



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

Supratim Ray^{*,a}, Bibhas Pandit^b, Sumit Das^b, Subrata Chakraborty^a

^aDivision of Pharmaceutical Chemistry, Dr. B C Roy College of Pharmacy and Allied Health Sciences, Bidhannagar, Durgapur, 713 206, India

^bDivision of Pharmaceutical Chemistry, Himalayan Pharmacy Institute, Majhitar, Rangpo, East Sikkim, 737 136, India

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 Zealand rabbit were used as lipid source for the models. Lipid peroxidation study was performed by measuring the malondialdehyde, 4-hydroxy-2-nonenal, reduced glutathione and nitric oxide content of tissue homogenates or 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. 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.

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

Received: July 29, 2010; **Accepted:** March 16, 2011

*Corresponding author: Supratim Ray
Dr. B C Roy College of Pharmacy and Allied Health Sciences,
Bidhannagar, Durgapur, India
Tel: +919434443946, Fax: +91343-2532679
E-mail: supratimray_in@yahoo.co.in

1. 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 $\omega 3$ and $\omega 6$ 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

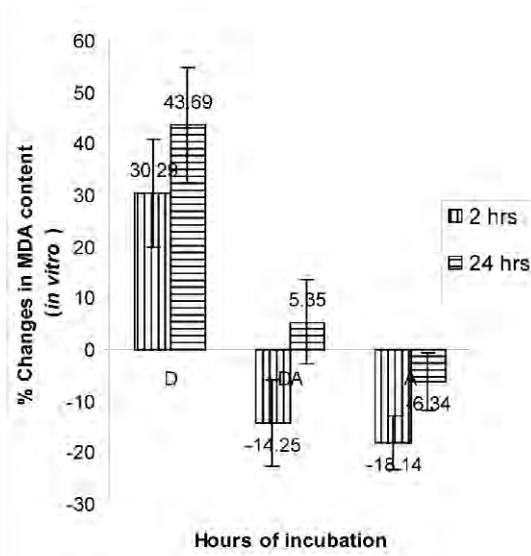


Figure 1. Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: changes in MDA profile in goat liver homogenates (*in vitro*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples.

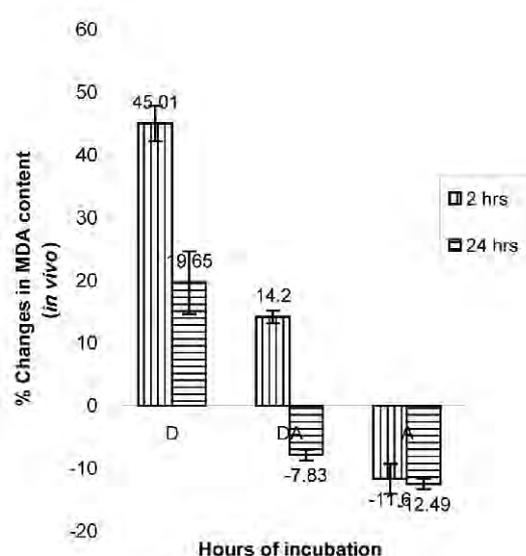


Figure 2. Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: changes in MDA profile in rabbit blood (*in vivo*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples.

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.

2. Materials and methods

2.1. Materials

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

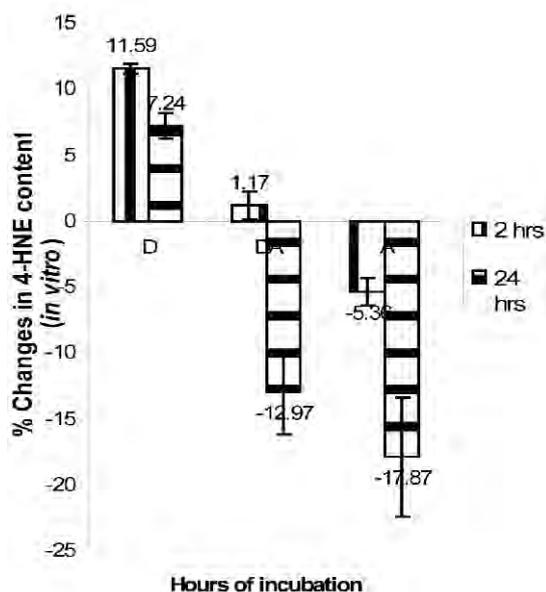


Figure 3. Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: changes in 4-HNE profile in goat liver homogenates (*in vitro*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione-treated samples.

