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Cyclophosphamide Induced Lipid Peroxidation and Changes in Cholesterol Content: Protective Role of Reduced Glutathione

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Abstract: The study was designed with an aim to evaluate the protective effects of reduced glutathione on cyclophosphamide induced lipid peroxidation and also changes in cholesterol content. Goat liver and white New Zeeland rabbit were used as lipid source for *in vitro* and *in vivo* model respectively. Lipid peroxidation study was performed by measuring the malondialdehyde, 4-hydroxy-2-nonenal, reduced glutathione and nitric oxide content of tissue homogenates / rabbit blood. In the cholesterol profile total cholesterol and high density lipoprotein cholesterol content of rabbit blood was determined. The data presented in this work demonstrate the lipid peroxidation induction potential of cyclophosphamide and the antiperoxidative potential of reduced glutathione on cyclophosphamide-induced lipid peroxidation. It was also observed that reduced glutathione has protective effect on cyclophosphamide-induced changes in cholesterol content. A significant correlation was also found between malondialdehyde, 4-hydroxy-2-nonenal with total cholesterol as well as between reduced glutathione and nitric oxide with HDL cholesterol.

Key words: Cyclophosphamide; Malondialdehyde; Reduced glutathione; 4-Hydroxy-2-nonenal; Nitric oxide; Cholesterol.

Introduction

Lipid peroxidation is a degenerative process that affects unsaturated membrane lipids under conditions of oxidative stress[1]. This complex process is believed to contribute to human aging and disease by disrupting the structural conformation, the packing of lipid components and ultimately the function of biological membranes. Oxidative stress may cause lipid peroxidation by damaging the DNA-sugar and oxidizing protein by introducing carbonyl group into the side chains of protein molecules[2, 3]. Reactive oxygen species and other pro-oxidants cause the decomposition of ω_3 and ω_6 polyunsaturated fatty acids of membrane phospholipids leading to the formation of aldehydic end products including malondialdehyde (MDA), 4-hydroxy-2-nonenals and 4-hydroxy-2-alkenals (HAKs) of different chain length. These aldehydic molecules have been considered as ultimate mediators of toxic effects elicited by oxidative stress occurring in biological membrane[4]. Oxidative stress in cells can be initiated by the addition of Fe^{2+} in the presence of dioxygen. This stress will result in lipid peroxidation and subsequent formation of lipid

radicals[5, 6]. Free radical mediated oxidative stress results usually from deficient natural antioxidant defenses and act as a main factor in the pathophysiology of various diseases and ageing[7]. Various antioxidants and free radical scavengers have been suggested to be general cytoprotective agents of therapeutic benefit[8]. In case of reduced or impaired defense mechanism and excess generation of free radicals that are not counter balanced by endogenous antioxidant defense exogenously administered antioxidants have been proven useful to overcome oxidative damage[9]. Lipid peroxidation induction capacity of drugs may be related to their toxic potential as exemplified by adriamycin induced cardiotoxicity, which is occurred through free radical mediated process[10]. So the evaluation of antioxidants as suppressor of drug induced lipid peroxidation provides a scope of further investigation for their co-administration with drugs to reduce drug-induced toxicities that are possibly mediated by free radical mechanism.

Cyclophosphamide, an alkylating agent widely used in cancer chemotherapy, is an inactive cytostatic, which is metabolized into active metabolites mainly in the liver. During bioactivation reactive oxygen species are also formed, which can modify the components of both healthy and neoplastic cell leading to decreased antioxidative capacity[11]. It has been reported that cyclophosphamide produces genotoxicity and oxidative stress in mice[12] and early lung injury in rats[13]. It also causes fatal cardiotoxicity[14]. Hemorrhagic cystitis is a major dose limiting side effect of Cyclophosphamide[15]. It was also found that cyclophosphamide has the ability to produce male germ cell toxicity[16].

Serum cholesterol or its fractions like low density lipoproteins (LDL), high density lipoproteins (HDL) content have been found responsible for many diseases. Cholesterol and lipoprotein levels correlate well with the risk of cardiovascular diseases[17]. Stress in the form of starvation was found to increase lipid peroxidation and alter lipid profile in rabbits[18].

In view of the above findings and the ongoing search of the present authors for antioxidant that may reduce drug induced lipid peroxidation [19-22], the present work has been carried out *in vitro* and *in vivo* to evaluate the antiperoxidative potential of reduced glutathione on cyclophosphamide-induced lipid peroxidation and also to evaluate the effect of reduced glutathione on cyclophosphamide induced changes in cholesterol content in rabbit blood sample.

Materials and Methods

Thiobarbituric acid (TBA), 2, 4-dinitrophenylhydrazine (DNPH), sodium nitrite and trichloroacetic acid (TCA) were purchased from Ranbaxy Fine Chemicals Ltd., New Delhi; 5, 5' dithiobis-2-nitrobenzoic acid was from SRL Pvt. Ltd., Mumbai; Sulfanilamide was from SD Fine Chem.Ltd., Mumbai; N-naphthylethylenediamine dihydrochloride was from Loba Chemie Pvt. Ltd., Mumbai; 1, 1, 3, 3-tetraethoxypropane, reduced glutathione were from Sigma Chemicals Co. St. Louis, MO, USA; The standard sample of 4-HNE was purchased from ICN Biomedicals INC., Ohio; Cyclophosphamide injection (Oncomide-200) (for *in vivo* work) was from Khandelwal Laboratories Pvt. Ltd., Mumbai, India and pure cyclophosphamide (for *in vitro* work) was form Dabur Research Foundation, Ghaziabud , India. Cholesterol test kit was from Span Diagnostic Ltd., Surat, India. All other reagents were of analytical grade.

