ORIGINAL ARTICLE

RESTORATION OF RENAL GLUTATHIONE LEVELS AND HISTOARCHITECTURE BY N-ACETYLCYSTEINE POST CYCLOPHOSPHAMIDE EXPOSURE IN RATS

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Objective: Optimal clinical efficacy of cyclophosphamide, an integral part of various antineoplastic and immunomodulatory regimens is limited by its numerous dose-related life threatening toxicities becoming as lethal as the cancer itself. This study was designed to evaluate the protective potential of N-acetylcysteine against cyclophosphamide induced nephrotoxicity in rats. **Methods:** This was a Laboratory-based randomized controlled trial carried out in Department of Pharmacology and Therapeutics in collaboration with Clinicopathologic Laboratory, Army Medical College, Rawalpindi, and National Institute of Health, Islamabad from March to July 2018. A total of 18 rats were divided into three equal groups. Group I served as the untreated control, Group II received a single intraperitoneal injection of cyclophosphamide (200 mg/Kg) and Group III was administered intraperitoneal injection of N-acetylcysteine (100 mg/Kg), once daily for 5 consecutive days followed by a single injection of cyclophosphamide one hour after the last dose. Twenty-four hours after cyclophosphamide administration, the animals were sacrificed and the kidneys were evaluated for glutathione levels and histopathological changes. The results were analyzed on SPSS-21, for quantitative analysis one-way ANOVA followed by post hoc Tukey test and for histopathologic analysis Chi-square test was applied. **Results:** In the renal tissue, cyclophosphamide depleted the glutathione levels from the normal mean of 1.17 ηg/ml to 0.34 ηg/ml (p<0.05), alongside notable histopathologic changes including multiple foci of haemorrhages and dense leukocytic infiltrates. N-acetylcysteine re-established the glutathione pools by raising the mean value upto 0.93 ηg/ml and preserved the normal histoarchitecture. **Conclusion:** N-acetylcysteine can attenuate cyclophosphamide induced nephrotoxicity by preserving the normal glutathione levels and histoarchitecture.

Keywords: Cyclophosphamide, Glutathione, Histoarchitecture, N-acetylcysteine, Nephrotoxicity

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INTRODUCTION

Cyclophosphamide (CP) is a globally renowned cytotoxic alkylating agent and an integral part of various antineoplastic and immunomodulatory regimens. However, its optimal clinical efficacy is limited by its numerous dose-related life threatening toxicities becoming as lethal as the cancer itself.[1] Amongst the various organ toxicities the rising incidence of acute kidney injury creates a major concern. This nephrotoxic potential of CP is attributed to its highly noxious major metabolic end product acrolein alongside its side chain byproduct chloroacetaldehyde.[2] Both acrolein and chloroacetaldehyde are uptaken by the renal cells via highly specific carriers situated on the basolateral membranes, i.e., organic anion (OAT) and cation (OCT) transporters.[3] Acrolein is a tremendously reactive aldehyde which diffuses in the cell and conjugates with the reduced glutathione (GSH) via covalent bonding consequently disrupting the structural layout of sulfhydryl groups and forming reactive oxygen species which then triggers lipid peroxidation and pro-inflammatory cytokines.[4] Meanwhile chloroacetaldehyde intensifies its action by causing further depletion of the antioxidants and altering the normal mitochondrial respiratory chain by plummeting the levels of ATP and coenzyme-A.[2] These changes cause direct cellular injury which can be recognized histologically as diminution of the brush border membrane in proximal and vacuolization in cortical tubules. Glomerular inflammation, interstitial edema and mild hemorrhagic changes may also appear in renal cortex.[5] Transmission electron microscopy reveals atypical mitochondrial dilatation with absent cristae. These changes can result in glomerular and tubular dysfunction, reduction of glomerular filtration rate and loss of regulation of ion concentrations. If left untreated these idiosyncrasies may result in osteomalacia, osteoporosis, hypokalemic nephropathy, and cardiac arrhythmias.[6,7]

N-acetylcysteine (NAC) is a widely acclaimed mucolytic and acetaminophen antidote which has lately been discovered to alleviate the signs of stress induced injury by ROS scavenging and suppression of resultant inflammatory cascade.[4,8] It can also replenish the endogenous GSH pools as it undergoes deacetylation by cytosolic enzymes to form cysteine which diffuses

through the cellular membranes and endorse the GSH synthesis by thiol exchange.[9] It also boosts the rate of translocation of Nrf-2 inside the nucleus where it binds to and intensifies the expression of antioxidant genes, which further aids in providing cytoprotection.[10]

This study was designed to evaluate the nephroprotective potential of NAC as an antioxidant against the CP induced acute kidney injury in rats by gauging and comparing the tissue GSH levels and histopathologic changes.

