FABAD J. Pharm. Sci., 33, 1–10, 2008

RESEARCH ARTICLE

Evaluation of Protective Effect of Reduced Glutathione on Flutamide-Induced Lipid Peroxidation and Changes in Cholesterol Content Using Common Laboratory Markers

Sarbani Dey RAY*, Partha CHOWDHURY**, Supratim RAY*°

Evaluation of Protective Effect of Reduced Glutathione on Flutamide-Induced Lipid Peroxidation and Changes in Cholesterol Content Using Common Laboratory Markers

Summary

This study was designed with an aim to evaluate the protective effects of reduced glutathione on flutamide-induced lipid peroxidation and also changes in cholesterol content. Blood of male white New Zealand rabbit was 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 rabbit blood. An attempt was also made to evaluate the effect of reduced glutathione on flutamide-induced changes in cholesterol content in 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 flutamide and the antiperoxidative potential of reduced glutathione on flutamide-induced lipid peroxidation. It was also observed that reduced glutathione had protective effect on flutamide-induced changes in cholesterol content.

Key Words: *Malondialdehyde, reduced glutathione, 4-hydroxy-2-nonenal, nitric oxide, cholesterol.*

Flutamid ile indüklenen lipit peroksidasyonuna ve kolesterol içeriğinin değişimine indirgenmiş glutatyonun koruyucu etkisinin değerlendirilmesi

Özet

Bu çalışma flutamit ile indüklenen lipit peroksidasyonuna ve kolesterol içeriğinin değişimine indirgenmiş glutatyonun koruyucu etkinliğini değerlendirmeyi amaçlamaktadır. Modeller için beyaz erkek Yeni Zellanda tavşanı lipit kaynağı olarak kullanılmıştır. Lipit peroksidasyon çalışması tavşan kanında malondialdehit, 4-hidroksi-2-nonenal, indirgenmiş glutatyon ve nitrik oksit içeriklerinin ölçülmesi şeklinde gerçekleştirilmiştir. Ayrıca indirgenmiş glutatyonun tavşan kanında kolesterol içeriğine etkisi de değerlendirilmiştir. Kolesterol profilinde ise yine tavşan yanında total kolesterol ve yüksek dansiteli lipoprotein miktarları tanımlanmıştır. Bu çalışma elde edilen sonuçlar flutamitin lipit peroksidasyonunu artırıcı potansiyeli ve indirgenmiş glutatyonunun flutamit ile indüklenen lipit peroksidasyonundaki antiperoksidatif etkisini göstermektedir. Ayrıca, indirgenmiş glutatyonun flutamit ile indüklenen kolesterol içeriğinin değişiminde de koruyucu etkinliğinin olduğu gözlenmiştir.

Anahtar kelimeler: *Malondialdehit, indirgenmiş glutatyon, 4-hidroksi-2-nonenal, nitrik oksit, kolesterol*

Received: 14.07.2009
Revised: 29.07.2009
Accepted: 13.10.2009

INTRODUCTION

Lipid peroxidation is a free radical related process that may occur in the biological system under enzymatic control or nonenzymatically (1-3). The latter form is mostly associated with cellular damage as a result of oxidative stress (4). Reactive oxygen species and other pro-oxidants cause the decomposition of w_3 and w_6 polyunsaturated fatty acids of membrane phospholipids leading to the formation of aldehydic end products including malondialdehyde (MDA), 4-hydroxy-2-nonenals (4-HNE) 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

* Division of Pharmaceutical Chemistry, Dr. B C Roy College of Pharmacy & Allied Health Sciences, Dr. Meghnad Saha Sarani, Bidhannagar, Durgapur, 713 206, India
** Division of Pharmaceutical Chemistry, Himalayan Pharmacy Institute, Majhitar, Rangpo, East Sikkim, 737 136, India.
° Corresponding author e-mail: supratimray_in@yahoo.co.in

membrane (5). Free radical mediated oxidative stress results usually from deficient natural antioxidant defense. 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 (6). Lipid peroxidation induction capacity of drugs may be related to their toxic potential as exemplified by adriamycin-induced cardiotoxicity, which occurred through free radical mediated process (7). Thus, 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.