Animals

The *in vitro* drug-lipid interaction studies were performed using goat liver as lipid source. The liver was collected from Durgapur Municipal Corporation (DMC) approved outlet. Goat liver was selected because of its easy availability and close similarity with human liver in its lipid profile[23]. The *in vivo* drug-lipid interaction studies were carried out using white New Zeeland rabbit (*Oryctolagus caniculus*) as experimental model. The *in vivo* animal experiment was carried out in accordance with the protocol of institutional animal ethics committee of Himalayan Pharmacy Institute, Majhitar, East Sikkim, India (sanctioned by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, Govt. of India, Chennai-600041; Registration no of the institute 1028/C/07/CPCSEA). Normal healthy rabbits weighing 1.5-2.0 kg were taken for the study. All the animals were housed in normal ambient temperatures (25-29^{0}C) and acclimatized in the laboratory for at least 72 hrs. They were maintained on a standard laboratory diet and water at *ad libitum*.

Preparation of tissue homogenate for in vitro lipid peroxidation studies

Goat liver perfused with normal saline through hepatic portal vein was harvested and its lobes were briefly dried between filter papers to remove excess

blood and thin cut with a heavy-duty blade. The small pieces were then transferred in a sterile vessel containing phosphate buffer (pH = 7.4) solution. After draining the buffer solution as completely as possible, the liver was immediately grinded to make a tissue homogenate (1gm / ml) using freshly prepared phosphate buffer (pH = 7.4). The homogenate was divided into four equal parts, which were then treated differently as mentioned below.

Incubation of tissue homogenate with cyclophosphamide and / or reduced glutathione for *in vitro* lipid peroxidation studies

The tissue homogenate was divided into four parts of 50 ml each. The first portion was kept as control (C), while the second portion was treated with cyclophosphamide (D) at a concentration of 0.015 mg / g tissue homogenate. The third portion was treated with cyclophosphamide at a concentration of 0.015 mg / g tissue homogenate and reduced glutathione at a concentration of 0.05 mg / g tissue homogenate (DA) and the fourth one was treated with reduced glutathione alone at a concentration of 0.05 mg /g tissue homogenate (A). After treatment with cyclophosphamide and / or reduced glutathione, the liver homogenates were shaken for 2 hrs and incubated at 18 ± 2^0C for a period of maximum 24 hrs for further work.

Group division of rabbits for *in vivo* lipid peroxidation and cholesterol profile studies

In each set there were twelve animals. Twelve animals were further subdivided into four groups. There were three animals in each group. The first group was control group (C), while the second group (D) was treated with cyclophosphamide intramuscularly at a dose of 15 mg / kg body weight. The third group (DA) was treated both with cyclophosphamide intramuscularly at a dose of 15 mg / kg body weight and reduced glutathione intramuscularly at a dose of 50 mg / kg body weight. The final group (A) received only an intramuscular injection of reduced glutathione at a dose of 50 mg / kg body weight.

Estimation of malondialdehyde (MDA) level from tissue homogenate / rabbit blood

The extent of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using thiobarbuturic acid (TBA) method[24]. The estimation was done at 2 and 24 hrs of incubation and repeated for five times in accordance with the protocol of Ray *et al*.[21]. The concentrations of MDA for various groups were determined from standard curve. The best-fit equation is A = 0.006502M, where M = nanomoles of MDA, A = absorbance, r = 0.9977, SEM = 0.0117 and F = 2029.42 (df = 1, 9).

Estimation of 4-hydroxy-2-nonenal (4-HNE) level from tissue homogenate / rabbit blood

4-HNE content was determined by reaction with 2, 4-dinitrophenyl hydrazine (DNPH) [25]. The estimation was done at 2 and 24 hrs of incubation and it was repeated for five times in accordance with the protocol of Ray *et al*.[21]. The concentrations of 4-HNE for various groups were determined from standard curve. The best-fit equation is: Nanomoles of 4-HNE = $(A_{350}$ - 0.005603185) / 0.003262215, where A_{350} = absorbance at 350 nm, r = 0.999, SEM = 0.007.

Estimation of reduced glutathione (GSH) level from tissue homogenate / rabbit blood

Reduced glutathione (GSH) was measured in accordance with Ellman's method[26]. The estimation was done at 2 and 24 hrs of incubation and repeated for five times in accordance with the protocol of Ray *et al*.[21]. The concentrations of GSH for various groups were determined from standard curve. The best-fit equation was A = 0.000495M, where M = nanomoles of reduced glutathione, A = absorbance, r = 0.9986, SEM = 0.0021 and F = 4405.78 (df = 1, 12).

Estimation of nitric oxide (NO) level from tissue homogenate / rabbit blood

NO content was determined by reaction with Griess reagent. Griess reagent was prepared by mixing equal volumes of sulphanilamide (1% w/v in 3N HCl) and (0.1% w/v N-naphthylethylenediamine dihydrochloride) [27]. The estimation was done at 2 and 24 hrs of incubation and it was repeated for five times in accordance with the protocol of Ray *et al*.[21]. The concentrations of GSH for various groups were determined from standard curve. The best-fit equation is A = 0.015846M, where M = nanomoles of NO, A = absorbance, r = 0.9973, SEM = 0.0033 and F=1960.35 (df = 1, 9).

Estimation of total cholesterol and HDL-cholesterol from rabbit blood

Determination of cholesterol concentration was performed in one step method[28] with the help of cholesterol test kit. The determinations were done at 2 and 24 hrs of incubation and it was repeated for five times. In each case there were three samples. After the specified hours of incubation, 2 ml of blood was withdrawn from the ear vein of rabbits. The blood samples were centrifuged at 2000 rpm for 15 minutes

and the supernatant (plasma) was separated out. After that total cholesterol and high density lipoprotein cholesterol of the rabbit blood were determined

Total cholesterol

The Total Cholesterol (TC) was calculated by using the following formula

Total Cholesterol (mg / dL) = (O.D. of Test / O.D. of Standard) x 200

HDL cholesterol

Step-I

HDL- cholesterol separation: 0.2 ml of the supernatant was transferred into a centrifuge tube and to it 0.2 ml of reagent 3 from test kit was added. Then it was shaken well to mix and the tubes were kept at room temperature for 10 minutes. It was centrifuged at 2000 rpm for 15 minutes to obtain a clear supernatant.

