

Counteracting adriamycin-induced oxidative stress by administration of N-acetyl cysteine and vitamin E

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Abstract

Adriamycin (ADR), a cytotoxic antineoplastic drug, is used in the treatment of various solid tumors. However, its efficacy continues to be challenged by significant toxicities including nephrotoxicity. In the present study, the effects of N-acetyl cysteine (NAC) and vitamin E, known antioxidants, were investigated on ADR-induced peroxidative damage in rat kidney. Adult male albino rats of Wistar strain were administered ADR as a single dose (10 mg/kg body weight, i.v.). Histopathological studies indicated that ADR-treated kidney sections show focal tubular necrosis and casts. ADR-injected rats showed a significant decline in the activities/levels of enzymic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase and glutathione-S-transferase) and non-enzymic antioxidants (thiols, vitamin C and vitamin E) with high malondialdehyde levels. The extent of nephrotoxicity was evident from the increased activities of urinary marker enzymes (alkaline phosphatase, lactate dehydrogenase and γ -glutamyltransferase). Treatment with NAC and vitamin E (50 mg/kg b.w., i.p.) 1 day prior to ADR administration maintained near normal activities of the enzymes, significantly reduced lipid peroxidation and prevented the necrosis caused by ADR, thereby proving to be an effective thiol replenishing agent and antioxidant.

Keywords: adriamycin; antioxidant; N-acetyl cysteine; nephrotoxicity; vitamin E.

Introduction

Adriamycin (ADR), an aminoglycosidic antibiotic, is a commonly used anticancer agent with proven efficacy

in acute leukemias, lymphomas and a number of solid tumors (1). The effect of ADR toxicity is both species- and organ-dependent (2). Nephrotoxicity is the major side effect of aminoglycoside antibiotics accounting for 10–15% of all cases of renal failure (3). ADR-induced nephrotoxicity involves various conditions such as nephropathy, renal insufficiency, nephrotic syndrome, glomerulosclerosis, necrosis, hemorrhage, tubular degeneration, swelling and vacuolation of epithelial cell cytoplasm with accompanying proteinuria (4). ADR nephropathy represents a model very close to human progressive chronic renal disease (5). The molecular mechanism by which ADR causes renal damage is unknown. However, numerous studies support the hypothesis that the ADR-induced nephrotoxicity may be the consequence of oxidative stress, i.e., oxidation and cross linking of cellular thiols and membrane lipid peroxidation (6). Several *in vitro* and *in vivo* studies have demonstrated that reactive oxygen species including superoxide radical, hydroxyl radical and hydrogen peroxide (H₂O₂) are important mediators of tissue injury (7).

Biological antioxidants contribute to the protection of cells and tissues against the deleterious effects of reactive oxygen species and other free radicals. Glutathione is involved in numerous vital processes where the reducing potential of the thiol is used. Several disorders are believed to be characterized by an increase in oxidative burden, potentially due to depleting glutathione. Hence, administration of precursors of glutathione may be effective in reducing the oxidative stress and thereby its consequences (8). N-acetyl cysteine (NAC), a precursor of glutathione, has a broad array of biological properties underlying its protective role in a variety of pathophysiological conditions. NAC is found to be effective against a variety of metabolic disorders involving oxidative stress, such as paracetamol toxicity, *in vitro* and *in vivo* (9), calcium oxalate urolithiasis (10), doxorubicin cardiac toxicity in mice (11) and radiographic-contrast agent-induced reductions in renal function (12).

Vitamin E, a powerful antioxidant, quenches free radicals and inhibits lipid peroxidation. Vitamin E reacts with lipid peroxy and alkoxy radicals, donates its liable hydrogen to them and scavenges the chain-propagating radicals. This process is important in maintaining the integrity of cell membranes (13). Vitamin E was shown to reduce the progression of injury and the development of glomerular diseases in rats (14). Supplementation of vitamin E has been shown to reduce the number and incidence of chemically-induced tumors in animals (15). The present study aimed to find out the efficacy of a combination therapy of NAC and vitamin E on ADR-induced nephrotoxicity in experimental rats.

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Materials and methods

Drugs and chemicals

ADR (doxorubicin hydrochloride-Adrim) was procured from Dabur Pharmaceuticals (New Delhi, India). NAC and vitamin E were obtained from Sisco Research Laboratories Pvt Ltd. (Mumbai, India). All other chemicals and solvents were of analytical grade.