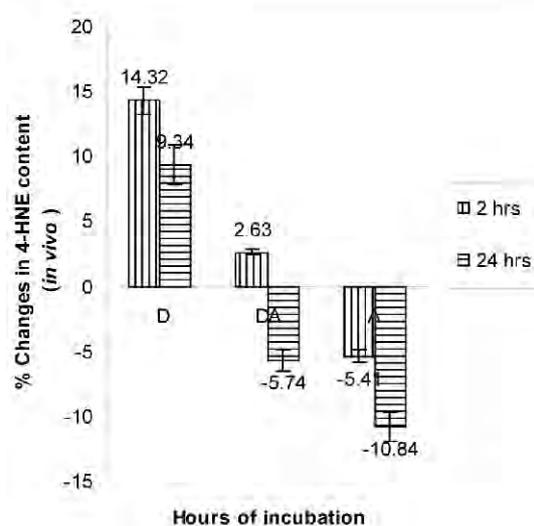


Figure 4. Effects of reduced glutathione on cyclophosphamide-induced lipid peroxidation: changes in 4-HNE profile in rabbit blood (*in vivo*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples.

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 from Dabur Research Foundation, Ghaziabad, India. Cholesterol test kit was from Span Diagnostic Ltd., Surat, India. All other reagents were of analytical grade.

2.2. 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 Zealand rabbit (*Oryctolagus cuniculus*) 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 °C) and acclimatized in the laboratory for at least 72 h. They were maintained on a standard laboratory diet and water *ad libitum*.

2.3. 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

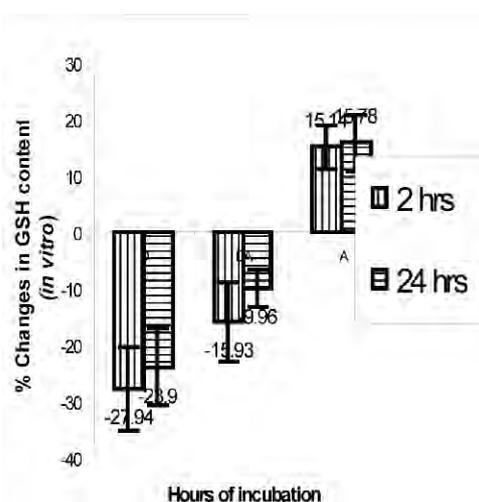


Figure 5. Effects cyclophosphamide on GSH content of goat liver homogenates (*in vitro*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples.

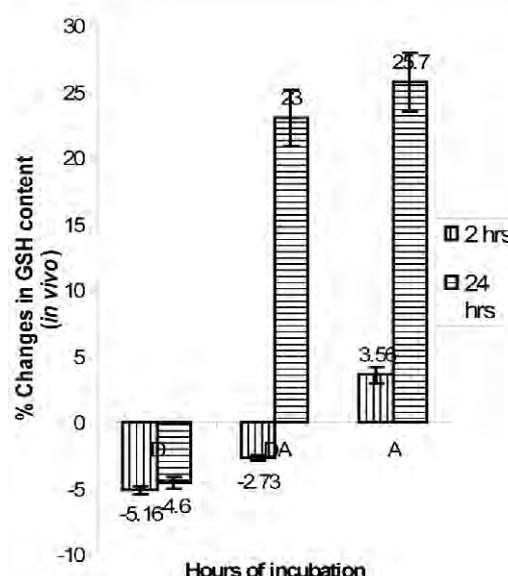


Figure 6. Effects cyclophosphamide on GSH level of rabbit blood (*in vivo*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples.

immediately grinded to make a tissue homogenate (1 g/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.

2.4. Incubation of tissue homogenate with drug and/or antioxidant 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 h and incubated at 18 ± 2 °C for

a period of maximum 24 h for further work.

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

Sixty rabbits were divided into five sets. 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.

