

Protective role of n-acetyl cysteine against cyclophosphamide-induced cardiotoxicity

K JYOTHI¹, A GOPALA REDDY², Y RAMANA REDDY³ and B ANIL KUMAR⁴

Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh 517 502 India

Received: 20 December 2009; Accepted: 14 May 2010

Key words: Cardiotoxicity, Cyclophosphamide - N-acetyl cysteine, Oxidative stress

Cyclophosphamide, an anti-cancerous and immuno-suppressant drug, gets activated by hepatic microsomal cytochrome P₄₅₀ mixed function oxidase (MFO) system to phosphoramidate mustard and acrolein (Georgia *et al.* 2005). Anti-neoplastic effects are associated with phosphoramidate mustard, while acrolein is linked to adverse effects (Kern and Kehrer 2002). One of the potential toxic effect of cyclophosphamide is cardiotoxicity and the toxic potential of cyclophosphamide is related to lipid peroxidation (Ray *et al.* 2010, Tripathi and Jena 2010). N-acetyl cysteine, the acetyl derivative of amino acid L-cysteine, acts like an electron donor for neutralizing free radicals and also enters into glutathione synthesis to act as an antioxidant (Gurbuz *et al.* 2009). In the present study, an attempt was made to assess the protective role of N-acetyl cysteine with reference to cyclophosphamide-induced cardiotoxicity.

Female albino rats of *Wistar kyoto* strain weighing 200±20 g procured from National Institute of Nutrition, Hyderabad, India, were used for the study. Rats were fed with commercial standard mash feed. Feed and water were provided *ad lib*. Animal experimental protocols were approved by Institutional Animal Ethics Committee. Rats were divided into 4 groups, with 6 animals in each group. Group 1 served as control. Group 2 (cyclophosphamide toxic control) was given cyclophosphamide dissolved in sterile distilled water intraperitoneally @ 40 mg/kg b wt on day 1, 3, 5 and 7. Groups 3 and 4 were administered cyclophosphamide as in group 2 and were also treated with N-acetyl cysteine orally @ 0.2% of feed from day 1 to 14 and 8 to 14, respectively.

Blood was collected at the end of second week for biochemical analysis of serum and RBC. The animals were then euthanized; hearts were immediately excised, rinsed with ice-cold physiological saline and stored at -20°C for further homogenization. The activity of creatine

phosphokinase (CPK) and lactate dehydrogenase (LDH) was assayed using diagnostic kits. The cardiac troponins were estimated as serum salt soluble proteins by the method of Indira and Rao (2002). The activity of superoxide dismutase (SOD) was determined as per Madesh and Balasubramanian (1998) and catalase according to Asru (1972).

The heart tissues were weighed and homogenized in 0.2 M tris HCl buffer (pH 7.4) and aliquots of this homogenate were used for estimating concentration of thiobarbituric acid reacting substances (TBARS) (Balasubramanian *et al.* 1988), reduced glutathione (GSH) (Moron *et al.* 1979) and protein carbonyls (Levine *et al.* 1990). For estimation of Na⁺-K⁺ ATPase activity of heart, tissue microsomal preparation was done by the method described by Wallace (2001) and then assay of Na⁺-K⁺ ATPase activity was done in the way described by Kiku *et al.* (1967), and the inorganic phosphate released was estimated by the method of Fiske and Subba Row (1956).

All chemicals and solvents used were of the highest purity and analytical grade. The data were subjected to statistical analysis by applying one way ANOVA using statistical package for social sciences (SPSS) version 15. Differences between means were tested using Duncan's multiple comparison test and significance was set at P<0.05.

The activities of CPK and LDH (IU/l) in serum increased significantly (P< 0.05) in cyclophosphamide-treated group 2 as compared to control (Table 1). N-acetyl cysteine treatment in groups 3 and 4 produced a non-significant decrease in their activity. The concentration of troponins (mg/dl), measured as serum salt soluble proteins, was raised significantly (P< 0.05) in cyclophosphamide group which decreased significantly (P< 0.05) on N-acetyl cysteine administration. The cellular mechanisms of toxicity by phosphoramidate mustard and acrolein, the principal metabolites of cyclophosphamide, are thought to be mediated through increased formation of free radicals and diminution of cellular antioxidant status (Ray *et al.* 2010, Tripathi and Jena 2010). In the present study, administration of cyclophosphamide induced heart damage as evinced by rise in the activity of serum LDH and CPK, and an increase in

Present address: ¹H. No. # 5-4-17/1, Bhavani Colony, Rajendranagar, Hyderabad 500 030 India.

²Professor and University Head (e mail: gopalarreddy123@rediffmail.com), ⁴Ph.D. Scholar, Department of Pharmacology and Toxicology, ³Department of Animal Nutrition, College of Veterinary Science, Rajendranagar, Hyderabad 500 030 India.