Animal model

Adult male albino rats of Wistar strain weighing 150–200 g (12–14 weeks old) were obtained from Madras Veterinary College (Chennai, India). The animals were maintained under standard conditions of humidity, temperature ($25 \pm 2^\circ\text{C}$) and light (12 h light/12 h dark). They were fed with a standard rat pelleted diet (M/s Pranav Agro Industries Ltd., India, under the trade name Amrut rat/mice feed) and had free access to water. The animal experiments were conducted according to the guidelines of the Institutional Animal Ethics Committee (IAEC).

Experimental design

The animals were divided into four groups of six rats each, as follows. Group I (control) received normal saline throughout the course of the experiment. Group II received a single dose intravenous injection of ADR through the tail vein (10 mg/kg body weight). Group III received intraperitoneal injections of NAC (50 mg/kg body weight) in saline and vitamin E (50 mg/kg body weight) 1 h prior to the administration of ADR as Group II. Group IV received intraperitoneal injection of NAC and vitamin E in the above mentioned concentration and served as drug controls.

At the end of the experimental period (after 48 h) the animals were housed in metabolic cages for 24-h urine collection, which was collected in ice-jacketed beakers at 0°C . The urine samples were centrifuged for 10 min at low speed to remove any sediment; the supernatant was dialyzed and used for enzyme assays. Animals were sacrificed by cervical decapitation. Kidneys were excised immediately and placed in ice-cold saline, trimmed free of connective tissues, blotted with filter paper and weighed.

Histopathological studies

Immediately after sacrifice a portion of the kidney tissue was fixed in 10% formalin. The washed tissue was dehydrated in descending grades of isopropanol and finally cleared in xylene. The tissue was then embedded in molten paraffin wax. Sections were cut at $5\text{-}\mu\text{m}$ thickness, stained with hematoxylin and eosin. The sections were then viewed under a light microscope for histopathological changes.

Biochemical analysis

Antioxidant enzymes were assayed in a 10% kidney homogenate of the control and experimental groups. Superoxide dismutase (SOD) was assayed as described (16), with one unit of enzyme activity defined as the amount required for 50% inhibition of pyrogallol auto-oxidation. Catalase (CAT) was assayed by the reduction of the dichromate in acetic acid to chromic acetate when heated in the presence of H_2O_2 ; the chromic acetate thus produced was measured colorimetrically at 610 nm (17). Glutathione peroxidase (GPX) was assayed by the method of Rotruck et al. (18), which is based on the reaction between glutathione remaining after the

action of GPX and 5,5'-dithiobis-(2-nitrobenzoic acid) to give a compound that absorbs light at 412 nm. Glutathione-S-transferase (GST) was assayed as previously described (19). Glutathione reductase (GR), which utilizes NADPH to convert oxidized glutathione (GSSG) to the reduced form (GSH), was assayed as previously described (20). Glucose-6-phosphate dehydrogenase (G6PD) was measured according to the method of Beutler (21) in which the increase in absorbance is measured when the reaction was started with the addition of glucose-6-phosphate. Non-enzymic antioxidants such as total glutathione (GSH) (22), total sulfhydryl groups (TSH) and non-protein sulfhydryl groups (NPSH) (23), and vitamin E (24) were estimated using standard protocols. Vitamin C (25) was oxidized by copper to form dehydroascorbic acid and diketoglutaric acid, and was treated with 2,4-dinitrophenyl hydrazine to form the derivative of bis-2,4-dinitrophenyl hydrazine. This compound in sulfuric acid undergoes a rearrangement to form a product that was measured at 520 nm. A mildly reducing medium with thiourea was used to prevent non-ascorbic chromogen interference. Lipid peroxidation (LPO) was assayed by the method of Devasagayam (26) in which the malondialdehyde (MDA) released serves as the index of LPO. Protein content was estimated by the method of Lowry et al. (27).

Urinary cytotoxic marker enzymes such as alkaline phosphatase (ALP), γ -glutamyltransferase (GGT), and lactate dehydrogenase (LDH) were assayed in the dialyzed 24-h urine sample. ALP was assayed as previously described (28) using disodium phenyl phosphate and the liberated phenol was measured colorimetrically using Folin-Ciocalteu's reagent. GGT was assayed as previously described (29). LDH (30) was assayed using lithium lactate as substrate and the color was developed using dinitrophenyl hydrazine. Creatinine was assayed by the method of Owens et al. (31).