2.6. 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

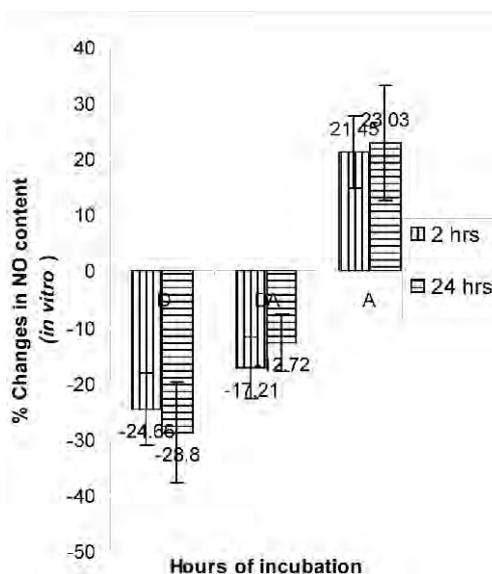


Figure 7. Effects of cyclophosphamide NO production in goat liver homogenates (*in vitro*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples.

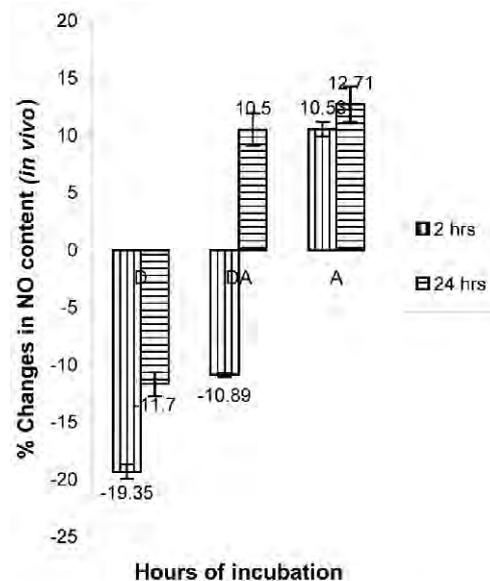


Figure 8. Effects of cyclophosphamide on NO production in rabbit blood (*in vivo*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples.

at 2 and 24 h of incubation and repeated for five times. In each case three samples of 2.5 ml of incubation mixture or 2.5 ml of blood withdrawn from ear vein (xylene treated) of rabbits were treated with 2.5 ml of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at room temperature at 3000 rpm for 30 min to precipitate protein. Then 2.5 ml of the supernatant was treated with 5 ml of 0.002 (M) TBA solutions and then volume was made up to 10 ml with distilled water. The mixture was heated on a boiling water bath for 30 min. Then tubes were cooled to a room temperature and the absorbance was measured at 530 nm against a TBA blank (prepared from 5 ml of TBA solution and 5 ml of distilled water) using Shimadzu UV-1700 double beam spectrophotometer. The concentrations of MDA were determined from standard curve, which was constructed as follows. Different aliquots from standard 1,1,3,3-tetrahydroxypropane (TEP) solution were taken in graduated stopper test tubes and volume of each solution was made up to 5 ml. To each solution, 5 ml of TBA solution was added and the mixture was heated in a steam bath for 30 min. The solutions were cooled to a room temperature and their absorbances were measured at 530 nm against TBA as blank. By plotting absorbance against concentrations a straight line passing through the origin of grid was obtained. The best-fit equation is $A=0.006502M$, where M =nanomoles of MDA, A =absorbance, $r=0.9977$, $SEM=0.0117$ and $F=2029.42$ ($df=1, 9$).

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

The estimation was done at 2 and 24 h of incubation and it was repeated for five times. In each case three samples of 2 ml of incubation mixture or 2 ml of blood withdrawn from ear vein (xylene treated) of rabbits were treated with 1.5 ml of 10% (w/v) TCA solution and centrifuged at 3000 rpm for

30 min. Then 2 ml of the filtrate was treated with 1 ml of 2, 4-dinitrophenyl hydrazine (DNPH) (100 mg/100 ml of 0.5 M HCl) and kept for 1 h at room temperature. After that, the samples were extracted with hexane, and the extract was evaporated to dryness under argon at 40 °C. After cooling to a room temperature, 2 ml of methanol was added to each sample and the absorbance was measured at 350 nm against methanol as blank [26]. The values were determined from the standard curve. The standard calibration curve was drawn based on the following procedure. A series of dilutions of 4-HNE in different concentrations of solvent (phosphate buffer) were prepared. From each solution 2 ml of sample pipette out and transferred into stopper glass tube. DNPH solution (1 ml) was added to all of the samples and kept at room temperature for 1 h. Each sample was extracted with 2 ml of hexane for three times. All extracts were collected in stopper test tubes. After that extract was evaporated to dryness under argon at 40 °C and the residue was reconstituted in 1 ml of methanol. The