MATERIAL AND METHODS

This laboratory-based randomized controlled trial was conducted in the department of Pharmacology and Therapeutics in collaboration with the department of Pathology, Army Medical College, Rawalpindi and National Institute of Health, Islamabad from March to July 2018 after acquiring approval from the ethics committee of Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College.

The study span consisted of a total of 6 days. A homogenous population of 18 Sprague Dawley rats aged between 6−8 weeks and weighing around 200–250 grams were procured from and retained in the animal house of NIH under optimal environmental conditions with free access to a standard diet and clean drinking water. The rats were indiscriminately assigned into three groups, each having equal number of animals (n=6). Prior to the intervention, all the rats underwent allocation concealment via stratified randomization technique where each animal was weighed for accurate dosage adjustment and rats aged less than 6 weeks and pregnant females were excluded. All the drugs were injected intraperitoneally (i.p.) with following designated regimens:

Group-I (Control group): Group-I served as the untreated control. It was housed along with other groups under similar settings for a similar period of time.

Group-II (Cyclophosphamide alone): Group-II received CP as a single dose i.p. at the rate of 200 mg/Kg of body weight.[11] After 24 hours, the anesthetized rats were sacrificed and both kidneys were excised proximately and preserved for estimation of oxidative stress markers and microscopic examination. This group served as a high toxic group.

Group-III (N-acetylcysteine and Cyclophosphamide): Group-III received pretreatment of NAC (100 mg/Kg i.p.) once daily for five days.[4] One hour after the last dose the rats were injected with a single dose of CP (200 mg/Kg, i.p.) and sample collection was carried out as in group II.

For oxidative stress analysis, half portion of each kidney was rinsed thoroughly and stored at -20 °C. The homogenates were prepared in an isotonic solution of phosphate saline buffer using tissue homogenizer (IKA Dispergierstation T8) at 25,000 rpm (revolutions per minute). These homogenates were further centrifuged at 10,000 rpm for 20 mins at 4 °C to obtain the supernatants. These supernatants were analyzed for renal tissue GSH levels on the standardized commercially available kits from Bio-Essay Technology by applying the principles of Sandwich ELISA (Enzyme-Linked Immunosorbent Assay). The optical density of each well was determined using a microplate reader at 450 nanometer (nm). The results were compared with the given standard solutions and expressed as nanogram per milliliter (ηg/ml).

For histopathologic analysis, the other half of each tissue was processed, sliced measuring around 5 micrometer (μm) and stained with haematoxylin and eosin. Ultrastructure of renal tissue was scrutinized. For qualitative assessment, the specimens were studied for haemorrhages, tubular degeneration, interstitial fibrosis, leukocyte infiltration, apoptosis and necrosis. For quantitative analysis, the inflammatory cell count was taken into consideration and a five-point scoring system was implemented where 0=No change, 1=Minimal changes with a few inflammatory cells, 2=Mild changes having a ring of cells with one cell layer, 3=Moderate changes having a ring of cells with 2–4 cell layers, 4=Severe changes having a ring of cells with more than four cell layers.[12]

For statistical analysis the values were presented as Mean±SEM. The one way ANOVA-followed by post hoc Tuckey test was used to compare the quantitative results obtained within the groups. For qualitative analysis Chi-square test was applied. A value of $p<0.05$ was accepted as indicative of significant difference among groups.

RESULTS

The values of tissue GSH obtained from the excised and homogenized kidneys of the control group had a mean of 1.17±0.05 ηg/ml. These values were remarkably reduced in the toxic group within 24 hours of a single intraperitoneal dose of CP (200 mg/Kg). The obtained mean value of renal GSH in group II was 0.34±0.05 ηg/ml which had a high statistical significance against the control group ($p<0.001$) (Table-1).

In group III, administration of intraperitoneal NAC (200 mg/Kg) once daily for five consecutive days prior to the CP administration significantly refurbished the endogenous pool of tissue antioxidant by bringing the renal GSH as high as the mean value of 0.93±0.1 ηg/ml (Table-2).

The value was close to the normal showing no statistical significance, however, once compared with the toxic group the *p*-value turned out to be <0.05 marking the nephroprotective potential of NAC. This value was close to the normal control and showed no significance on comparison (Table-3).