Statistical analysis

Values are expressed as mean \pm SD for six animals. The results were computed statistically using the SPSS software package for Windows (SPSS Inc., Chicago, IL, USA). One-way analysis of variance, post-hoc testing was performed for inter-group comparisons by the least significant difference (LSD) test. A p-value < 0.001 was considered significant.

Results

In the present experimental study, a single high dose injection of ADR (10 mg/kg body weight) induced severe biochemical changes as well as oxidative damage in kidneys. There was no instance of death in any of the experimental groups during the study period.

Histological examination on control and experimental rats confirmed marked foci of proximal tubular necrosis, hyaline casts and tubular dilatations along with mild focal interstitial inflammatory cell infiltration in ADR-administered rat kidney (Figure 1B), whereas in control (Figure 1A) and Group IV (Figure 1D) a normal architecture was observed. Kidneys of rats pretreated with NAC and vitamin E along with ADR administration (Group III) showed very little damage to the tubular epithelium (Figure 1C).

Table 1 shows the activities of antioxidant enzymes in the control and experimental rats. Administration of ADR to the experimental rats (Group II) showed a

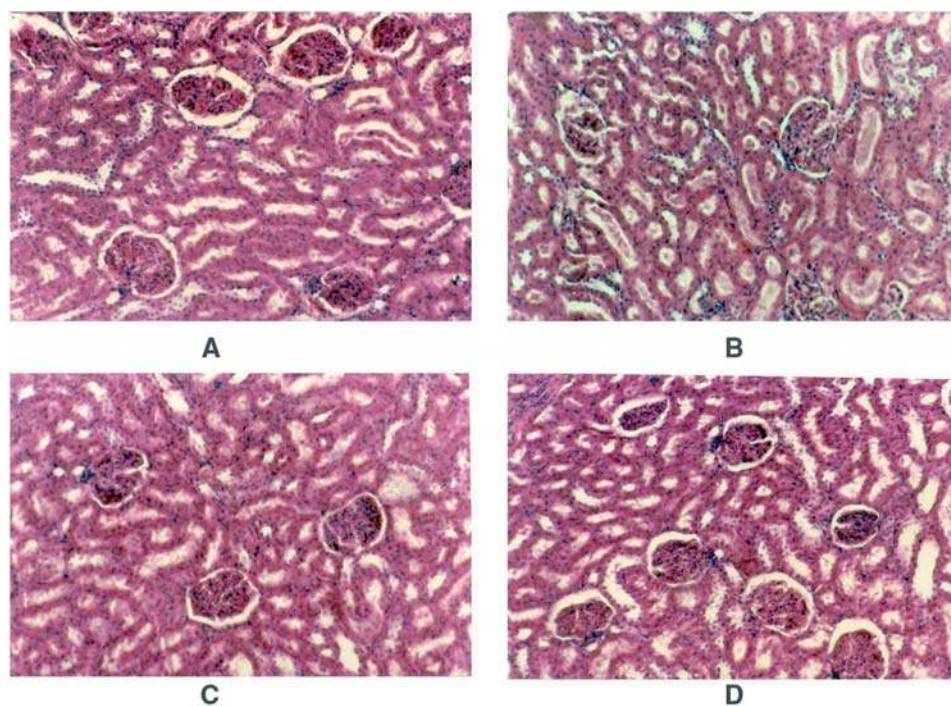


Figure 1 Histopathological observations in the rat kidney. (A) Control rat kidney section showing normal glomeruli and tubules; (B) ADR-administered rat kidney sections showing numerous foci of proximal tubular necrosis, hyaline casts and mild interstitial inflammatory cell infiltration; (C) ADR-administered and Vitamin E + NAC co-supplemented rat kidney section showing near normal tubules with minimal epithelial damage; (D) Vitamin E- and NAC-treated rat kidney section showing normal architecture as control rat kidney sections. Images were taken under light microscopy using hematoxylin and eosin as staining agents (100 \times).

significant decrease ($p < 0.001$) in the activities of enzymic antioxidants like SOD, CAT, GPX, GR, G6PD and GST when compared to the control rats (Group I), whereas their levels were maintained near normal in the Group III rats.

Table 2 delineates the activities of non-enzymic antioxidants like GSH and thiols (protein and non-protein) in control and experimental rats. In Group II rat kidneys, thiol levels were markedly decreased ($p < 0.001$) when compared to the control rats, while they were maintained near normal in Group III rats.