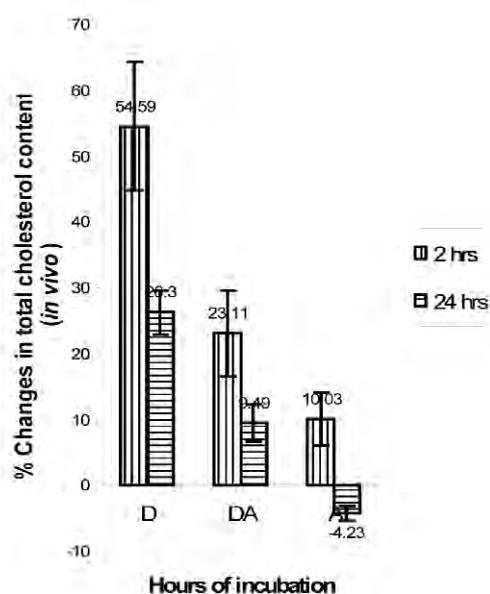


Figure 9. Effects of cyclophosphamide on total cholesterol in rabbit blood (*in vivo*) (n=5); D, DA & A indicate only cyclophosphamide-treated, cyclophosphamide & reduced glutathione -treated and only reduced glutathione -treated samples.

absorbance was measured at 350 nm using the 0 μ M standard as blank. 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.

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

Reduced glutathione (GSH) was measured in accordance with Ellman's method [25]. The estimation was done at 2 and 24 h of incubation and repeated for five times. In each case, three samples of 1 ml of incubation mixture or 1 ml of blood withdrawn from ear vein (xylene treated) of rabbits were treated with 1 ml of 5% (w/v) TCA in 1 mM EDTA centrifuged at 2000 g for 10 min. After that, 1 ml of the filtrate was mixed with 5 ml of 0.1 M phosphate buffer (pH=8.0) and 0.4 ml of 5,5'-dithiobis-2-nitrobenzoic acid (0.01% in phosphate buffer pH=8.0) (DTNB) was added to it. The absorbances of the solutions were measured at 412 nm against blank (prepared from 6.0 ml of phosphate buffer and 0.4 ml of DTNB). The concentrations of reduced glutathione were determined from standard curve, which was constructed as follows. Different aliquots of standard reduced glutathione stock solution were taken in 10 ml volumetric flasks. To each solution 0.4 ml of DTNB solution was added and volume was adjusted up to the mark with phosphate buffer (pH=8.0). The absorbance of each solution was measured at 412 nm against a blank containing 9.6 ml of phosphate buffer (pH=8.0) and 0.4 ml DTNB solution. By plotting absorbance against concentration a straight line passing through the origin of grid was obtained. The best-fit equation was $A=0.000495 M$, where M =nanomoles of reduced glutathione, A =absorbance, $r=0.9986$, SEM=0.0021 and $F=4405.78$ (df=1, 12).

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

The estimation was done at 2 and 24 h of incubation and it was repeated for five times. NO content was determined by reaction with Griess reagent. Griess reagent was prepared by mixing equal volumes of sulphanilamide (1% w/v in 3 N HCl) and (0.1% w/v N-naphthylethylenediamine dihydrochloride) [27]. In each case three samples of 4 ml of tissue homogenate or 4 ml of blood withdrawn from ear vein (xylene treated) of rabbits were treated with 2.5 ml of 10% (w/v) TCA solution and centrifuged at 3000 rpm for 30 min. Then 5 ml of the filtrate were treated with 0.5 ml Griess reagent. After 10 min. the absorbances of the solutions were measured at 540 nm against blank (prepared from 5.0 ml of distilled water and 0.5 ml of Griess reagent). The values were calculated from standard curve, which was constructed as follows. Different aliquots from standard sodium nitrite solution were taken in 5 ml volumetric flasks. To each solution 0.5 ml of Griess reagent was added and volume was adjusted up to the mark with phosphate buffer. The absorbances of each solution were noted at 540 nm against a blank containing the buffer and Griess reagent. By plotting absorbance against concentration a straight line passing through the origin was obtained. The best-fit equation is $A=0.015846 M$, where M =nanomoles of NO, A =absorbance, $r=0.9973$, SEM=0.0033 and $F=1960.35$ (df=1, 9).