Flutamide, a phototoxic anticancer drug acts as an androgen receptor antagonist and used mainly as an anticancer drug in certain type of prostrate cancer. It specifically inhibits androgen uptake and / or nuclear binding of androgen in target tissues. When used as monotherapy, it causes a gradual increase in plasma testosterone due to the blockage of feedback inhibition of the hypothalamus and pituitary by testosterone. On the other hand, it shows a photo hemolytic effect on human erythrocytes, and photo induces lipid peroxidation (8).

The protective effect of various antioxidants on anticancer drug-induced lipid peroxidation had been reported earlier by us (9-12). In continuation of ongoing search for antioxidants, the present work has been carried out *in vivo* to evaluate the antiperoxidative potential of reduced glutathione on flutamide-induced lipid peroxidation and also to evaluate the effect of reduced glutathione on flutamide-induced changes in cholesterol content in rabbit blood sample.

EXPERIMENTAL

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 4-HNE was purchased from ICN Biomedicals INC., Ohio;. Flutamide tablet (Cytomid-250) was purchased from Cipla Ltd. Mumbai, India. Reduced glutathione was purchased from S.D. Fine Chem.Ltd., Mumbai, India. Cholesterol test kit was from Span Diagnostic Ltd., Surat, India. All other reagents were of analytical grade.

Animal Experiments

The *in vivo* experiments were carried out using male white New Zealand rabbit (*Oryctolagus caniculus*) as experimental model. The 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 consisting of several ingredients such as barley, wheat and rice bran, soya-bean and mustard meal, several minerals like sodium, potassium etc. and water at *ad libitum*; but no extra cholesterol was added to the diet.

Methods

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. The first group (C) was the control group (not treated with flutamide and / or reduced glutathione), while the

second group (D) was treated with drug (flutamide orally at a dose of 12.5 mg / Kg-body weight). The third group (DA) was treated both with drug and antioxidant (flutamide orally at a dose of 12.5 mg / Kg-body weight and reduced glutathione orally at a dose of 50 mg / Kg-body weight. The final group (A) received only reduced glutathione orally at a dose of 50 mg / Kg-body weight). After the administration of flutamide and / or reduced glutathione, rabbits were kept for 5 h of incubation.

Estimation of malondialdehyde (MDA) level from rabbit blood

The extent of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using thiobarbuturic acid (TBA) method (13). 2.5 mL of blood sample was withdrawn from ear vein (xylene treated) of each rabbit and treated with 2.5 mL of 10% (w/v) trichloroacetic acid (TCA) then centrifuged at room temperature at 3000 rpm for 30 min to precipitate protein. 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 Shimadju 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 stoppered 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 room temperature and their absorbances were measured at 530 nm against TBA as blank. By plotting absorbances against concentrations, a straight line passing through the origin of the grid was obtained. The best-fit equation is A = 0.005631M, where M = nanomoles of MDA, A = absorbance, r = 0.991, SEM = 0.0289 and F = 490.83 (df = 1, 9).

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

2 mL of blood sample was withdrawn from ear vein (xylene treated) of each rabbit and treated with 1.5 mL of 10% (w/v) TCA solution then centrifuged at 3000 rpm for 30 min. 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 40^0 C. After cooling to room temperature, 2 mL of methanol was added to each sample and the absorbance was measured at 350 nm against methanol as blank (14). 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 stoppered glass tube. 1 mL of DNPH solution was added to all 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 stoppered test tubes. After that extract was evaporated to dryness under argon at 40^0C and the residue was dissolved in 1 mL of methanol. The absorbance was measured at 350 nm using the 0 mM standard as blank. The best-fit equation is: Nanomoles of 4-HNE = (A_{350} - 0.005603185) / 0.003262215, where A_{350} = absorbance at 350nm, r = 0.999, SEM = 0.007.