Step-II

HDL-cholesterol determination: The test sample was prepared by mixing 3 ml of reagent 1 from test kit with 0.12 ml of the supernatant obtained from the step-I. The centrifuge tubes were shaken well and the tubes were kept in the boiling water bath exactly for 90 sec. The tubes were cooled immediately at room temperature under running tap water. The O.D. of Standard (S) & Test (T) were measured at 560 nm against reagent 1 as blank. The content of HDL-Cholesterol was calculated by using the following formula:

HDL-Cholesterol (mg / dL) = (O.D. of Test / O.D. of Standard) x 50

Statistical analysis

For both *in vitro* and *in vivo* model of experiments, interpretation of the result is supported by analysis of variance (ANOVA) and multiple comparison analysis using least significant different procedure[29, 30].

Results

The percent changes in MDA, 4-HNE, GSH, NO, total cholesterol and HDL-cholesterol content of different samples at different time of incubation were calculated with respect to the control of the corresponding time of incubation and was considered as indicator of the extent of lipid peroxidation and changes in cholesterol content. Average of percent changes of five sets is shown in bar diagram along with corresponding standard error of mean. The results of the studies on cyclophosphamide-induced lipid peroxidation both *in vitro* and *in vivo* model and its inhibition with reduced glutathione were shown in Figure 1-8. The results of the studies on cyclophosphamide-induced changes in cholesterol content (*i.e.* changes in total cholesterol and HDL-cholesterol) and the effects of reduced glutathione on these changes were also shown in Figure 9-10.

The data generated from both *in vitro* and *in vivo* models indicate that incubation of the liver homogenates / rabbit blood with cyclophosphamide resulted an increase in MDA content (30.29 and 43.69 % for *in vitro* model; 45.01 and 19.65 % for *in vivo* model) and 4-HNE content (11.59 and 7.24% for *in vitro* model; 14.32 and 9.34 % for *in vivo* model) (Figure 1-4) in comparison to control to a significant extent after incubation for varying period of time. These observations suggest that cyclophosphamide has the ability to induce lipid peroxidation process. So the lipid peroxidation induction capacity of the drug may be related to its toxic potential. But the MDA contents (-14.25 and 5.35% for *in vitro* model; 14.2 and -7.83% for *in vivo* model) and 4-HNE contents (1.17 and -12.97% for *in vitro* model; 2.63 and -5.74% for *in vivo* model) were significantly reduced with respect to cyclophosphamide-treated group, when the liver tissue homogenates / rabbit blood were treated with cyclophosphamide in combination with reduced glutathione. This implies that reduced glutathione had the ability to suppress cyclophosphamide-induced lipid peroxidation. This may be due to the protective effects against free radicals that may have been generated within the system due to presence of cyclophosphamide. Again the liver tissue homogenates / rabbit blood were treated only with the reduced glutathione then the MDA contents (-18.14 and -6.34% for *in vitro* model; -11.6 and -12.49% for *in vivo* model) and 4-HNE levels (-5.36 and -17.87% for *in vitro* model; -5.41 and -10.84% for *in vivo* model) were reduced in comparison to the control as well as cyclophosphamide-treated group. This decrease may be due to the free radical scavenging property of the reduced glutathione.

Figure 5-8 indicate that incubation of the liver tissue homogenates / rabbit blood with cyclophosphamide caused a decrease in GSH content (-27.94 and -23.9% for *in vitro* model; -5.16 and -4.6% *in vivo* model) and NO content (-24.66 and -28.8% for *in vitro* model; -19.35 and -11.7% for *in vivo* model) with respect control to a significant extent. The decrease in GSH and NO content was associated with an increase in lipid peroxidation. These observations also suggest lipid peroxidation induction potential of the cyclophosphamide. When the liver tissue homogenates / rabbit blood were treated with cyclophosphamide along with reduced glutathione then

the GSH level (-15.93 and -9.96% for *in vitro* model; -2.73 and 23% for *in vivo* model) and NO levels (-17.21 and -12.72% for *in vitro* model; -10.89 and 10.5 for *in vivo* model) were increased in comparison to cyclophosphamide-treated group of that corresponding time. Again, when the liver tissue homogenates / rabbit blood were treated only with reduced glutathione the GSH contents (15.14 and 15.78% for *in vitro* model; 3.56 and 25.7% for *in vivo* model) and NO contents (21.45 and 23.03% for *in vitro* model; 10.53 and 12.71% for *in vivo* model) were also increased in comparison to control as well as cyclophosphamide-treated samples. The increase in GSH and NO level suggest the antiperoxidative potential of reduced glutathione.

It was observed from Figure 9-10 that rabbits treated with cyclophosphamide caused an increase in total cholesterol content (54.59 and 26.32 %) with respect to corresponding control. But the HDL-cholesterol level (-19.37 and -12.17%) was reduced in comparison to control group. These observations suggest that cyclophosphamide can change the cholesterol profile. It was further found that incubation of blood sample with cyclophosphamide and reduced glutathione produce a decrease in total cholesterol (23.11 and 9.49%), but the HDL-cholesterol contents (-7.78 and -3.85%) were increased in comparison to cyclophosphamide-treated group respectively. Incubation of blood samples only with reduced glutathione also shows a tendency of decrease in total cholesterol (10.03 and -4.23%), but HDL-cholesterol contents (5.71 and 6.81%) were increased in comparison to control or cyclophosphamide-treated group respectively. These results suggest that reduced glutathione could inhibit cyclophosphamide-induced changes in cholesterol profile.