2.10. Estimation of total cholesterol and HDL-cholesterol from rabbit blood

The estimation of cholesterol content was determined in one step method [28] with the help of cholesterol test kit. The kit contain three reagents such as, reagent 1 (cholesterol reagent), reagent 2 (working cholesterol standard, 200%) and reagent 3 (precipitating reagent). The estimation was done at 2 and 24 h of incubation and it was repeated for five times. In each case there were three samples.

After the specified h of incubation, 2 ml of blood was withdrawn from the ear vein of (xylene treated) rabbits. The blood samples were centrifuged at 2000 rpm for 15 min and the supernatant (plasma) was separated out. After that the following procedures were performed for the estimation of total cholesterol and high density lipoprotein cholesterol of the rabbit blood.

2.10.1. Total cholesterol

The standard solution was prepared by mixing 3 ml of reagent 1 and 0.015 ml of reagent 2 and the test sample was prepared by mixing 3 ml of reagent 1 and 0.015 ml of supernatant in cleaned glass test tubes. Then both the tubes were shaken well and the tubes were immediately kept in the boiling water bath exactly for 90 s. Then tubes were cooled immediately at room temperature under running tap water. The optical density (O.D.) of Standard (S) and Test (T) were measured on the spectrophotometer at 560 nm against reagent 1 as blank. The Total Cholesterol (TC) was calculated by using the following formula

$$\text{Total Cholesterol (mg/dl)} = (\text{O.D. of Test/O.D. of Standard}) \times 200$$

2.10.2. 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 was added. Then it was shaken well to mix properly and the tubes were kept at room temperature for 10 min. It was centrifuged at 2000 rpm for 15 min. to obtain a clear supernatant.

Step-II

HDL-cholesterol estimation: the test sample was prepared by mixing 3 ml of reagent 1 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 seconds.

The tubes were cooled immediately at room temperature under running tap water. The O.D. of Standard (S) and Test (T) were measured at 560 nm against reagent 1 as blank. The content of HDL- Cholesterol was calculated by using the following formula:

$$\text{HDL-Cholesterol (mg/dl)} = (\text{O.D. of Test/O.D. of Standard}) \times 50$$

2.11. Statistical analysis

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] was also performed on the percent changes data of various groups with respect to control group of corresponding time.

3. 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 and cholesterol peroxidation. Average of percent changes of five sets is shown in figures 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 are shown in Figures 1 to 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 are also shown in Figures 9 and 10.

The data generated from both *in vitro* and *in vivo* models indicate that incubation of the liver homogenates or rabbit blood with cyclophosphamide resulted an increase in MDA and 4-HNE content (Figures 1 to 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 and 4-HNE contents were significantly reduced with respect to cyclophosphamide-treated group when the liver tissue homogenates or rabbit blood were treated with cyclophosphamide in combination with reduced glutathione. This implies that the 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 or rabbit blood were treated only with the reduced glutathione then the MDA and 4-HNE level were reduced in comparison to the control and the cyclophosphamide-treated group. This decrease may be due to the free radical scavenging property of the antioxidants.

Figures 5 to 8 indicate that incubation of the liver tissue homogenates or rabbit blood with cyclophosphamide result in a decrease in GSH and NO content 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 or rabbit blood were treated with cyclophosphamide along with reduced glutathione then the GSH and NO levels were increased in comparison to cyclophosphamide-treated group of the corresponding time point. Again, when the liver tissue homogenates or rabbit blood were treated only with reduced glutathione the GSH and NO contents were also increased in comparison to the control samples. The increase in GSH and NO level suggest the antiperoxidative potential of reduced

glutathione.

It was observed from Figures 9 and 10 that rabbit treated with cyclophosphamide caused an increase in total cholesterol content with respect to corresponding control. But the HDL-cholesterol level 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/increase in total cholesterol / HDL-cholesterol content respectively in comparison to cyclophosphamide-treated group. Incubation of blood samples only with reduced glutathione also shows a tendency of decrease/increase in total cholesterol/HDL-cholesterol content, respectively in comparison to control and cyclophosphamide-treated group. 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 with respect to corresponding h. It is seen that there are 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 in both *in vitro* and *in vivo* models. In case of GSH/NO content of *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 of *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.

