GFX treated, GSH treated and CFX+GSH treated groups. (Number of rats = 10/group. $p < 0.05$ considered significant, * significant when compared to the control group, • significant when compared to the GSH group, # significant when compared to the CFX group).

GPx and GSR mRNA RQ% were significantly lower, while CYP4F1 was significantly higher ($P < 0.05$) in the CFX treated group (32.39 ± 2.91 and 44.32 ± 3.92 , 145.62 ± 12.75 % respectively), when compared to the corresponding values in the control or the GSH treated groups. There was significant up-regulation ($P < 0.05$) in the mRNA RQ% of GPx and GSR genes, while CYP4F1 was down-regulated (183.87 ± 17.94 , 196.31 ± 18.23 , 105.23 ± 13.47 % respectively) in the CFX+GSH treated group, when compared to the corresponding values in the CFX treated group. GPx and GSR mRNA levels were significantly higher, while there was no significant difference in CYP4F1 mRNA level when comparing the CFX+GSH treated group to the control or the GSH treated groups (Figure 2).

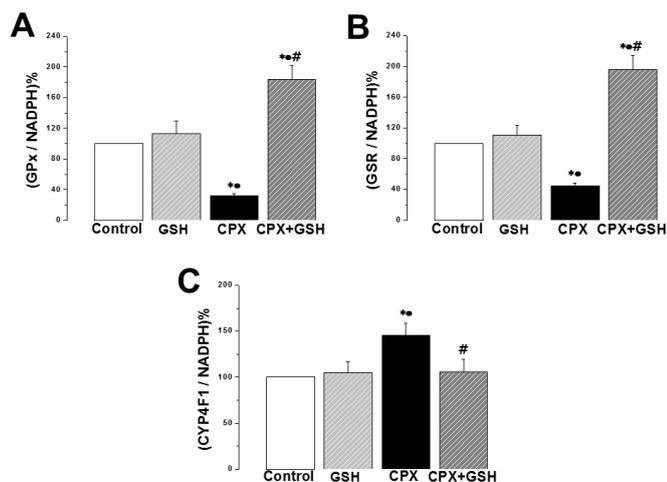


Figure 2: Effect of exogenous GSH on antioxidant enzymes gene expression. (A) GPx mRNA RQ in control, GFX treated, GSH treated and CFX+GSH treated groups. (B) GSR mRNA RQ in control, GFX treated, GSH treated and CFX+GSH treated groups. (C) CYP4F1 mRNA

RQ in control, GFX treated, GSH treated and CFX+GSH treated groups. (Number of rats = 10/group. $p < 0.05$ considered significant, * significant when compared to the control group, • significant when compared to the GSH group, # significant when compared to the CFX group).

DISCUSSION

AKI is clinical syndrome with unfavourable outcomes on a substantial number of hospitalized and non-hospitalized patients. AKI can be defined as a rapid (usually within hours to 7 days) decrease in the renal functions. AKI comprises structural damage as well as loss of function, with ill-defined single clear pathophysiology²³. Although most of the reported cases of CFX-induced ARF occur in older patients with pre-existing renal disease or with high doses of the medication, young and healthy patients using therapeutic doses of the CFX could still be at risk^{24,25}. Consequently, the impact of CFX on the kidney functions remain an important area of research. This study was an attempt to explore the potential therapeutic effect of exogenous GSH in CFX-induced AKI.