To compare means of more than two samples, multiple comparison analysis along with analysis of variance was performed on the percent changes data of various groups. It is seen that there is significant differences among various groups (F1) such as cyclophosphamide-treated, cyclophosphamide and reduced glutathione-treated and only reduced glutathione-treated. But within a particular group, differences (F2) are insignificant which shows that there is no statistical difference in animals in a particular group. It was found that the content of MDA / 4-HNE in cyclophosphamide-treated group is statistically significantly different from the cyclophosphamide and reduced glutathione-treated group and only reduced glutathione-treated group for both *in vitro* and *in vivo* models (Table 1-2). In case of GSH / NO content for *in vitro* model suggest that cyclophosphamide-treated group as well as cyclophosphamide and reduced glutathione-treated groups were statistically different from only reduced glutathione-treated group. But the GSH / NO content for *in vivo* model indicate that the cyclophosphamide-treated group is statistically significantly different from the cyclophosphamide and reduced glutathione-treated group as well as only reduced glutathione-treated group (Table 3-4).

From Tables 5-6, it is seen that the content of total cholesterol / HDL-cholesterol in cyclophosphamide-treated group, cyclophosphamide and reduced glutathione-treated group and only reduced glutathione-treated groups are statistically significantly different from each other with the exception of total cholesterol content (2 hrs) in cyclophosphamide-treated group which is statistically significantly different from the cyclophosphamide and reduced glutathione-treated group and only reduced glutathione- treated group.

Discussion

The results from both *in vitro* and *in vivo* models presented in this work clearly demonstrate the lipid peroxidation induction potential of cyclophosphamide, which may be related to its toxic potential. This is an analogy to cardiotoxicity of doxorubicin[10] and indomethacin-induced gastric mucosal injury[31]. MDA is a highly reactive three-carbon dialdehyde produced as a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism[32]. Increase in the accumulation of MDA in cells can result into cellular degradation, some biochemical changes and even cell death[33]. 4-Hydroxy-2-nonenal (4-HNE), a lipid aldehydes that form due to lipid peroxidation occurring during episodes of oxidant stress, readily forms adducts with cellular proteins; these adducts can be assessed as a marker of oxidant stress in the form of lipid peroxidation[34]. 4-HNE can be produced from arachidonic acid, linolenic acid or their hydroperoxide in concentration of 1 μM to 5 nM in response to oxidative stress[35]. It can diffuse within or even escape from the cell and attack targets far from the site of the original free radical event[36]. 4-hydroxy-2-nonenal (HNE) can also modify and inactivate proteins and responsible for age related muscular degeneration[37]. So the decrease in MDA and 4-HNE content of liver tissue homogenates / rabbit blood, when treated with cyclophosphamide and reduced glutathione as well as only with reduced glutathione implies the free radical scavenging property of reduced glutathione. Glutathione is a small protein composed of three amino acids, such as cysteine, glutamic acid and glycine[38]. It is an important antioxidant and plays a very important role in the defence mechanism for

tissue against the reactive oxygen species[39]. The depletion of GSH is associated with increase in lipid peroxidation. The decrease in GSH level may be a consequence of enhanced utilization of this compound by the antioxidant enzymes, glutathione peroxidase and glutathione-S-transferase. NO plays a very important role in host defense[40]. Nitric oxide has versatile role in biology because it can be a signaling molecule in vasodilatation[41-44], a toxin[44], a pro-oxidant[45] and a potential antioxidant[46-50]. So the increase in GSH and NO content of liver tissue homogenates / rabbit blood, when treated with drug and antioxidant as well as only with antioxidant implies the free radical scavenging activity of the antioxidant. It has been proposed that NO causes chain termination reactions during lipid peroxidation as observed in low-density lipoprotein oxidation as well as in chemical systems[48-50].

Increase in total cholesterol level / decrease in HDL-cholesterol level in cyclophosphamide- treated group indicates that cyclophosphamide has the ability to change cholesterol profile may be by inducing oxidation of cholesterol. An increase in the lipid peroxidation level was observed in hyperlipidemic and hypercholesterolemic patients[51, 52]. But the increase in HDL-cholesterol level in cyclophosphamide along with reduced glutathione-treated group as well as only reduced glutathione-treated group implies that reduced glutathione and HDL-cholesterol has protective effect against lipid peroxidation. It was also reported that antioxidant like vitamin C prevents oxidation of LDL-cholesterol, decreases total and LDL-cholesterol and triglyceride and also raises HDL-cholesterol level[53-55].

To explore possibility of any mathematical relationship between MDA / 4-HNE / GSH / NO with total cholesterol / HDL-cholesterol and to interpret the data in a better way, regression analysis have been performed between % changes of MDA / 4-HNE with % changes of total cholesterol and % changes of GSH / NO with % changes of HDL-cholesterol content of rabbit blood. The results (Table 7) indicates that for cyclophosphamide-treated group MDA / 4-HNE with total cholesterol have a correlation coefficient 0.89 / 0.769 at 2 hrs of incubation and 0.778 / 0.308 at 24 hrs of incubation. So it can be said that MDA / 4-HNE and total cholesterol have a direct correlation. It was also observed that MDA / 4-HNE and total cholesterol have detrimental activity towards living system. Several studies also show that lipid peroxidation increases the total cholesterol level. In rabbits, on cholesterol feeding, increase in serum and blood MDA levels was noticed[56, 57]. The correlation coefficients between GSH and HDL-cholesterol for antioxidant treated group are 0.898 and 0.628 at 2 and 24 hrs of incubation respectively. For NO and HDL-cholesterol, the correlation coefficients for drug-treated group are 0.842 and 0.576 at 2 and 24 hrs of incubation, where as for only antioxidant treated group the correlation coefficients are 0.968 and 0.218 respectively. There are also similar links between GSH / NO with HDL-cholesterol and they are capable of inhibiting cyclophosphamide-induced changes in lipid and cholesterol profile. The quality of some of the equations is not in the acceptable range, such is unavoidable while working directly with raw biological data.