Estimation of reduced glutathione (GSH) level from rabbit blood

Reduced glutathione (GSH) was measured in accordance with Ellman's method (15). 1 mL of blood sample was withdrawn from ear vein (xylene treated) of each rabbit and treated with 1 mL of 5% (w/v) TCA in 1 mM EDTA then centrifuged at 2000 g for 10 min. After that 1 mL of the filtrate was mixed with 5 mL of 0.1M 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 = 0.000531M, where M = nanomoles of reduced glutathione, A = absorbance, r = 0.991, SEM = 0.0059 and F = 574.07 (df = 1, 10).

Estimation of nitric oxide (NO) level from 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) (16). 4 mL of blood sample was withdrawn from ear vein (xylene treated) of each rabbit and treated with 2.5 mL of 10% (w/v) TCA solution then centrifuged at 3000 rpm for 30 min. Then 5 mL of the filtrate was 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 the 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 = 0.014061M, where M = nanomoles of NO, A = absorbance, r = 0.9994, SEM = 0.0029 and F=8122.28 (df = 1, 9).

Estimation of total cholesterol and HDL-cholesterol from rabbit blood

The estimation of cholesterol content was determined in one step method (17) with the help of cholesterol test kit. The kit contains three reagents such as, reagent 1 (cholesterol reagent), reagent 2 (working cholesterol standard, 200%) and reagent 3 (precipitating reagent). 2 mL of blood sample was withdrawn from the ear vein of (xylene treated) each rabbit and centrifuged at 2000 rpm for 15 min to enable the separation of the supernatant (plasma). After that the following procedures were performed for the estimation of total cholesterol and high density lipoprotein cholesterol of the rabbit blood.

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 tubes were shaken well, then immediately emerged into the boiling water bath and kept there exactly for 90 sec. Then the tubes were cooled immediately at room temperature under running tap water. The optical density (O.D.) of Standard (S) & 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

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 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 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

Interpretation of the result is supported by student "*t*" test. Analysis of variance (ANOVA) and multiple comparison analysis using least significant different procedure (18, 19) were also performed on the percent changes data of various groups such as flutamide-treated (D), flutamide and reduced glutathione-treated (DA) and only reduced glutathione-treated (A) with respect to the control group of the corresponding time.

Results and Discussion

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 group of the corresponding time of incubation and was considered as the indicator of the extent of lipid and cholesterol peroxidation. The results of the studies on flutamide-induced lipid peroxidation and its inhibition with reduced glutathione were shown in Tables 1-4. The results of the studies on flutamide-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 listed in Tables 5-6.

From Table 1-2, it was evident that rabbits treated with flutamide showed an increase in MDA (41.47 %) and 4-HNE (7.40%) content in blood samples in comparison tohose of the control group to a significant extent. The observations suggest that flutamide could significantly induce the lipid peroxidation process. Increase in the accumulation of MDA in cells can result in cellular degradation, some biochemical changes and even cell death (20). 4-Hydroxy-2-nonenal (4-HNE), a lipid aldehydes that form due to lipid peroxidation occurring during the 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[21]. But the MDA (34.39%) and 4-HNE (-5.77%) contents were significantly reduced in

Table 1. Effects of reduced glutathione on flutamide-induced lipid peroxidation: changes in MDA profile

Animal sets	% Changes in MDA content (with respect to corresponding control) due to treatment with flutamide and or reduced glutathione			Analysis of variance and multiple comparison
	Samples			
	D	DA	A	
An 1	39.04[a]	30.77[a]	-7.44[a]	F1=1018.34 [df=(2,8)]
An 2	43.43[a]	33.36[a]	-7.59[b]	F2=2.83 [df=(4,8)]
An 3	43.15[a]	35.44[b]	-4.59[b]	Φ =26.04
An 4	37.88[a]	36.42[a]	-7.59[a]	Pooled variance (S^2)*=3.274
An 5	43.84[a]	36.00[a]	-4.66[b]	Critical difference (p=0.05)[#] LSD =3.407
Av. (±SEM)	**41.47** (±1.25)	**34.39** (±1.05)	**-6.37** (±0.71)	Ranked means[**] (D) (DA) (A)