In the present study, CFX treatment resulted in rise in serum levels of creatinine and urea, which was in agreement with previously published reports²⁶. Elevated levels of serum creatinine is commonly associated with elevated levels of blood urea nitrogen (BUN), and could reflect a substantial reduction in the glomerular filtration rate (GFR)²⁷. The vulnerability of the renal tubular cells, in particular the proximal tubule cells, to CFX or other drugs toxic effects could be primarily due to their exposure to higher levels of the circulating toxins subsequent to their involvement in the glomerular filtrate concentration and reabsorption episodes. The possible underlying mechanisms include mitochondrial dysfunction, oxidative stress, altered tubular transport, or generation of ROS^{26,27}. One of the advantages of measuring cystatin C production in the body is the stability of its production process. Cystatin C production is not influenced by extra-renal conditions such as protein catabolism or diet. Additionally, unlike creatinine, it is not altered by age or muscle mass. Hence, the serum level of cystatin C could be an ideal endogenous biomarker reflecting GFR status, as well as an early and accurate detector of nephropathy²⁸. In the present study, CFX treatment resulted in an increased level of cystatin C, while in the CFX-GSH treated group serum cystatin C level was normal. It has been reported recently that cystatin C level is correlated with oxidative stress status in cardiopulmonary bypass related AKI²⁹. GGT is not only a prognosticator for the involvement of oxidative stress and inflammatory reactions in disease pathophysiology, but also a valid and economic detector of AKI^{30,31}. It was logic then to evaluate serum GGT levels in the studied groups. CFX treatment significantly increased GGT level, which could be countered by the concomitant treatment with GSH. Intriguingly, GGT, as part of the gamma glutamyl cycle, is reported to maintain GSH homeostasis. GGT restores GSH, and therefore during oxidative stress, GGT activity is associated with increased GSH³².

The fact that GSH was capable to counter the deleterious effects of CFX on renal functions in the present study encouraged us to explore the effect of CFX on GSH homeostasis and relate that to AKI pathophysiology. In fact, GSH was selected for the present study due to its ROS scavenging capacity, that enables it to protect the cell both directly and indirectly³³. The preservation of renal functions seen in the CFX+GSH treated rats, marked by normal serum creatinine and urea levels, could then be explained, at least in part, by GSH antioxidant properties.

We studied then the gene expression of GPx and GSR, GSH homeostasis related gene, to test the hypothesis that exogenous GSH can activate the endogenous GSH replenishing machinery. Stimulation of GSH synthesis significantly opposed oxidative stress-induced

apoptosis in human retinal pigment epithelial cells from the ARPE-19 cell line³⁴. CFX induced significant decrease in both GPx and GSR mRNA genes expression, while co-treatment with GSH significantly enhanced CPx and GSR mRNA levels. GSH is a thiol compound that acts as an intracellular antioxidant. Under normal physiological conditions, reduction of GSSG by GSR produces GSH at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH). Thereby, the peroxidase/glutathione reductase redox cycle is crucial for maintaining normal GSH concentration³⁵. One of the explanations of fluoroquinolones side effects their tendency to produce singlet oxygen and superoxide anion. ROS have detrimental effects on DNA, proteins and lipids, and mediate various cellular processes that lead to cell death^{36,37}. Despite the continuous efforts of endogenous GPx and GSR, marked by initial increase in mRNA level, to eliminate the over-production of free radicals, eventually the enzymes could be depleted by CFX with subsequent cellular damage³⁸.

Previously published data demonstrated that the administration of CFX in patients with urinary tract infection resulted in excessive ROS generation via CYP450-mediated metabolism with subsequent substantial attenuation of the antioxidants defence mechanism³⁹. It has been shown that the induction of P450s by phenobarbital or clofibrate in mice resulted in increased generation of ROS parameters in isolated liver microsomes⁴⁰. We tested the possibility that CYP4F1, which is highly expressed in rat renal cortex, could be involved in the depletion of endogenous GSH in response to CFX. CFX treated rats had higher levels of expression of CYP4F1 mRNA, while CFX+GSH treated rats resisted CFX-mediated CYP4F1 induction. Our results support the possibility that the attenuation of the peroxidase/glutathione reductase redox cycle could be linked to induction of CYP4F1.

CONCLUSION

The present study demonstrated that exogenous GSH can improve CFX-induced AKI and preserve kidney functions. Our data revealed that oxidative stress could be the underlying mechanism in nephrotoxicity. Hence, the present study supports the significance of using exogenous GSH, or possibly similar antioxidants, as an adjuvant with CFX.

Authorship Contribution: Yahya M. Naguib carried out the PCR experiments, participated in the study design and coordination, analysed the results, performed the statistical analysis, and drafted the manuscript. Eman I. Algizawy carried out the animal experiments and biochemical assays and participated in the study design. All authors have read and approved the final version of the manuscript.

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Competing Interest: None.

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