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Comparative protective assessments of some antioxidants against cyclophosphamide-induced kidney toxicity in albino rats

Elias Adikwu[1*] , Ebinyo C Nelson[2], Abraham Singesi Yambozibe[2]

[1]Department of Pharmacology, Faculty of Basic Medical Sciences, University of Port Harcourt, Choba, Rivers State
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Bayelsa State

A B S T R A C T

Introduction: Nephrotoxicity is one of the adverse effects of cyclophosphamide (CP).
Objectives: The aim of this study is to comparatively investigate the protective effects of melatonin (MT), alpha-lipoic acid (ALA) and N-acetylcysteine (NAC) on CP-induced nephrotoxicity in albino rats.
Materials and Methods: Sixty adult albino rats used for this study were divided into four groups (A-D). Rats in group A were treated with water intraperitoneally (ip) while rats in group B (B1-B4) were treated with NAC (10 mg/kg), ALA (10 mg/kg), MT (10 mg/kg) and MT+ALA ip respectively for 5 days. Rats in group C were treated with CP (150 mg/kg) ip on day 5. Rats in group D (D1-D4) were pretreated with NAC, MT, ALA and MT+ALA ip for 5 days before treatment with CP on day 5. Rats were sacrificed on the 6th day. Serum was extracted from blood and evaluated for renal function parameters. Kidneys were removed and used for light microscopic and biochemical studies.
Results: CP-treated rats showed significant ($P < 0.001$) increases in serum creatinine, urea, uric acid, potassium, sodium, chloride bicarbonate and kidney malondialdehyde (MDA) levels while kidney superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPX) levels were significantly ($P < 0.001$) decreased when compared to control. Nephrotic changes characterized by tubular necrosis and infiltrations by inflammatory cells were observed in CP-treated rats. However, effects observed in CP-treated rats were significantly abrogated in ALA ($P < 0.05$), MT ($P < 0.05$), NAC ($P < 0.01$) and MT+ALA ($P < 0.001$) pretreated rats when compared to CP-treated rats.
Conclusion: The finding in this study showed that the nephroprotective effects of NA, MT, ALA, and MT+ALA can be ranked as MT+ALA> NAC>MT>ALA.

Original

Implication for health policy/practice/research/medical education:
This experimental study showed that N-acetyl cysteine, melatonin and alpha lipoic acid had ameliorative impact on cyclophosphamide induced-nephrotoxicity and their nephroprotective effects can be ranked as MT+ALA >NAC>MT>ALA.
Please cite this paper as: Adikwu E, Nelson EC, Yambozibe AS. Comparative protective assessments of some antioxidants against cyclophosphamide-induced kidney toxicity in albino rats. J Nephropharmacol. 2019;8(2):e22. DOI: 10.15171/npj.2019.22

Introduction

Cyclophosphamide (CP) is an alkylating agent belonging to the class of oxazaphosphorines (1). It is a potent anticancer agent that is effective against a wide spectrum of malignancies, such as leukemia, lymphoma, breast, lung, prostate, and ovarian cancers (2). Its metabolic activation through microsomal cytochrome P450 mixed functional oxidase pathway yields 4-hydroxy CP that exist in equilibrium with aldophosphamide, which is degraded by β-elimination to form phosphoramide mustard and the toxic byproduct, acrolein (3). Phosphoramide mustard brings about inter-strand cross-links between opposite DNA strands and prevents their replication and transcription process which characterize the clinical anti-cancer activity of CP (4) whereas acrolein has been associated with CP toxicities especially nephrotoxicity. Numerous studies have shown that acrolein can disrupt redox balance in the kidney leading to kidney damage (5). Nephrotoxicity due to CP can result in glomerular dysfunction, tubular dysfunction, and decrease in glomerular filtration rate (6).

Melatonin (N-acetyl-5-methoxytryptamine) (MT),

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a circadian hormone mainly secreted by the pineal gland, is a derivative of tryptophan and predominantly secreted at night (7). It is also produced in the retina, thymus, bone marrow, respiratory epithelium, skin, lens, and intestine (8). It modulates a diverse number of physiological processes through receptor-mediated and receptor-independent mechanisms, thus, manifesting enormous functional versatility and diversity (9). MT and its metabolites have potent antioxidant and anti-inflammatory properties and have been proven to be highly effective in a variety of disorders linked to inflammation and oxidative stress (10). MT can increase gene expression and enzyme activities of glutathione peroxidase (GPX), glutathione (GSH) reductase, superoxide dismutase (SOD), and catalase (CAT) (11). It is an efficient protector of DNA, protein, and lipids in cellular membrane from endogenous and exogenous free radical generated during cellular processes (12,13).