Percent changes with respect to control groups of corresponding hours are shown. D, DA & A indicate only flutamide-treated, flutamide & reduced glutathione-treated and only reduced glutathione-treated samples respectively; Av. = Averages of five sets of animal; SEM = Standard error of mean (df=4); Significant of '*t*' values of the changes of MDA content (df=2) are shown as: a>99%; b=97.5-99%; 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; Φ = Power of ANOVA at p=0.05 level * Error mean square, # Critical difference according to least significant procedure[18, 19] **Two means not included within the same paranthesis are statistically significantly different at p=0.05 level.

Table 2. Effects of reduced glutathione on flutamide-induced lipid peroxidation: changes in 4-HNE profile

Animal sets	% Changes in 4-HNE content (with respect to corresponding control) due to treatment with flutamide and or reduced glutathione			Analysis of variance and multiple comparison
	Samples			
	D	DA	A	
An 1	8.87[a]	-7.21[a]	-11.79[a]	F1=31.42 [df=(2,8)]
An 2	4.23[b]	-2.39[b]	-4.17[a]	F2=0.503 [df=(4,8)]
An 3	6.76[a]	-3.44[b]	-5.64[a]	Φ =14.34
An 4	11.67[a]	-9.63[a]	-11.26[a]	Pooled variance (S^2)*=11.502
An 5	5.48[a]	-6.19[a]	-9.63[a]	Critical difference (p=0.05)[#]
				LSD =6.38
Av.	**7.40**	**-5.77**	**-8.50**	Ranked means[**]
(±SEM)	(±1.31)	(±1.30)	(±1.53)	(D) (DA, A)

Percent changes with respect to control groups of corresponding hours are shown. D, DA & A indicate only flutamide-treated, flutamide & reduced glutathione-treated and only reduced glutathione-treated samples respectively; Av. = Averages of five animal sets; SEM = Standard error of mean (df=4); Significant of '*t*' values of the changes of 4-HNE content (df=2) are shown as: a>99%; b=97.5-99%; 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; Φ = Power of ANOVA at p=0.05 level * Error mean square, # Critical difference according to least significant procedure[18, 19] **Two means not included within the same paranthesis are statistically significantly different at p=0.05 level.

Table 3: Effects of reduced glutathione on flutamide-induced lipid peroxidation: changes in GSH profile

Animal sets	% Changes in GSH content (with respect to corresponding control) due to treatment with flutamide and or reduced glutathione			Analysis of variance and multiple comparison
	Samples			
	D	DA	A	
An 1	-4.96[a]	2.15[b]	7.36[a]	F1=157.61 [df=(2,8)]
An 2	-1.43[c]	3.56[a]	7.36[a]	F2=1.667 [df=(4,8)]
An 3	-2.86[b]	2.14[b]	7.38[a]	Φ =7.38
An 4	-2.58[a]	1.64[b]	5.87[a]	Pooled variance (S^2)*=0.7715
An 5	-3.29[a]	3.05[a]	6.10[a]	Critical difference (p=0.05)[#]
				LSD =1.653
Av.	**-3.02**	**2.51**	**6.81**	Ranked means[**]
(±SEM)	(±0.57)	(±0.35)	(±0.34)	(D) (DA) (A)

Percent changes with respect to control groups of corresponding hours are shown. D, DA & A indicate only flutamide-treated, flutamide & reduced glutathione-treated and only reduced glutathione-treated samples respectively; Av. = Averages of five animal sets; SEM = Standard error of mean (df=4); Significant of '*t*' values of the changes of GSH content (df=2) are shown as: a>99%; b=97.5-99%; c=95-97.5%; 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; Φ = Power of ANOVA at p=0.05 level * Error mean square, # Critical difference according to least significant procedure[18, 19] **Two means not included within the same paranthesis are statistically significantly different at p=0.05 level.