Table-1: Comparison of renal tissue GSH levels between the control and the toxic group (Mean±SEM)

Variable	Group I (n=6)	Group II (n=6)
Renal Tissue GSH (ηg/ml)	1.17±0.08	0.34±0.05*

*p<0.001

Table-2: Comparison of effects of cyclophosphamide alone (CP) and the combination of cyclophosph-amide and N-acetylcysteine (NAC+CP) on the renal tissue GSH (Mean±SEM)

Variable	Group II (n=6)	Group III (n=6)
Renal Tissue GSH (ηg/ml)	0.34±0.05	0.93±0.10*

*p<0.001

Table-3: Comparison of effects of cyclophosphamide alone (CP) and the combination of cyclophosph-amide and N-acetylcysteine (NAC+CP) on the renal tissue GSH (Mean±SEM)

Variable	Group I (n=6)	Group III (n=6)
Renal Tissue GSH (ηg/ml)	1.17±0.08	0.93±0.10#

*p<0.05

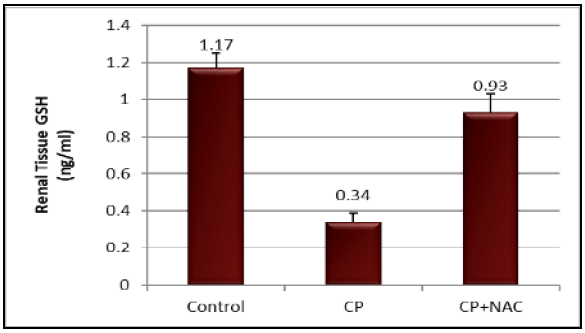
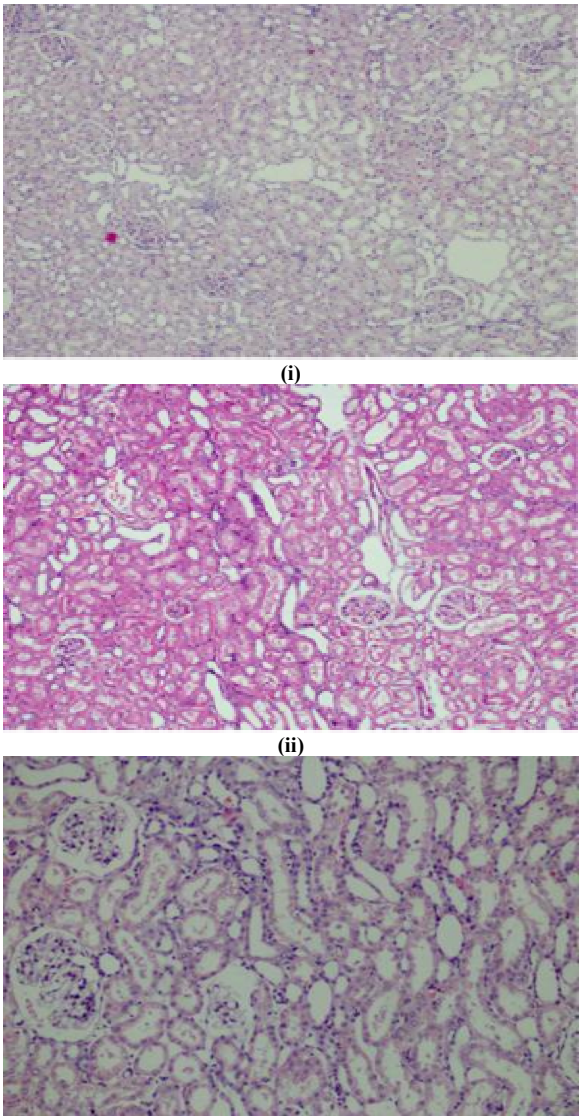


Figure-1: Effects of cyclophosphamide (CP) and combination of cyclophosphamide and N-acetyl-cysteine on the renal tissue GSH of rats. (Mean±SEM) (n=6)

Individual slides were graded under the light microscope with a magnification power of 10× in a blinded fashion. In untreated control group all the slides fell into grade zero as there were no inflammatory cells seen, meanwhile the histoarchitecture remained preserved (Figure-2, i). In the toxic group, a single dose of CP gave the impression of significant inflammation as 5 out of 6 slides had moderate changes with multiple foci of inflammatory cells arranged as 2−4 cellular layer deep rings (grade 3) alongside several prominent haemorrhagic foci predominantly in the glomeruli (Figure-2, ii). The remaining one slide of the same group had mild changes (grade 2). In group III, NAC markedly reduced the pathologic changes induced by CP. The inflammatory foci were comparatively sparse, with one layer deep rings falling into grade 2 of the applied scoring system with marked reduction in the number of hemorrhagic sites in the renal ultrastructure, in conjunction with that the glomeruli were absolutely unaltered (Figure-2, iii). On applying Chi-square test on all the pre-graded values of experimental sets the results were statistically significant (p<0.05).