There was a significant decrease ($p < 0.001$) in the concentrations of vitamins E and C in ADR-administered rats when compared to the control and NAC + vitamin E pretreated rats (Figure 2). In Figure 3, which represents the LPO level in control and experimental

rats, a 2.02-fold increase in the LPO was observed in ADR-administered rats when compared to the control rats. Table 3 shows a significant increase ($p < 0.001$) in the activities of urinary cytotoxic marker enzymes like ALP, LDH and GGT in Group II rats when compared to the control rats, whereas their levels were maintained near normal in Group III rats.

Discussion

The nephrotoxic effect of ADR has been well documented in all tumor-associated diseases. Since its introduction for the treatment of cancer in 1969, ADR has been widely employed in clinical practice (1). Effective anticancer therapy with ADR and other qui-

Table 1 Antioxidant enzymes in the kidney of control and experimental animals.

Particulars	Group I	Group II	Group III	Group IV
SOD	19.93 \pm 2.01	10.21 \pm 0.98 ^{a***}	18.54 \pm 1.87 ^{b***}	19.13 \pm 1.72
CAT	2.53 \pm 0.21	1.64 \pm 0.13 ^{a***}	2.15 \pm 0.20 ^{a*** b***}	2.59 \pm 0.18
GPX	18.16 \pm 1.63	12.81 \pm 1.47 ^{a***}	17.43 \pm 1.65 ^{b***}	18.39 \pm 2.06
GST	1.32 \pm 0.11	0.65 \pm 0.06 ^{a***}	1.24 \pm 0.13 ^{b***}	1.37 \pm 0.12
GR	9.42 \pm 0.83	5.32 \pm 0.57 ^{a***}	8.41 \pm 0.73 ^{a* b***}	9.72 \pm 0.83
G6PD	1.29 \pm 0.14	0.84 \pm 0.08 ^{a***}	1.16 \pm 0.13 ^{b***}	1.28 \pm 0.12

Values are mean \pm SD for six rats. One unit of enzyme activity expressed as SOD-units/mg. Protein (1 U = amount of enzyme required to bring about 50% inhibition of auto-oxidation of pyrogallol); CAT, μ mol of H₂O₂ utilized/min/mg protein; GPX, μ g of GSH utilized/min/mg protein; GST, nmol of 1-chloro-2,4-dinitro benzene-GSH conjugate formed/min/mg protein; GST, μ mol of CDNB-GSH conjugate formed/min/mg protein; GR, μ mol of NADPH oxidized/min/mg protein; G6PD, μ mol of NADP reduced/min/mg protein. Comparisons are made between: ^a Group I and Groups II, III, IV; ^b Group II and Group III. The symbols represent statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 2 Non-enzymic antioxidant thiol in the kidneys of control and experimental animals.

Particulars	Group I	Group II	Group III	Group IV
GSH	2.93±0.24	1.36±0.12 ^{a***}	2.70±0.24 ^{b***}	3.06±0.21
TSH	8.87±0.76	4.46±0.44 ^{a***}	8.01±0.74 ^{b***}	8.95±0.78
NPSH	4.93±0.44	2.67±0.28 ^{a***}	4.72±0.51 ^{b***}	5.01±0.33

Values are mean±SD for six rats. Units: GSH, TSH and NPSH, $\mu\text{g}/\text{mg}$ protein. Comparisons are made between: ^a Group I and Groups II, III, IV; ^b Group II and Group III. The symbols represent statistical significance: *** $p < 0.001$.

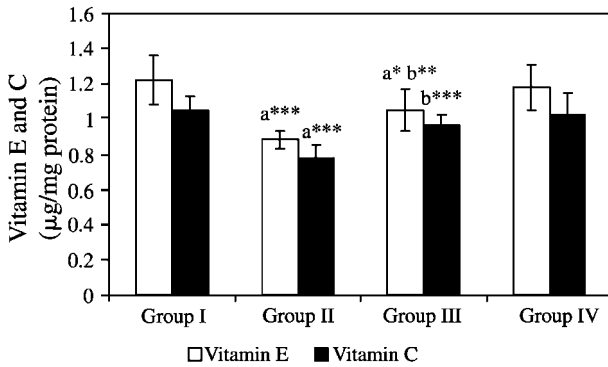


Figure 2 Levels of vitamin E and C in control and experimental animals. Values are mean±SD for six rats. Comparisons are made between: ^aGroup I and Groups II, III, IV; ^bGroup II and Group III. The symbols represent statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