Alpha liopic acid is a natural antioxidant that palys a fundamental role in metabolism. It has been shown to affect cellular processes, alter redox status of cells, and interact with thiols and other antioxidants (14). It has beneficial effects on energy production, and is also an essential cofactor of mitochondrial complexes. It acts as coenzyme of pyruvate and alpha-ketoglutarate dehydrogenase multi-enzyme complex of tricarboxylic acid cycle (15). Moreover, ALA acts as an antioxidant that is fat and water soluble in both oxidized and reduced forms which allows it to concentrate in cellular and extracellular environments (16). Furthermore, ALA can regenerate other endogenous antioxidants (17) and has a unique property of neutralizing free radicals without it being consumed in the process (18). It is capable of preventing or treating many diseases associated with oxidative stress, such as diabetes, chronic liver diseases, and neurodegenerative processes (19).

N-acetylcysteine (NAC) is a water soluble antioxidant that is a source of sulfhydryl groups in cells and due to its interaction with reactive oxygen species; it is a scavenger of free radicals (20). NAC is one of the large groups of exogenous antioxidants that protects against oxidative tissue injury. This effect may be directly related to its free radical scavenging property or to the secondary induction of GSH production (21). It protects mitochondrial from damage, inhibits lipid peroxidation (LPO) and cellular damage (22). It enhances many cellular defense mechanisms and enriches cellular GSH level by acting as a precursor in the GSH synthesis pathway (22). Furthermore, NAC is capable of restoring impaired pro-oxidant/antioxidant balance and has been widely used as an effective antioxidant against oxidative stress both in vivo and in vitro (23).

Objectives
The present study aimed to assess the comparative nephroprotective effects of NAC, MT, ALA against CP-induced kidney damage in a rat model.

Materials and Methods
Experimental animals
Adult albino rats with an average weight of 200±5g used for this research were housed in cages (6 per cage) and allowed to acclimatize for 2 weeks in a well ventilated room. The rats were fed with standard rodents chow and given tap water *ad labium*. All animals used for this study were handled in accordance with the regulations promulgated by the Canadian Council of Animal Care (2009) (24).

Drugs and chemicals
The dose of CP (150 mg/kg), MT (10 mg/kg), NAC (10 mg/kg) and ALA (10 mg/kg) were used for this study (25-28). All other chemicals used for this study are of analytical grade.

Grouping of animals
Sixty adult rats were grouped into four groups A to D. Group A contained six rats while group B was divided into subgroups B1to B4 of six rats each. Group C contains six rats while group D was divided into subgroups D1 to D4 of six rats each.

Experimental protocol
Group A was treated with water intraperitoneally (ip) while rats in group B (B1-B4) were treated with NAC (10 mg/kg/d), ALA (10 mg/kg/d), MT (10 mg/kg/d) and MT+ALA ip respectively for 5 days. Rats in group C were treated with CP (50 mg/kg/d) ip on day 5. Rats in group D (D1-D4) were pretreated with NAC, MT, ALA and MT+ALA ip for 5 days before treatment with CP ip on day 5.

Sacrifice and sample collection
At the end of drug administration, the rats were sacrificed using diethyl ether as anesthesia. Blood samples were collected from the heart and centrifuged at 1500 rpm for 20 minutes and serum extracted and evaluated for markers of renal function. The kidneys were collected and rinsed in an ice cold 1.15% KCl solution. The kidneys were homogenized with 0.1M phosphate buffer (pH 7.2) and centrifuged at 1200 rpm for 20 minutes. The supernatant was decanted and evaluated for markers of oxidative stress

Evaluation of renal function parameters and oxidative stress indices
Serum creatinine, urea, uric acid, bicarbonate, albumin and total protein content were assayed using standard laboratory technique and reagents. Potassium and sodium were determined using flame photometric methods, while chloride levels were determined using titrimetric methods. SOD was evaluated according to Sun and Zigma (29), CAT was evaluated as described by Sinha et al, 1972

(30). Reduced GSH was measured as reported by Sedlak and Lindsay (31), while the method of Rotruck et al (32) was used for the evaluation of GPX. Malondialdehyde (MDA) was evaluated according to Buege et al (33).