Table 1. Serum and tissue biochemical parameters

Parameter	Group 1: Control	Group 2: Cyclophosphamide control (d 1,3,5,7)	Group 3: Cyclophosphamide (d1,3,5,7)+N-acetyl cysteine (1–14 d)	Group 4: Cyclophosphamide (d1,3,5,7)+N-acetyl cysteine (8–14 d)
CPK (IU/l)	165.54±7.46 ^a	248.31±43.88 ^b	170.58±19.23 ^{ab}	196.26±16.94 ^{ab}
LDH (IU/l)	242.41±10.24 ^a	345.49±17.29 ^b	303.54±19.89 ^b	328.53±18.19 ^b
Troponins (mg/dl)	14.31±0.98 ^a	38.07±1.74 ^c	32.94±2.28 ^b	32.75±1.37 ^b
SOD (U/mg protein)	0.25±0.03 ^c	0.12±0.01 ^a	0.19±0.01 ^b	0.19±0.02 ^b
Catalase (µg of H ₂ O ₂ decomposed/mg protein/min)	9.52±0.77 ^b	5.48±0.40 ^a	7.90±0.54 ^{ab}	6.50±1.58 ^a
TBARS (µ mol MDA/mg protein)	0.11±0.01 ^a	0.27±0.02 ^c	0.19±0.01 ^b	0.24±0.01 ^c
Protein carbonyls (n mol/100 mg protein)	1.34±0.07 ^a	1.94±0.14 ^b	1.59±0.12 ^a	2.17±0.10 ^b
GSH (µ mol/mg protein)	22.18±1.62	10.16±0.85	14.73±1.57	13.29±0.52
Na ⁺ -K ⁺ ATPase (µ mol of Pi released/mg protein/30 min)	19.11±1.48 ^c	4.01±0.71 ^a	17.50±0.63 ^{bc}	16.03±0.80 ^b

Values are mean±S.E (n = 6); Means with different alphabets differ significantly (P< 0.05) with other groups; One-way ANOVA.

the concentration of troponins as compared to control group. This might be due to direct free radical damage or due to release of lysosomal enzymes that further aggravate injury. N-acetyl cysteine treatment significantly decreased troponins elevation, while a moderate decrease was observed in the activity of CPK and LDH that might be due to its free radical scavenging and anti-apoptotic properties (Gurbuz *et al.* 2009).

The activity of antioxidant enzymes namely SOD (units/mg protein) and catalase µg of H₂O₂ decomposed/mg protein/min measured in RBCs decreased significantly (P< 0.05) in cyclophosphamide-treated group compared to control group. N-acetyl cysteine treatment could improve activity of SOD significantly (P< 0.05) but had no significant effect on catalase in groups 3 and 4. The concentration of malondialdehyde (MDA), a thiobarbituric acid reacting substance (TBARS; µ mol MDA/mg protein), and protein carbonyls (n mol/100 mg protein) increased (0.27±0.02 and 1.94±0.14, respectively) in cyclophosphamide group as compared to control group (0.11±0.01 and 1.34±0.07, respectively). N-acetyl cysteine treatment for 14 days reduced concentration of MDA significantly (P< 0.05). GSH concentration (µmol/mg protein) in group 2 showed a significant (P< 0.05) decline as compared to control, while it showed significant (P< 0.05) improvement with N-acetyl cysteine administration for 14 days in group 3. Marked oxidative stress by cyclophosphamide in this study was indicated by an increase in the concentration of TBARS and protein carbonyls, the markers of peroxidative damage of lipids and proteins, respectively when compared to those of control group. N-acetyl cysteine treatment for 14 days could significantly reverse these changes. The thiol group of N-acetyl cysteine reacts with 4-OH CP (the intermediate metabolite of cyclophosphamide) and forms alkyl-thio derivative, which does not spontaneously form

phosphoramidate mustard and acrolein. This might be the reason for decreased concentration of TBARS and protein carbonyls in the N-acetyl cysteine treated groups 3 and 4. Reduced glutathione (GSH), the thiol containing cellular antioxidant, functions to scavenge free radicals, to donate electrons to OH and superoxide and to metabolize xenobiotics. The concentration of GSH was significantly lowered in cyclophosphamide toxic group 2, which may be due to its increased participation in metabolism of 4-OH CP. N-acetyl cysteine treatment for 14 days significantly improved the GSH level, while 7 days treatment produced nonsignificant improvement, which suggests enhanced glutathione synthesis following 14 day administration with N-acetyl cysteine.

The Na⁺-K⁺ ATPase activity (µ mol of Pi released/mg protein/30 min) of heart tissue was significantly (P< 0.05) reduced by cyclophosphamide in group 2 as compared to control, while it revealed a significant (P< 0.05) improvement following N-acetyl cysteine treatment in groups 3 and 4. When the proteins get oxidized by ROS, the SH groups of proteins get converted into disulphide bonds. This decrease in SH group content might be responsible for the fall in Na⁺-K⁺ ATPase activity found in cyclophosphamide group as the functional ability is very much dependent on SH groups. This decline was significantly reversed by N-acetyl cysteine in groups 3 and 4.