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 h) in cyclophosphamide-treated group which is statistically significantly different from the cyclophosphamide and reduced glutathione-treated group and only reduced glutathione-treated group.

4. 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 μ M 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 or 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 1 in supplementary material section) indicates that for cyclophosphamide-treated group MDA/4-HNE with total cholesterol have a correlation coefficient 0.89/0.769 at 2 h of incubation and 0.778/0.308 at 24 h 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 h 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 h 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.

5. 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.

Acknowledgements

This work was supported by All India Council for Technical Education (AICTE), New Delhi, for a financial assistance to one of us (SR) under research promotion scheme (RPS, F.No.: 8023/BOR/RPS-176/2006-07). The authors also wish to thank Dabur Research Foundation, Ghaziabad (U.P), for the free gift sample of cyclophosphamide.

References

- [1] Girotti AW. Lipid hydroperoxide generation, turnover and effector action in biological systems. *J Lipid Res* 1998; 39: 1529-42.
- [2] Cerruti PA. In: Colburn NH, Moses HL, Stanbridge EJ, (editors.). Growth Factors, Tumor Promoters and Cancer Genes. New York: *Alan R Liss Inc*, 1998; pp: 239-47.
- [3] Goidstein BD, Witz G, Amomso M, Troll W. Protease inhibitors antagonize the activation of polymorphonuclear leukocyte oxygen consumption. *Biochem Biophys Res Commun* 1979; 88: 854-60.
- [4] Parola M., Belloma G, Robino G, Barrera G, Dianzani, MU. 4-Hydroxynonenal as a biological signal: molecular basis and pathophysiological