Ethical issues

This project was approved by Ethics Committee of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, Niger Delta University, Bayelsa State, Nigeria. Prior to the experiment, the protocols were confirmed to be in accordance with the guidelines of Animal Ethics Committee of Niger Delta University.

Statistical analysis

Statistical analysis was performed using SPSS 18 software (SPSS Inc, Chicago, IL). Results are expressed as mean ± standard error of mean (SEM). Mean of groups were compared using ANOVA and Tukey's *post hoc* test. Significance was set at $P < 0.05$; 0.01.

Results

The administration of NAC, MT and ALA did not produce significant ($P > 0.05$) effects on body, kidney weighs, serum urea, uric acid, creatinine, total protein, and albumin levels in comparison to control (Tables 1 and 2). In contrast, serum levels of urea, uric acid, creatinine were increased significantly ($P < 0.001$) whereas total protein, and albumin were significantly ($P < 0.001$) decreased in CP-treated rats when compared to control. However rats pretreated with individual doses of MT and ALA showed significant ($P < 0.05$) decreases in serum levels of urea, uric acid, creatinine whereas total protein, and albumin were significantly ($P < 0.05$) increased when compared to CP-treated rats. Interestingly, effects observed on the above parameters most evident in rats pretreated with NAC ($P < 0.01$) and MT +ALA ($P < 0.001$) when compared to CP- treated rats (Table 2). Furthermore, the administration of NAC, MT and ALA had no significant ($P > 0.05$) effects on serum electrolytes (Na^+, Cl^-, K^+, HCO_3^-) when compared to control. However, there were significant ($P < 0.001$) alterations in serum electrolytes in CP-treated rats when compared to control. Notwithstanding, serum electrolytes were significantly ($P < 0.05$) restored in individual doses of MT and ALA pretreated rats in comparison to CP-treated rats. Most significant restorations in serum electrolytes were observed in rats pretreated with NAC ($P < 0.01$) and MT + ALA ($P < 0.001$) in comparison to CP-treated rats (Table 3). Furthermore, the administration of NAC, MT and ALA had no significant ($P > 0.05$) effects on kidney MDA, CAT, SOD, GSH, and GPX levels when compared to control. On the other hand, there was significant ($P < 0.001$) increase in MDA level whereas CAT, SOD, GSH, and GPX levels were significantly ($P < 0.001$) decreased in CP- treated rats when compared to control. In contrast, pretreatment with individual doses of MT and ALA significantly ($P < 0.05$) decreased MDA levels with significant ($P < 0.05$) increases in CAT, SOD, GSH and GPX levels when compared to CP-treated rats. It is fascinating that effects on the above parameters were most pronounced in rats pretreated with NAC ($P < 0.01$) and MT+ALA ($P < 0.001$) when compared to CP-treated rats (Table 4). Furthermore, the kidney of the control rats showed normal histology (Figure 1A) whereas the kidney of rat treated with CP showed tubular necrosis and infiltrations by inflammatory cells (Figure 1B). On the other hand, the kidneys of rats pretreated with NAC, MT, ALA and MT+ALA respectively before treatment with CP showed normal histology (Figures 1C to 1F).

Discussion

Oxidative stress can be defined as an imbalance between oxidants and antioxidants characterized by disturbance in the pro-oxidant–antioxidant balance in favor of the oxidant, leading to biomolecular damage (34). Studies have demonstrated that oxidative stress could be a key mechanism in drug-induced renal toxicity (35). This study aimed at investigating the comparative protective effects

Table 1. Effects of NAC, MT, and ALA on body and kidney weights of CP-treated albino rats

Treatment	Initial body weight (g)	Final body weight (g)	Kidney weight (g)	Relative kidney weight (%)
Control	220.6±13.0	225.9±10.7	0.76±0.06	0.34±0.02
NAC	200.8±11.7	212.5±10.0	0.80±0.08	0.37±0.06
MT	210.6±12.1	221.1±14.6	0.76±0.02	0.34±0.01
ALA	218.8±10.9	225.6±11.7	0.81±0.08	0.36±0.03
MT+ALA	220.1±14.6	229.0±12.2	0.78±0.07	0.34±0.04
CP	215.4±13.6	220.4±12.6	0.88±0.02	0.40±0.08
CP + NAC	220.6±14.3	226.8±10.1	0.79±0.09	0.35±0.03
CP + MT	209.8±10.7	217.7±13.3	0.87±0.07	0.40±0.01
CP + ALA	221.9±11.9	230.2±10.7	0.88±0.05	0.38±0.02
CP + MT+ALA	210.2±13.2	221.2±13.5	0.83±0.09	0.38±0.05

CP; cyclophosphamide, NAC; N-acetylcysteine, MT; melatonin, ALA; alpha lipoic acid.
Data are expressed as mean ± SEM (n=6).