Conclusion

These findings from both *in vitro* as well as *in vivo* models indicate the lipid peroxidation induction potential of cyclophosphamide which may be related to its toxic potential. The results also suggest the antiperoxidative effects of reduced glutathione and demonstrate its potential to reduce cyclophosphamide-induced lipid peroxidation and thus to increase therapeutic index of the drug by way of reducing toxicity that may be mediated through free radical mechanisms. It is also observed that cyclophosphamide also has the ability to change the cholesterol profile and reduced glutathione has a protective effect on these changes. However a detailed study of total lipid profile is required in this regard.

Table 1: ANOVA & Multiple comparison for changes of MDA content

Type of model	Time of incubation (hrs)	Analysis of variance and multiple comparison
In vitro	2	F1=19.90 [df=(2, 8)], F2=3.59 [df=(4, 8)], Pooled variance (S^2)*=182.04, Critical difference (p=0.05)# LSD=25.40, Ranked means** (D) (DA, A)
	24	F1=13.52 [df=(2, 8)], F2=2.82 [df=(4, 8)], Pooled variance (S^2)*=238.18, Critical difference (p=0.05)# LSD=29.06, Ranked means** (D) (DA, A)
In vivo	2	F1=284.18 [df=(2, 8)], F2=3.23 [df=(4, 8)], Pooled variance (S^2)*=14.14, Critical difference (p=0.05)# LSD=7.079, Ranked means** (D) (DA) (A)
	24	F1=30.55 [df=(2, 8)], F2=0.653 [df=(4, 8)], Pooled variance (S^2)*=49.37, Critical difference (p=0.05)# LSD=13.23, Ranked means** (D) (DA, A)

Theoretical values of F: p=0.05 level F1=4.46 [df=(2,8)], F2=3.84 [df=(4,8)] P=0.01 level F1=8.65 [df=(2,8)], F2=7.01 [df=(4,8)], F1 and F2 corresponding to variance ratio between groups and within groups respectively; * Error mean square, # Critical difference according to least significant procedure (LSD)[29, 30] **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

Table 2: ANOVA & Multiple comparison for changes of 4-HNE content

Type of model	Time of incubation (hrs)	Analysis of variance and multiple comparison
In vitro	2	F1=170.51 [df=(2, 8)], F2=3.54 [df=(4, 8)], Pooled variance (S^2)*=2.145, Critical difference (p=0.05)# LSD=2.76, Ranked means** (D) (DA) (A)
	24	F1=32.35 [df=(2, 8)], F2=3.69 [df=(4, 8)], Pooled variance (S^2)*=27.38, Critical difference (p=0.05)# LSD=9.852, Ranked means** (D) (DA, A)
In vivo	2	F1=189.26 [df=(2, 8)], F2=0.646 [df=(4, 8)], Pooled variance (S^2)*=2.60, Critical difference (p=0.05)# LSD=3.036, Ranked means** (D) (DA) (A)
	24	F1=115.66 [df=(2, 8)], F2=2.49 [df=(4, 8)], Pooled variance (S^2)*=4.76, Critical difference (p=0.05)# LSD=4.11, Ranked means** (D) (DA) (A)

Theoretical values of F: p=0.05 level F1=4.46 [df=(2,8)], F2=3.84 [df=(4,8)] P=0.01 level F1=8.65 [df=(2,8)], F2=7.01 [df=(4,8)], F1 and F2 corresponding to variance ratio between groups and within groups respectively; * Error mean square, # Critical difference according to least significant procedure (LSD)[29, 30] **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

Table 3: ANOVA & Multiple comparison for changes of GSH content

Type of model	Time of incubation (hrs)	Analysis of variance and multiple comparison
In vitro	2	F1=19.32 [df=(2, 8)], F2=2.69 [df=(4, 8)], Pooled variance (S^2)*=127.80, Critical difference (p=0.05)# LSD=21.28, Ranked means** (D, DA) (A)
	24	F1=11.45 [df=(2, 8)], F2=0.38 [df=(4, 8)], Pooled variance (S^2)*=208.26, Critical difference (p=0.05)# LSD=27.17, Ranked means** (D, DA) (A)
In vivo	2	F1=108.91 [df=(2, 8)], F2=0.438 [df=(4, 8)], Pooled variance (S^2)*=0.906, Critical difference (p=0.05)# LSD=1.792, Ranked means** (D) (DA) (A)
	24	F1=80.96 [df=(2, 8)], F2=0.859 [df=(4, 8)], Pooled variance (S^2)*=17.37, Critical difference (p=0.05)# LSD=7.84, Ranked means** (D) (DA, A)

Theoretical values of F: p=0.05 level F1=4.46 [df=(2,8)], F2=3.84 [df=(4,8)] P=0.01 level F1=8.65 [df=(2,8)], F2=7.01 [df=(4,8)], F1 and F2 corresponding to variance ratio between groups and within groups respectively; * Error mean square, # Critical difference according to least significant procedure (LSD)[29, 30] **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