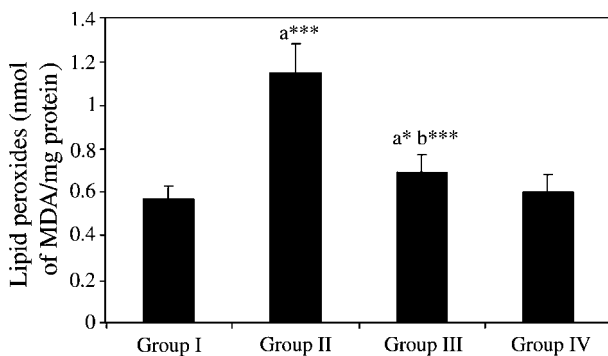


Figure 3 Levels of lipid peroxidation in control and experimental animals. Values are mean±SD for six rats. Comparisons are made between: ^aGroup I and Groups II, III, IV; ^bGroup II and Group III. The symbols represent statistical significance: * $p < 0.05$, *** $p < 0.001$.

nine anthracyclines is severely limited by its nephrotoxicity, which has been well established in a variety of animal species (32). ADR is known to generate superoxide radicals, thus, the formation of free radicals as well as the accumulation of lipid peroxides in

response to the treatment with ADR has been well documented (6). Hence, identification of compounds that could counteract the free radicals produced due to ADR toxicity could undoubtedly increase the medical usefulness of ADR.

Histopathological results confirm the nephrotoxic effect of ADR in rat kidney. A marked proximal tubular necrosis with casts and mild interstitial inflammatory cell infiltration are seen in ADR-administered rats when compared to control sections, whereas these changes are not observed in NAC- and vitamin E-pretreated rats. Similar changes have been reported by Malarkodi et al. (33) in ADR-induced nephrotoxicity.

In this study, ADR administration decreased the activities of enzymic antioxidants like SOD, CAT, GPX and GST. Oxidative stress is found to play a critical role in nephrotoxicity. Decreases in the antioxidant enzymes have already been reported in the kidneys of doxorubicin- and other anticancer agents like cisplatin-treated rats (34, 35). SOD is responsible for the catalytic dismutation of the potentially toxic superoxide anion radical to H_2O_2 . It is an effective defense of the cells against endogenous and exogenous generation of reactive oxygen species (36). CAT is present in peroxisomes and catalyzes the decomposition of H_2O_2 to yield O_2 and water (36). GPX is also one of the important enzymes responsible for the conversion of H_2O_2 into water. Decline in the activities of these antioxidant enzymes may be due to their inactivation caused by excess ROS production (37). In the present study, NAC and vitamin E pretreatment significantly increased the activities of these enzymes to near normalcy. Vitamin E has also been shown to restore the activity of SOD and CAT in the kidneys of ADR-treated rats (34).

GST is a group of multifunctional proteins encoded by a multigene family. They perform functions ranging from catalyzing the detoxification of electrophilic compounds to protecting against peroxidative damage. The decreased activity of GST after ADR administration might be due to enhanced production of H_2O_2 and subsequent GSH depletion. Increase in H_2O_2

Table 3 Urinary marker enzymes in control and experimental animals.

Particulars	Group I	Group II	Group III	Group IV
ALP	0.19±0.02	0.30±0.03 ^{a***}	0.20±0.03 ^{b***}	0.19±0.02
LDH	0.39±0.03	0.78±0.06 ^{a***}	0.42±0.04 ^{b***}	0.38±0.04
GGT	2.16±0.19	3.71±0.31 ^{a***}	2.21±0.20 ^{b***}	2.12±0.18

Values are mean±SD for six rats. Units: ALP, μmol of phenol liberated/h/mg creatinine; LDH, μmol of pyruvate liberated/h/mg creatinine; GGT, μmol of p-nitroaniline released/h/mg creatinine. Comparisons are made between: ^a Group I and Groups II, III, IV; ^b Group II and Group III. The symbols represent statistical significance: *** $p < 0.001$.

leads to the production of hydroxyl radicals, which are deleterious to the cell (7). NAC and vitamin E administration improves the situation by increasing the activity of GST.

GSH is a non-protein endogenous thiol that detoxifies reactive oxygen species formed during intermediate metabolism and drug detoxification. Depletion of cellular GSH has been reported to play an important role in tissue injury. GSH levels are maintained by GSH generating enzyme, GR, and GSH utilizing enzymes, GST and GPX. The reduced activity of GR in ADR-treated groups might be one of the reasons for decreased GSH levels. ADR is also capable of reducing GSH synthesis (38). The observed decrease in GSH levels in ADR-treated groups was brought to near normal values by the administration of the NAC, which is the precursor of the GSH.