In conclusion, the results of the study revealed that cyclophosphamide exerted cardiotoxicity by free radical (ROS) generation or by inhibiting the major antioxidant defenses and N-acetyl cysteine treatment could produce moderate protective effect on heart, which could be attributed to its ability to augment glutathione synthesis and quenching of free radicals. Further, simultaneous administration of N-acetyl cysteine along with cyclophosphamide was found to be superior.

SUMMARY

A study was conducted to evaluate the mechanism of cyclophosphamide-induced cardiotoxicity and protective role of N-acetyl cysteine in Wistar rats. The rats were divided into 4 groups: Group 1 served as control, groups 2, 3 and 4 received 4 doses of cyclophosphamide (40 mg/kg b.wt. i/p on alternate days), while groups 3 and 4 also received N-acetyl cysteine (0.2% of feed) from day 1 to 14 and day 8 to 14 of experiment, respectively. The concentration of troponins, protein carbonyls, TBARS and GSH, and the activity of CPK, LDH, SOD, catalase and Na⁺- K⁺ ATPase were assessed. Significant (P<0.05) alterations were recorded in CPK, LDH, troponins, protein carbonyls, SOD, catalase, TBARS, GSH and Na⁺- K⁺ ATPase in cyclophosphamide toxic group 2. N-acetyl cysteine treatment for 14 days produced improvement on heart, while 7 day treatment has no recognizable effect. It is concluded that cyclophosphamide induced cardiac toxicity by stimulating oxidative stress and 14 day treatment with N-acetyl cysteine offered protection. It is desirable to co-administer N-acetyl cysteine to avoid undue cardiotoxicity due to cyclophosphamide.

REFERENCES

- Asru K S. 1972. Colorimetric assay of catalase. *Analytical Biochemistry* **47**: 389–94.
- Balasubramanian K A, Manohar M and Mathan V I. 1988. An unidentified inhibitor of lipid peroxidation in intestinal mucosa. *Biochimica et Biophysica Acta* **962**: 51–58.
- Fiske and Subba Row. 1956. *Hawk's Physiological Chemistry*. 14th edn. Oser, Summerson. Editors. McGraw Hill.
- Georgia JP, Dianne C, Michael B, Sally L, Eric W, Brian H, Colin J H and Roland C W 2005. Role of hepatic cytochrome P450s in the pharmacokinetics and toxicity of cyclophosphamide: Studies with the hepatic cytochrome P450 reductase Null mouse. *Cancer Research* **65**: 4211–17.
- Gurbuz N, Ozkul A and Burgaz S. 2009. Effects of vitamin C and N-acetylcysteine against cyclophosphamide-induced genotoxicity in exfoliated bladder cells of mice *in vivo*. *Official Journal of Balkan Union of Oncology* **14**: 647–52.
- Indira B and Rao S B. 2002 New, simple and cheap alternative to troponin test for diagnosis of acute myocardial infarction. *Indian Journal of Experimental Biology* **40**: 628–30.
- Kern J C and Kehrer J P. 2002. Acrolein-induced cell death: a caspase-influenced decision between apoptosis and oncosis/necrosis. *Chemico-Biological Interactions* **139**: 79–95.
- Kiku N, Shiusuke K and Makoto N. 1967. Adenosine triphosphatase activity of erythrocyte membrane in hereditary spheocytosis. *Life Sciences* **6**: 595–600.
- Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn B W, Shaltiel S and Stadtman E R. 1990. Determination of carbonyl content in oxidatively modified proteins. *Methods in Enzymology* **186**: 464–78.
- Madesh M and Balasubramanian K A. 1998. Microtiter plate assay for superoxide dismutase using MTT reduction by superoxide. *Indian Journal of Biochemistry and Biophysics* **35**: 184–88.
- Moron M S, Depierre J W and Mannervik B. 1979. Levels of glutathione, glutathione reductase and glutathione S transferase in rat lung and liver. *Biochimica et Biophysica Acta* **582**: 67–68.
- Ray S, Pandit B, Ray S D, Das S, Chakraborty S. 2010. Cyclophosphamide induced lipid peroxidation and changes in cholesterol content: protective role of reduced glutathione. *International Journal of PharmTech Research* **2**: 704–18.
- Tripathi D N and Jena D B. 2010. Astaxanthin intervention ameliorates cyclophosphamide-induced oxidative stress, DNA damage and early hepatocarcinogenesis in rat: Role of Nrf2, p53, p38 and phase-II enzymes. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **696**: 69–80.
- Wallace H A. 2001. *Principles and Methods of Toxicology*. 4th edn. Taylor & Francis.