- implications. *Antioxid Redox Signal* 1999; 1: 255-84.
- [5] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford: *Oxford University Press*, 1989; pp. 195-205.
- [6] Wagner BA, Buerner GR, Burns CP. Increased generation of lipid-derived and ascorbate free radicals by L1210 cells exposed to the ether lipid edelfosine. *Cancer Res* 1993; 53: 711-3.
- [7] Kampkotter A, Nkwonkam CG, Zurawski RF, Timpel C, Chovolou Y, Watjen W, Kahl R. Investigations of protective effects of the flavonoids quercetin and rutin on stress resistance in the model organism *Caenorhabditis elegans*. *Toxicology* 2007; 234: 113-23.
- [8] Ratty AK, Sunamoto J, Das NP. Interaction of flavonoids with 1, 1-diphenyl-2-picrylhydrazyl free radical, liposomal membranes and soybean lipoxygenase-1. *Biochem Pharmacol* 1988; 37: 989-95.
- [9] Halliwell B. Drug antioxidant effects-A basis for drug selection. *Drugs* 1991; 42: 569-605.
- [10] Luo X, Evrovsky Y, Cole D, Trines J, Benson LN, Lehotay DC. Doxorubicin- induced acute changes in cytotoxic aldehydes, antioxidant status and cardiac function in the rat. *Biochim Biophys Acta* 1997; 1360: 45-52.
- [11] Stankiewicz A, Skrzydlewska E, Makiela M. Effects of amifostine on liver oxidative stress caused by cyclophosphamide administration to rats. *Drug Metabol Drug Interact* 2002; 19: 67-82.
- [12] Premkumar K, Pachiappan A, Abraham SK, Santhiya ST, Gopinath PM, Ramesh A. Effect of *Spirulina fusiformis* on cyclophosphamide and mitomycin-C induced genotoxicity and oxidative stress in mice. *Fitoterapia* 2001; 72: 906-11.
- [13] Venkatesan N, Chandrakasan G. *in vivo* administration of taurine and niacin modulate cyclophosphamide-induced lung injury. *Eur J Pharmacol* 1994; 292: 75-80.
- [14] Sudharsan PT, Mythili Y, Selvakumar E, Varalakshmi P. Cardioprotective effect of pentacyclic triterpene, lupeol and its ester on cyclophosphamide- induced oxidative stress. *Hum Exp Toxicol* 2005; 24: 313-8.
- [15] Bhatia K, Ahmad F, Rashid H, Raisuddin S. Protective effect of S-allylcysteine against cyclophosphamide-induced bladder hemorrhagic cystitis in mice. *Food Chem Toxicol* 2008; 46: 3368-74.
- [16] Tripathi DN, Jena GB. Astaxanthin inhibits cytotoxic and genotoxic effects of cyclophosphamide in mice germ cells. *Toxicology* 2008; 248: 96-103.
- [17] Eliot RS. Stress and the heart. New York: *Futura Publishing Company*, 1974; pp. 41-63.
- [18] Lata H, Ahuja GK, Narang APS. Effect of starvation stress on lipid peroxidation and lipid profile in rabbits. *Indian J Physiol Pharmacol* 2002; 46: 371-4.
- [19] Ray S, Roy K, Sengupta C. Cisplatin-induced lipid peroxidation and its inhibition with ascorbic acid. *Indian J Pharm Sci* 2006; 68: 199-204.
- [20] Ray S, Roy K, Sengupta C. Evaluation of protective effects of water extract of *Spirulina platensis* (blue green algae) on cisplatin-induced lipid peroxidation. *Indian J Pharm Sci* 2007; 69: 378-83.
- [21] Ray S, Sengupta C, Roy K. *in vitro* evaluation of protective effects of ascorbic acid and water extract of *Spirulina platensis* (blue green algae) on 5-fluorouracil-induced lipid peroxidation. *Acta Pol Pharm-Drug Res* 2007; 64: 335-44.
- [22] Ray S, Roy K, Sengupta C. Exploring the protective effect of ascorbic acid and water extract of *Spirulina platensis* (blue green algae) on methotrexate-induced lipid peroxidation. *Iranian J Pharm Sci* 2007; 3: 217-28.
- [23] Hilditch TP, Williams PN. The chemical constituents of fats. London: *Chapman & Hall*, 1964; pp. 100-33.
- [24] Ohkawa H, Ohishi N, Yagi K. Assay of lipid peroxidation in animal tissue by thiobarbituric acid reaction. *Anal Biochem* 1979; 95: 351-8.
- [25] Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 1959; 82: 70-7.
- [26] Kinter M. Quantitative analysis of 4-hydroxy-2-nonenal. In: Punched NA, Kelly GJ, (editors). Free radicals- a practical approach. Oxford: *Oxford University Press*, 1996; pp. 130-6.
- [27] Sastry KVH, Moudgal RP, Mohan J, Tyagi JS, Rao GS. Spectrophotometric determination of serum nitrite and nitrate by copper-cadmium alloy. *Anal Biochem* 2002; 306: 79-82.
- [28] Wybenga DR, Pileqqi VJ, Dirstine PH, Di Giorquio J. Direct manual determination of serum total cholesterol with a single stable reagent. *Clin Chem* 1970; 16: 980-4.
- [29] Snedecor GW, Cochran WG. Statistical methods. New Delhi: Oxford & IBH Publishing Co Pvt Ltd, 1967; pp. 375-85.
- [30] Bolton S. Statistics. In: Gennaro AR, (editor). Remington: The science and practice of pharmacy. Philadelphia: *Lippincott Williams & Wilkins*, 2001; pp. 124-58.
- [31] Naito Y, Yoshikawa T, Yoshida N, Kondo M. Role of oxygen radical and lipid peroxidation in indomethacin-induced gastric mucosal injury. *Dig Dis Sci* 1998; 43: 30-4S.
- [32] Yahya MD, Pinnsa JL, Meinke GC, Lung CC.