Table 4: ANOVA & Multiple comparison for changes of NO content

Type of model	Time of incubation (hrs)	Analysis of variance and multiple comparison
In vitro	2	F1=12.06 [df=(2, 8)], F2=0.25 [df=(4, 8)], Pooled variance (S^2)*=254.21, Critical difference (p=0.05)# LSD=30.02, Ranked means** (D, DA) (A)
	24	F1=13.03 [df=(2, 8)], F2=1.92 [df=(4, 8)], Pooled variance (S^2)*=270.79, Critical difference (p=0.05)# LSD=30.98, Ranked means** (D, DA) (A)
In vivo	2	F1=447.62 [df=(2, 8)], F2=2.39 [df=(4, 8)], Pooled variance (S^2)*=2.832, Critical difference (p=0.05)# LSD=3.169, Ranked means** (D) (DA) (A)
	24	F1=88.77 [df=(2, 8)], F2=0.698 [df=(4, 8)], Pooled variance (S^2)*=10.27, Critical difference (p=0.05)# LSD=6.035, Ranked means** (D) (DA, A)

Theoretical values of F: p=0.05 level F1=4.46 [df=(2,8)], F2=3.84 [df=(4,8)] P=0.01 level F1=8.65 [df=(2,8)], F2=7.01 [df=(4,8)], F1 and F2 corresponding to variance ratio between groups and within groups respectively; * Error mean square, # Critical difference according to least significant procedure (LSD)[29, 30] **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

Table 5: ANOVA & Multiple comparison for changes of total cholesterol content

Type of model	Time of incubation (hrs)	Analysis of variance and multiple comparison
In vivo	2	F1=30.44 [df=(2, 8)], F2=6.84 [df=(4, 8)], Pooled variance (S^2)*=86.19, Critical difference (p=0.05)# LSD=17.48, Ranked means** (D) (DA, A)
	24	F1=37.58 [df=(2, 8)], F2=1.28 [df=(4, 8)], Pooled variance (S^2)*=31.11, Critical difference (p=0.05)# LSD=10.50, Ranked means** (D) (DA) (A)

Theoretical values of F: p=0.05 level F1=4.46 [df=(2,8)], F2=3.84 [df=(4,8)] P=0.01 level F1=8.65 [df=(2,8)], F2=7.01 [df=(4,8)], F1 and F2 corresponding to variance ratio between groups and within groups respectively; * Error mean square, # Critical difference according to least significant procedure (LSD)[29, 30] **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

Table 6: ANOVA & Multiple comparison for changes of HDL-cholesterol content

Type of model	Time of incubation (hrs)	Analysis of variance and multiple comparison
In vivo	2	F1=93.53 [df=(2, 8)], F2=4.56 [df=(4, 8)], Pooled variance (S^2)*=8.425, Critical difference (p=0.05)# LSD=5.46, Ranked means** (D) (DA) (A)
	24	F1=116.01 [df=(2, 8)], F2=7.88 [df=(4, 8)], Pooled variance (S^2)*=3.903, Critical difference (p=0.05)# LSD=3.72, Ranked means** (D) (DA) (A)

Theoretical values of F: p=0.05 level F1=4.46 [df=(2,8)], F2=3.84 [df=(4,8)] P=0.01 level F1=8.65 [df=(2,8)], F2=7.01 [df=(4,8)], F1 and F2 corresponding to variance ratio between groups and within groups respectively; * Error mean square, # Critical difference according to least significant procedure (LSD)[29, 30] **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

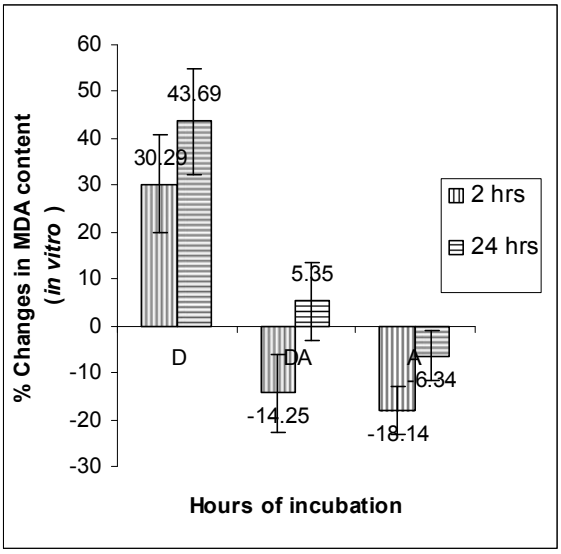

Figure 1: Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: Changes in MDA profile (*In vitro*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples

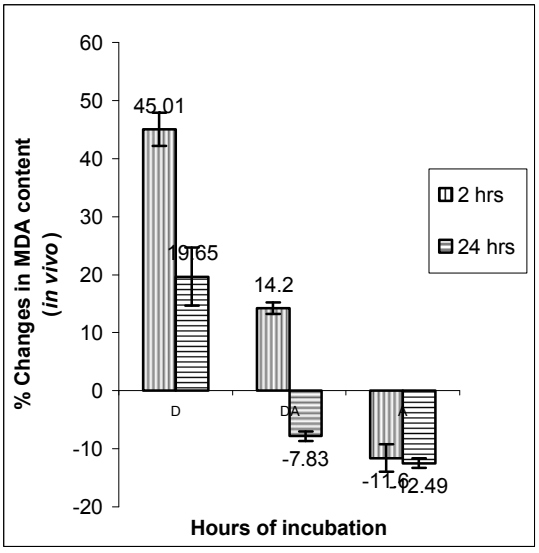

Figure 2: Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: Changes in MDA profile (*In vivo*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples

Table7: Correlation between % Changes of MDA / 4-HNE with TC and GSH / NO with HDL