GR requires reducing equivalents (NADPH) for its activity, which is provided by the action of G6PD. GR, the key enzyme of the GSH-redox cycle, was significantly decreased on ADR administration (39). This might be due to the reduced availability of NADPH. The decrease in G6PD activity and a fall in NADPH might occur as a result of impaired flux of glucose-6-phosphate through the hexose monophosphate shunt (40). NAC pretreatment resulted in an increased availability of reducing equivalents (NADPH) due to improved G6PD activity, thereby increasing the activity of GR, thus regenerating the GSH pool. Similar observations have been made by Sandhya et al. (41), who reported an increase in the activities of GR and G6PD in the kidneys on antioxidant supplementation in gentamycin-induced nephrotoxicity. Hence, restoration of G6PD levels by NAC and vitamin E treatment is a good indication for normalization and prevention of nephrotoxicity.

The principal high molecular weight thiol-containing compounds are proteins and their thiol groups are in equilibrium with low molecular weight thiol species such as glutathione, and the maintenance of an appropriate concentration of these species in their reduced state is essential for numerous cellular functions. GSH depletion leads to a decrease or reduction in protein thiol content and alters the membrane integrity and increases the susceptibility to lipid peroxidative damage. NAC and vitamin E supplementation is found to be effective in replenishing the protein thiols and preventing damage to kidneys. Similar studies were carried out by Tepel et al. (12), confirming the prevention of chronic renal insufficiency by the administration of NAC in radiographic-contrast agent-induced reductions in renal function.

Decreases in the levels of vitamin C and vitamin E were observed in the kidneys of ADR-treated rats. Vitamin E, an excellent chain-breaking fat-soluble antioxidant, functions as a trap for lipid peroxy radicals. Reduction of vitamin E levels might be deleterious to the cell membrane integrity. Vitamin E, vitamin C and GSH are interrelated with each other for the recycling process. Recycling of tocoperoxyl radical to tocopherol is achieved by reaction with ascorbic acid

(42). Dehydroascorbic acid formed in the above reaction is reduced to ascorbic acid by a non-enzymatic reaction with GSH (43). The reduced status of GSH, vitamin C and vitamin E are essential for maintaining the reduced milieu of the cell. Hence restoration of these reducing equivalents by NAC, by the generation of GSH and vitamin E treatment is shown to be effective in attenuating the nephrotoxicity induced by the oxy radicals on ADR administration.

LPO not only plays an important role in the genesis of many chronic disease conditions, but also evokes adverse effects on the normal organs in which they become distributed. It also leads to oxidation of protein thiol groups, decrease in the relative content of polyunsaturated fatty acids and changes in membrane receptor structure and function (7). The increase in lipid peroxides in the ADR-treated rats might result from increased production of free radicals and/or a decrease in antioxidant status. The ADR-induced LPO observed in our study is in line with other reports (6, 40). Restoration of enzymic and non-enzymic antioxidants by NAC and vitamin E afforded protection against LPO. NAC and vitamin E have been reported to be effective in reducing the amount of hydroxyl radical generated by Fenton-type reactions and also act as a scavenger of peroxide and superoxide radicals (44). The above reports corroborate well with our findings.

ADR is reported to accumulate more in the kidney than in any other organs (45). Renal GGT is an extrinsic brush-border membrane protein and hence, the alteration in GGT activity might be an indicator of nephrotoxicity. Increased excretion of GGT in urine might be due to increased tubular lesion, since the brush-border of the proximal tubules can be a toxic target of ADR. A similar increase in the GGT activity was reported by Saner et al. (46). Increased levels of LDH and ALP in urine have been observed in ADR-administered rats, indicating brush-border membrane damage (40). NAC and vitamin E supplementation prevented the increased excretion of these enzymes, suggesting a membrane protective effect.

In conclusion, ADR-induced increase in the lipid peroxides and the subsequent decrease in the antioxidant status of the cell, as demonstrated by the decreased activities of SOD, CAT, GR and G6PD, are attenuated upon pretreatment with NAC and vitamin E. The role of NAC and vitamin E in the regulation of thiol status and other intracellular antioxidants may be significant in the restoration of redox status. The present study provides evidence that the peroxidative damage brought about by this antineoplastic aminoglycoside can be counteracted by co-administration of NAC and vitamin E.

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