Table 2. Effects of NAC, MT, and ALA on serum renal function parameters of CP- treated albino rats

Treatment	Urea (mg/dL)	Creatinine (mg/dL)	Uric acid (mg/dL)	Total protein (g/dL)	Albumin (g/dL)
Control	22.3±2.20	2.36±0.13	2.72±0.32	9.40±0.71	5.55±0.20
NAC	20.0±2.11	2.21±0.14	2.50±0.21	9.61±0.53	5.57±0.11
MT	21.6±2.00	2.30±0.11	2.63±0.73	9.52±0.44	5.65±0.30
ALA	22.0±2.62	2.35±0.10	2.69±0.44	9.48±0.21	5.55±0.72
MT+ALA	20.5±2.43	2.25±0.24	2.55±0.41	9.78±0.55	5. 78±0.43
CP	99.12±5.13[a]	7.94±0.35 [a]	7.53±0.10[a]	2.03±0.41[a]	1.87±0.09[a]
CP+NAC	30.3± 3.22[b]	3.75±0.08[b]	3.10±0.09[b]	6.18±0.71[b]	4.14± 0.92[b]
CP + MT	62.8±2.04[c]	4.75±0.32[c]	4.45±0.32[c]	4.47±0.52[c]	3.55±0.73[c]
CP + ALA	65.7±3.19[c]	4.73±0.60[c]	4.99±0.41[c]	3.99±0.42[c]	3.41±0.42[c]
CP+ MT+ALA	35.2±2.45[b]	2.10±0.22[d]	2.31±0.22[d]	8.00±0.11[d]	5.07±0.40[d]

CP; cyclophosphamide, NAC; N-acetylcysteine, MT; melatonin, ALA; alpha lipoic acid.
Data are expressed as mean ± SEM (n=6).
[a] Significant ($P < 0.001$) difference when compared to control; [b] Significant ($P < 0.01$) difference when compared to CP-treated rats; [c] Significant ($P < 0.05$) difference when compared to CP-treated rats; [d] Significant ($P < 0.001$) difference when compared to CP-treated rats.

Table 3. Effects of NAC, MT, and ALA on serum electrolytes of CP-treated albino rats

Treatment	K+ (mmo/L)	Cl-(mmo/L)	Na+ (mmo/L)	HCO3- (mmo/L)
Control	3.54±0.61	120.4±6.11	170.5±7.42	20.6±2.21
NAC	3.50±0.72	121.0±7.29	175.2±6.51	21.4±2.32
MT	3.52±0.67	123.4±6.43	174.1±8.43	19.8±1.30
ALA	3.51±0.71	125.2±7.54	172.7±9.23	20.7±1.25
MT+ALA	3.63±0.44	122.9±9.53	170.6±7.43	18.2±1.47
CP	9.67±0.30[a]	679.8±8.77[a]	698.9±8.32[a]	40.9±3.30[a]
CP + NAC	4.70±0.25[b]	300.0±6.14[b]	340.8±8.42[b]	20.9±2.13[b]
CP+ MT	6.12±0.77[c]	435.3±9.40[c]	421.4±7.00[c]	28.0±2.11[c]
CP+ALA	6.33±0.68[c]	456.4±7.33[c]	435.5±8.17[c]	30.2±2.00[c]
CP+MT+ALA	3.82±0.56[d]	235.6±8.42[d]	330.8±9.22[b]	21.8±2.22[b]

CP; cyclophosphamide, NAC; N-acetylcysteine, MT; melatonin, ALA; alpha lipoic acid.
Data are expressed as mean ± SEM (n=6).
[a] Significant ($P < 0.001$) difference when compared to control; [b] Significant ($P < 0.01$) difference when compared to CP-treated rats; [c] Significant ($P < 0.05$) difference when compared to CP-treated rats; [d] Significant ($P < 0.001$) difference when compared to CP-treated rats.