Time of incubation (hrs)	Correlation Parameters	R	S.E.M	F	Equation
2	MDA-TC (D)	**0.89**	3.335	**11.58** (df=1,4)	MDA= -0.2627TC + 59.36
	MDA-TC (DA)	0.48	2.295	0.883 (df=1,4)	MDA= -0.0744TC + 15.92
	MDA-TC (A)	**0.91**	2.49	**15.30** (df=1,4)	MDA= -0.542TC – 6.16
24	MDA-TC (D)	0.308	12.23	0.315 (df=1,4)	MDA= 0.457TC + 7.623
	MDA-TC (DA)	0.07	2.20	0.0147 (df=1,4)	MDA= 0.021TC – 8.03
	MDA-TC (A)	0.26	2.09	0.218 (df=1,4)	MDA= 0.209TC – 11.606
2	4-HNE-TC (D)	**0.769**	1.72	**4.36** (df=1,4)	4-HNE= -0.0828TC + 18.84
	4-HNE-TC (DA)	0.046	0.594	0.0066 (df=1,4)	4-HNE= -0.00167TC + 2.664
	4-HNE-TC (A)	0.159	1.23	0.0786 (df=1,4)	4-HNE= -0.0192TC – 5.215
24	4-HNE-TC (D)	**0.778**	2.49	**4.61** (df=1,4)	4-HNE= -0.3567TC + 18.72
	4-HNE-TC (DA)	0.168	2.03	0.0877 (df=1,4)	4-HNE= 0.0476TC – 6.19
	4-HNE-TC (A)	**0.728**	1.99	**3.39** (df=1,4)	4-HNE= 0.7848TC – 7.516
2	GSH-HDL (D)	0.454	0.643	0.779 (df=1,4)	GSH= -0.05589HDL-6.242
	GSH-HDL (DA)	0.435	0.437	0.702 (df=1,4)	GSH= -0.06225HDL-2.245
	GSH-HDL (A)	**0.898**	0.682	**12.57** (df=1,4)	GSH= 0.2658HDL + 2.04
24	GSH-HDL (D)	0.208	1.10	0.136 (df=1,4)	GSH= -0.0438HDL-5.135
	GSH-HDL (DA)	0.236	5.46	0.177 (df=1,4)	GSH= -0.495HDL + 21.09
	GSH-HDL (A)	**0.628**	4.87	1.960 (df=1,4)	GSH= -0.9239HDL +31.99
2	NO-HDL (D)	**0.842**	0.891	**7.31** (df=1,4)	NO= -0.2369HDL-23.94
	NO-HDL (DA)	0.169	0.443	0.0883 (df=1,4)	NO= -0.0223HDL-11.05
	NO-HDL (A)	**0.968**	1.35	1.43 (df=1,4)	NO= -0.1775HDL+11.54
24	NO-HDL (D)	**0.576**	2.13	1.488 (df=1,4)	NO= 0.28HDL-8.29
	NO-HDL (DA)	0.376	3.41	0.494 (df=1,4)	NO= -0.515HDL+8.51
	NO-HDL (A)	0.218	3.97	0.149 (df=1,4)	NO= 0.236HDL +11.17

D, DA, A indicate cyclophosphamide-treated, cyclophosphamide and reduced glutathione-treated, only reduced glutathione-treated respectively. MDA, GSH, 4-HNE, NO, TC, HDL indicate malondialdehyde, 4-hydroxy-2-nonenal, reduced glutathione, nitric oxide, total cholesterol, HDL-cholesterol respectively; R = correlation coefficients; SEM = standard error of estimate; F = Variance ratio (df = 1, 4). The significant values for R and F are in bold face.

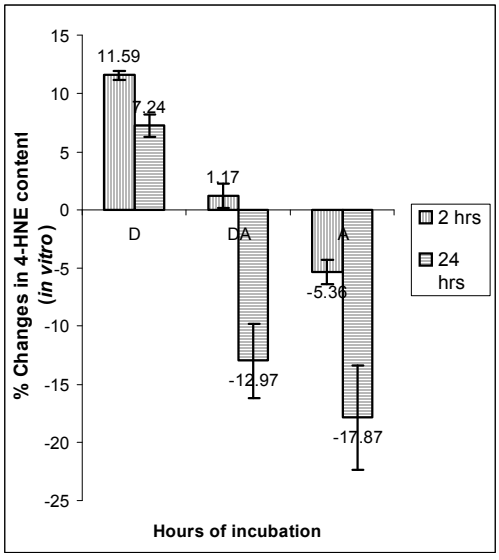

Figure 3: Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: Changes in 4-HNE profile (*In vitro*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated

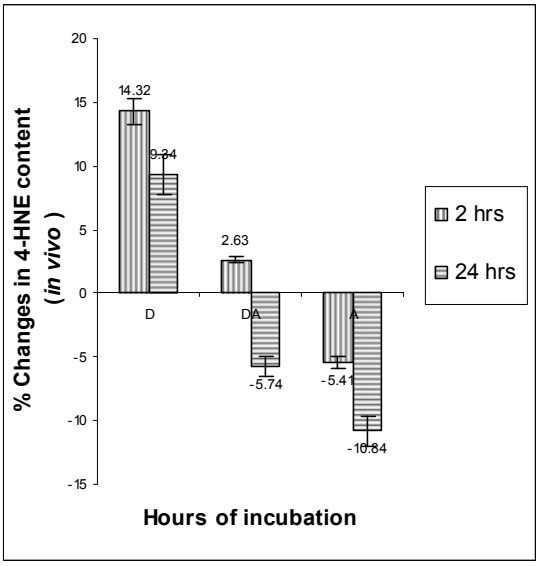

Figure 4: Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: Changes in 4-HNE profile (*In vivo*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples

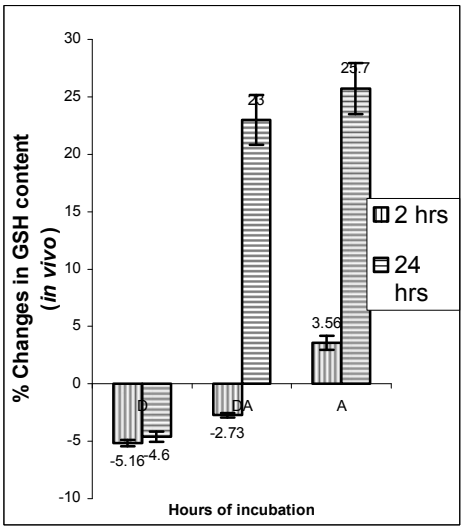

Figure 6: Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: Changes in GSH profile (*In vivo*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples

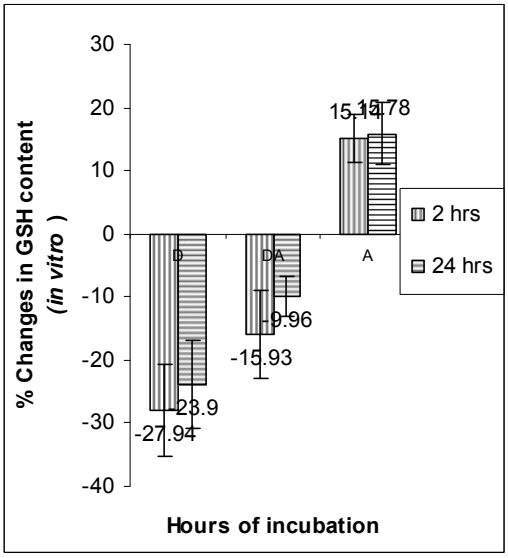

Figure 5: Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: Changes in GSH profile (*In vitro*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples

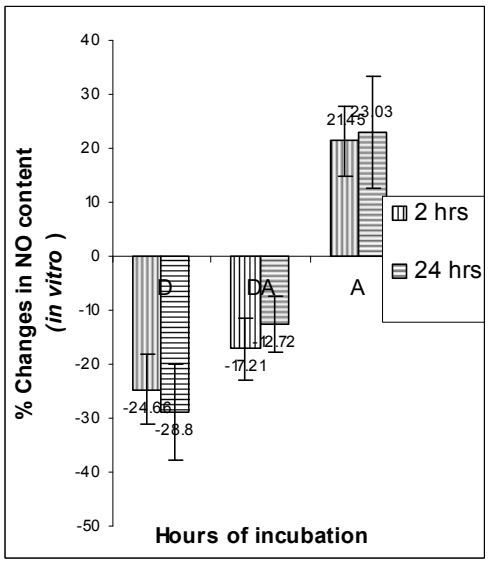

Figure 7: Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: Changes in NO profile (*In vitro*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples

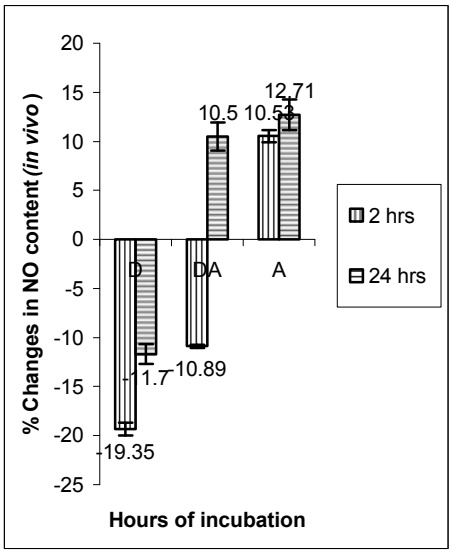

Figure 8: Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: Changes in NO profile (*In vivo*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione - treated and only reduced glutathione - treated samples

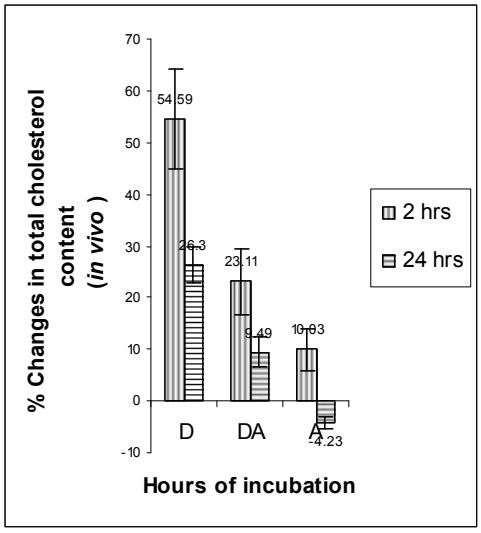

Figure 9: Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: Changes in total cholesterol profile (*In vivo*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione - treated and only reduced glutathione -treated samples

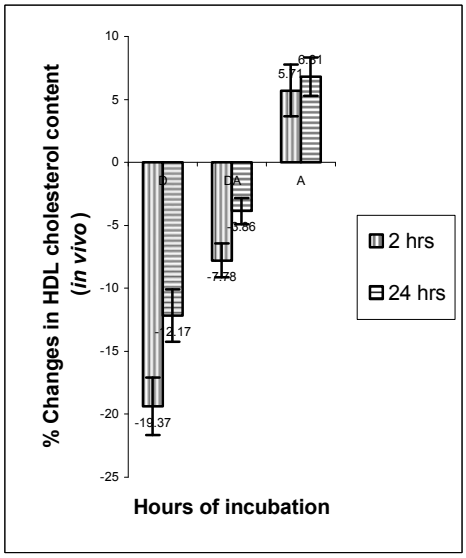

Figure 10: Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: Changes in HDL-cholesterol profile (*In vivo*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione - treated and only reduced glutathione -treated samples

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