(i)

(ii)

(iii)

Figure-2: Photomicrographs of renal tissue of experimental animals.

(i): normal histoarchitecture (Group-I); (ii): multiple rings with 2−4 layered density of leukocytes and numerous hemorrhagic foci (Group-II); (iii): sparse foci of leukocytes forming a single layered ring with normal renal morphology (Group-III)

DISCUSSION

In our study, within 24 hours of a single intraperitoneal injection of CP (200 mg/Kg), a marked reduction in the levels of reduced GSH in the renal tissue was observed indicating the advent of acute nephrotoxicity under the influence of stress induced injury. These results were quantified in comparison with the control group. The data obtained was in precise consonance with that of Kalantar and his associates who also reported a fall in

the level of renal GSH after following the similar dosage schedule of CP. They further validated their claims by associated derangements in the renal function tests including the levels of urea and creatinine.[13] Later on, Sadeghi *et al*[14] carried on with the similar string of experiments with the formation of an analogous nephrotoxic murine model and noted a remarkable drop in the antioxidant levels including GSH, superoxide dismutase and catalase 24 hours post CP administration. These findings may be attributed to the metabolic end products of CP particularly acrolein, which has the capability to disrupt the dynamic equilibrium between antioxidants and pro-oxidants, as the conjugation of acrolein with free or protein bound sulfhydryl groups in thiol compounds also depletes the GSH levels, consequently causing initiation and propagation of free oxygen radicals. Moreover, it also causes direct negative impact on the integrity of cellular membranes by triggering lipid peroxidation which further enhances the GSH consumption.[15] The previous literature also substantiates the dissemination of acrolein allied toxicodynamics to liver[12], lungs, brain, bladder and gonads[16].

The histopathologic finding of the toxic group were in correlation with that of the oxidative stress marker as the slides exhibited multiple dense rings of inflammatory cell alongside numerous hemorrhagic foci around glomeruli and capillaries with no signs of necrosis and fibrosis. These factors could be endorsed by post lipid peroxidation triggering of pro-inflammatory cytokines.[4] These outcomes were cited previously with a similar CP dosage schedule and amount by Goudarzi *et al*[12], their findings regarding necrosis, haemorrhage, tubular degeneration and leukocyte cell count in the renal tissue also helped us to formulate the histopathologic grading score. Alongside the aforementioned finding, Mansour *et al*[17] also mentioned vacuolization and desquamation of the tubular epithelium by extending the post treatment duration upto 48 hours, their results were attributed to the stimulation of cyclooxygenase cascade following the advent of oxidative stress injury due to prolong toxin exposure.

NAC is gaining the limelight as a potent thiol antioxidant which has the capability to synthesize and restock the GSH pools, alongside scavenging ROS.[18] Earlier studies have recorded its shielding effect against the cardio-[4] and gonadotoxic[19] effects of cyclophosphamide while emerging as a frontline antioxidant alongside alpha tocopherol, ascorbic acid and melatonin[20]. In current scenario, it has auxiliary proven its nephroprotective potential once it is administered prior to the CP challenge. Its consecutive administration for 5 days restored the GSH levels in the renal cells showing a statistically significant mean value as compared to the toxic group, while this value was close to that of the untreated control. Correspondingly, the normal ultrastructure of the tissue remained preserved with some scantily distributed inflammatory cells and complete attenuation of prominent hemorrhagic sites. A study conducted in Istanbul had parallel outcomes once a similar five days prophylactic regimen was followed with NAC with an even lower dose of 150 mg/Kg in rats, against organochlorine pesticide. This study detailed the complete remission of stress induced injury by recovery of normal GSH levels and renal interstitium.[21] Ifosfamide is an equally nephrotoxic isomer of CP, its co-supplementation with NAC for eight days were stated to have astonishing results as there was an absolute remission of the untoward effects of ifosfamide on biochemical, oxidative stress and histopathologic markers.[22] Though limited by the fact that this study further warrants extensive human trials to establish a detailed dosing and pharmacokinetic-dynamic profile, it is evidently concluded that NAC is a potent antioxidant which can overcome the stress by direct ROS depletion or indirectly via replenishing thiols and triggering cysteine, and therefore can be safely suggested as a potent nephroprotective agent to be introduced in CP containing regimens.

CONCLUSION

The protective effects of NAC on CP-induced nephrotoxicity were due to its antioxidant properties which helped to replenish endogenous glutathione levels and the ultrastructure of renal tissue. NAC can be employed to broaden the therapeutic window of cyclophosphamide.

CONFLICT OF INTEREST

There is no conflict of interest to be declared.

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