Table 4. Effects of NAC, MT, and alpha lipoic acid on kidney oxidative stress markers of cyclophosphamide-treated albino rats

Treatment	MDA (nmol/mg protein)	GSH (µg/mg protein)	CAT (U/mg protein)	SOD (U/mg protein)	GPX (U/mg protein)
Control	0.27±0.09	10.1±0.70	30.7±2.00	24.0±2.00	26.3±2.10
NAC	0.25±0.06	10.5±0.84	34.6±3.14	27.4±2.40	29.0±1.24
MT	0.26±0.02	10.3 ±1.0	32.2±3.28	25.7±2.51	27.3±1.00
ALA	0.25±0.02	10.2±0.52	32.3±3.11	25.6±2.56	26.4±2.13
MT+ALA	0.24±0.07	10.4±0.67	34.0±3.15	26.3±2.17	29.1±2.23
CP	2.68±0.13[a]	2.46±0.18[a]	9.34±0.82[a]	7.29±0.33[a]	8.05±1.00[a]
CP+ NAC	0.31±0.07[b]	6.98±0.64[b]	20.8±2.11[b]	18.6±2.86[b]	18.0 ± 1.25[b]
CP+MT	1.54±0.01[c]	4.99±0.37[c]	16.7±1.43[c]	13.4±1.60[c]	14.2±1.32[c]
CP+ALA	1.60±0.06[c]	4.75±0.55[c]	15.5±1.22[c]	12.2±0.17[c]	14.9±2.17[c]
CP+MT+ALA	0.33±0.01[b]	9.57±0.52[d]	25.1±3.34[d]	23.2±2.00[d]	22.2±3.37[d]

CP; cyclophosphamide, NAC; N-acetylcysteine, MT; melatonin, ALA; alpha lipoic acid.
Data are expressed as mean ± SEM (n=6).
[a] Significant ($P < 0.001$) difference when compared to control; [b] Significant ($P < 0.01$) difference when compared to CP-treated rats; [c] Significant ($P < 0.05$) difference when compared to CP-treated rats; d Significant ($P < 0.001$) difference when compared to CP-treated rats..
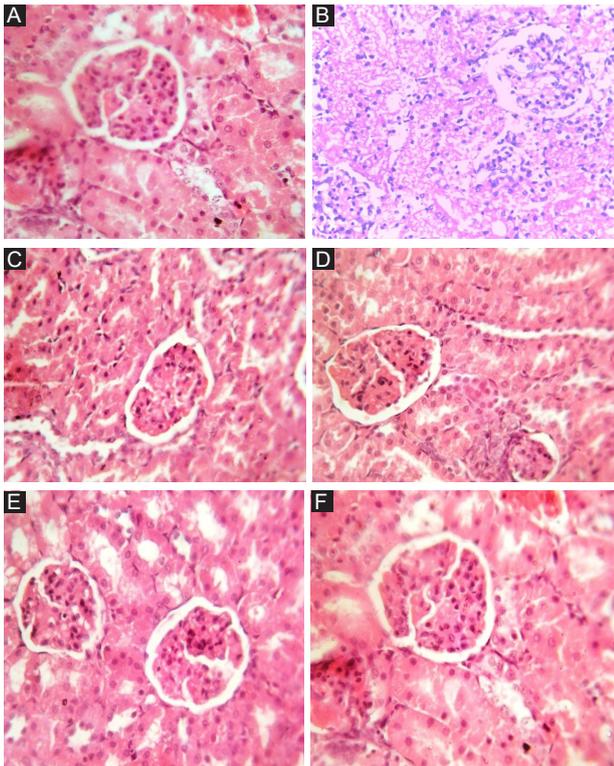
Figure 1. (A) Kidney of control rat showing normal histology. **(B)** Kidney of rat treated with cyclophosphamide showing tubular necrosis and infiltrations by inflammatory cells. **(C to F)** Kidney of rats treated with NAC, melatonin, and alpha lipoic acid respectively before the administration of cyclophosphamide showing normal histology. **(F)** Kidney of rats treated with a combination of melatonin, and alpha lipoic acid before the administration of cyclophosphamide showing normal histology.