- Antibodies against malondialdehyde (MDA) in MLR/lpr/lpr mice: evidence for an autoimmune mechanism involving lipid peroxidation. *J Autoimmunity* 1996; 9: 3-9.
- [33] Winrow VR, Winyard PG, Morris CJ, Black DR. Free radicals in inflammation: second messengers and mediators of tissue destruction. *Br Med Bull* 1993; 49: 506-22.
- [34] Esterbauer H, Benedetti A, Lang J, Fulceri R, Fauler G, Comporti M. Studies on the mechanism of formation of 4-hydroxynonenal during microsomal lipid peroxidation. *Biochim Biophys Acta* 1986; 876: 154-66.
- [35] Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 1991; 11: 81-128.
- [36] Uchida K, Szweda LI, Chae HZ, Stadtman ER. Immunochemical detection of 4-hydroxynonenal protein adducts in oxidized hepatocytes. *Proc Natl Acad Sci USA* 1993; 90: 8742-6.
- [37] Ethen CM, Reilly C, Fenq X, Olsen TW, Ferrington DA. Age-related macular degeneration and retinal protein modification by 4-hydroxy-2-nonenal. *Invest Ophthalmol Vis Sci* 2007; 48: 3469-79.
- [38] Sen CK. Nutritional biochemistry of cellular glutathione. *J Nutr Biochem* 1997; 8: 660-72.
- [39] Kosower EM, Kosower NS. Glutathione metabolism and function. New York: Raven Press, 1976; pp. 133-9.
- [40] Nathan C. Nitric oxide as a secretory product of mammalian cells. *Faseb J* 1992; 6: 3051-64.
- [41] Waldman SA, Murad F. Biochemical mechanisms underlying vascular smooth muscle relaxation: the guanylate cyclase-cyclic GMP system. *J Cardiovasc Pharmacol* 1988; 12: S115-8.
- [42] Ignarro LJ, Byrns RE, Buga GM, Wood KS. Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. *Circ Res* 1987; 61: 866-79.
- [43] Cohen GA, Hobbs AJ, Flich RM, Zinner MJ, Chaudhuri G, Ignarro LJ. Nitric oxide regulates endothelium-dependent vasodilator responses in rabbit hindquarters vascular bed *in vivo*. *Am J Physiol Heart Circ Physiol* 1996; 271: H133-9.
- [44] Moncada S, Higgs A. The L-arginine-nitric oxide pathway. *N Engl J Med* 1993; 329: 2002-12.
- [45] Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci USA* 1990; 87: 1620-4.
- [46] Hogg N, kalyanaraman B, Joseph J, Struck A, Parthasarathy S. Inhibition of low density lipoprotein oxidation by nitric oxide: potential role in atherogenesis. *FEBS Lett* 1993; 334: 170-4.
- [47] Rubho H, Parthasarathy S, Barnes S, Kirk M, kalyanaraman B, Freeman BA. Nitric oxide inhibition of lipoxygenase dependent liposomes and low-density lipoprotein oxidation: Termination of radical chain propagation reactions and formation of nitrogen containing oxidized lipid derivatives. *Arch Biochem Biophys* 1995; 324: 15-25.
- [48] Yamanaka N, Oda O, Nagao S. Nitric oxide released from zwitterionic polyamine/NO adducts inhibit Cu²⁺ -induced low-density lipoprotein oxidation. *FEBS Lett* 1996; 398: 53-60.
- [49] Odonnel VB, Chumley PH, Hogg N, Bloodsworth A, Freeman BA. Nitric oxide inhibition of lipid peroxidation: kinetics of reaction with lipid peroxy radicals and comparison with alpha-tocopherol. *Biochemistry* 1997; 36: 15216-23.
- [50] Kelly EE, Wagner BA, Buettner GR, Patrick Burns C. Nitric oxide inhibits iron-induced lipid peroxidation in HL-60 cells. *Arch Biochem Biophys* 1999; 370: 97-104.
- [51] Yalcin AS, Sabuncu N, Kilinc A. Increased plasma and erythrocyte lipid peroxidation in hyperlipidemic individuals. *Atherosclerosis* 1989; 80: 169-70.
- [52] Mehmetcik G, Toker G, Uysal M. Endogenous and copper-induced lipid peroxidation and antioxidant activity of serum in hypercholesterolemic subjects. *Horm Metab Res* 1997; 29: 63-5.
- [53] Anderson JW, Gowri MS, Turner J. Antioxidant supplementation effects on low-density lipoprotein oxidation for individuals with type 2 diabetes mellitus. *J Am Coll Nutr* 1999; 18: 451-61.
- [54] Kurowska EM, Spence JD, Jordan J, Wetmore S, Freeman DJ, Piche LA, Serratore P. HDL-cholesterol-raising effect of orange juice in subjects with hypercholesterolemia. *Am J Clin Nutr* 2000; 72: 1095-1100.
- [55] Owu DU, Antai AB, Udofia KH, Obembe AO, Obasi KO, Eteng MU. Vitamin C improves basal metabolic rate and lipid profile in alloxan-induced diabetes mellitus in rats. *J Biosci* 2006; 31: 575-9.
- [56] Das S, Snehlata, Srivastava LM. Effect of ascorbic acid on lipid profile and lipid peroxidation in hypercholesterolemic rabbits. *Nutr Res* 1997; 17: 231-41.
- [57] Prasad K, Karla J. Oxygen free radicals and hypercholesterolemic atherosclerosis: effect of vitamin E. *Am Heart J* 1993; 125: 958-73.