of ALA, NAC and MT on CP-induced nephrotoxicity in albino rats. Perturbations in body and organ weights are signs that could characterize CP-induced renal toxicity (36). However, the nephrotoxic effect of CP-observed in this study was not marked by alterations in body and kidney weights. Renal function assessment involves the measurement of serum creatinine, urea and uric acid concentrations (37). In the present study, the serum levels of creatinine, urea, and uric acid were not altered in ALA, MT and NAC treated rats. The observations are in agreement with previous reports (38). On the other hand, the serum levels of creatinine, urea and uric acid were elevated in CP administered rats. The observations are consistent with previous findings (39). The elevated levels of these parameters are evidence of renal dysfunction in CP-treated rats. This could be attributed to decreased excretion of creatinine, urea and uric acid due to decreased glomerular filtration rate in CP-treated rats (40). However, the serum levels of creatinine, urea and uric acid were restored in MT, ALA and NAC pretreated rats. Serum total protein and albumin are biochemical indexes for the assessment of kidney function especially in malnourished situations (41,42). CP-treated rats showed

decreased serum levels of albumin and total protein. This is an evidence of compromise kidney function which has been earlier reported (43). Interestingly, the serum levels of albumin and total protein were restored with MT, NAC and ALA pretreatments. CP associated renal toxicity is often characterized by perturbations in kidney antioxidant defense and LPO (44). In this study, CP-treated rats showed perturbations in kidney antioxidants characterized by decreases in the levels of SOD, GPX, CAT and GSH with increases in MDA levels. This finding is an evidence of oxidative kidney damage (45). However, the kidney levels of SOD, GPX, CAT, GSH and MDA levels were restored in ALA, MT and NAC pretreated rats. Furthermore, serum electrolytes play an important role in many body processes, such as controlling fluid levels, acid-base balance, nerve conduction, blood clotting and muscle contraction (46). Renal toxicity due to CP is often associated with perturbations in serum electrolytes (47). In this study, serum electrolytes were elevated in CP-treated albino rats. However, serum electrolytes were restored in MT, ALA and NAC pretreated rats. Kidney histological examination contributes to diagnostic accuracy in drug-induced kidney damage and remains a valuable tool in assessing drug-induced kidney damage (48). CP renal pathology is often characterized by necrotic changes in kidney structure (49). This study observed kidney pathologic changes characterized by tubular necrosis, and infiltrations by inflammatory cells in CP-treated rats. However, these pathologic changes were ameliorated in rats pretreated with MT, ALA and NAC.

The precise mechanism by which CP causes renal injury is poorly known. However, studies have attributed it to its cytotoxic metabolite; acrolein (50). Acrolein is a highly reactive, unsaturated aldehyde that induces LPO and changes intracellular redox balance (51). Acrolein, when filtered in the kidney into urine, induces a cascade of pro-inflammatory processes, and stimulates oxidative and nitrosative stress which can lead to kidney damage (52,53). MT, ALA and NAC might have inhibited CP-induced oxidative kidney damage through scavenging, neutralizing and mopping-up of free radicals generated by CP and its metabolites. NAC is a small water-soluble molecule containing a thiol group, which has antioxidant property. It replenishes GSH store, increases SOD activity, scavenges hydroxyl free radical and inhibits LPO (54). MT is an effective antioxidant that scavenges free radicals. It scavenges highly toxic hydroxyl radical, and peroxynitrite anion. Also, it scavenges superoxide anion radical, quenches singlet oxygen and inhibits oxidative stress induced biomolecular damage. MT can increase mRNA and protein levels of antioxidant enzymes through nuclear-related factor 2 activation (55). It is capable of penetrating into mitochondria and exerting protection against various pathological conditions induced by oxidative stress (56,57). ALA is a naturally occurring

dithiol compound that functions as an essential cofactor for mitochondrial bio-energetic enzymes (58). ALA and its reduced form, (dihydrolipoic acid), have antioxidant activity in fat- and water-soluble media. ALA can react with superoxide, hydroxyl radicals, hypochlorous acid, peroxyl radicals, and singlet oxygen thereby inhibiting their detrimental actions. Experimentally, it has been used for the treatment of various pathological conditions associated with oxidative stress (59).

Conclusion
This study demonstrated that the nephroprotective effects of NAC, MT, ALA and MT+ALA against CP-intoxicated rats can be ranked as MT+ALA>NAC>MT> ALA.

Authors' contribution
EA; Concept and design of the study, literature search, collection of data, statistical analysis, and manuscript preparation. ECN; Concept, collection of data, review of the literature, and preparation of first manuscript draft. ASY; Concept, collection of data, review of the literature, preparation of first manuscript draft and critical revision of the manuscript

Conflicts of interest
Authors declare no conflicts of interest.

Ethical considerations
Ethical issues (including plagiarism, data fabrication, double publication) have been completely observed by the authors.

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