Table 4. Effects of reduced glutathione on flutamide-induced lipid peroxidation: changes in NO profile

Animal sets	% Changes in NO content (with respect to corresponding control) due to treatment with flutamide and or reduced glutathione			Analysis of variance and multiple comparison
	Samples			
	D	DA	A	
An 1	-7.45[a]	-3.51[b]	2.94[b]	F1=159.33 [df=(2,8)]
An 2	-4.58[a]	-2.34[b]	3.73[a]	F2=3.23 [df=(4,8)]
An 3	-3.97[b]	-3.11[a]	6.12[a]	Φ =74.64
An 4	-5.32[a]	-2.34[b]	4.47[a]	Pooled variance (S^2)*=0.8606
An 5	-7.50[a]	-3.80[a]	4.55[b]	Critical difference (p=0.05)[#]
				LSD =1.747
Av.	**-5.76**	**-3.02**	**4.36**	Ranked means[**]
(±SEM)	(±0.73)	(±0.29)	(±0.53)	(D) (DA) (A)

Percent changes with respect to control groups of corresponding hours are shown. D, DA & A indicate only flutamide-treated, flutamide & reduced glutathione-treated and only reduced glutathione-treated samples respectively; Av. = Averages of five animal sets; SEM = Standard error of mean (df=4); Significant of '*t*' values of the changes of NO content (df=2) are shown as: a>99%; b=97.5-99%; 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; Φ = Power of ANOVA at p=0.05 level * Error mean square, # Critical difference according to least significant procedure[18, 19] **Two means not included within the same paranthesis are statistically significantly different at p=0.05 level.

comparison to flutamide-treated group when the rabbits were treated with flutamide in combination with the reduced glutathione. Again the rabbits were treated only with the reduced glutathione then the MDA (-6.37%) and 4-HNE (-8.50%) levels were reduced in comparison to those of the control and the flutamide treated groups. This decrease may be due to the free radical scavenging property of the reduced glutathione. So the decrease in MDA and 4-HNE content of rabbit blood, when treated with flutamide and reduced glutathione as well as only with reduced glutathione implies the free radical scavenging property of reduced glutathione.

It was evident from Table 3-4, that rabbits treated with flutamide experienced a decrease in GSH (-3.02%) and NO (-5.76%) content in comparisonto the control group to a significant extent. The decrease in GSH and NO content was associated with an increase in lipid peroxidation. When the rabbits were treated both with flutamide and reduced glutathione, the GSH (2.50%) and NO (-3.02%) levels increased in comparison to the flutamide treated group of the corresponding time. The GSH (6.81%) and NO (4.36%) contents of the rabbits treated only with the reduced glutathione also increase in comparison to those of the control samples. The increase in GSH and NO level suggest the antiperoxidative potential of reduced glutathione. GSH is an important antioxidant and plays a very important role in the defense mechanism of tissues against the reactive oxygen species (22). 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 (23). So the increase in GSH and NO content of rabbit blood, when treated with flutamide and reduced glutathione as well as only with reduced glutathione implies the free radical scavenging activity of reduced glutathione.

The experimental data in Table 5-6 indicated that treatment of rabbit with flutamide caused an increase in the total cholesterol content (21.80%) in comparison tothe corresponding control group. But the HDL-cholesterol level (-20.50%) was reduced in comparison to the control group. These observations suggest that flutamide can change the cholesterol profile. It was further found that rabbits treated with flutamide and

Table 5. Effects of reduced glutathione on flutamide -induced changes in cholesterol content: changes in Total Cholesterol profile

Animal sets	% Changes in Total Cholesterol content (with respect to corresponding control) due to treatment with flutamide and or reduced glutathione			Analysis of variance and multiple comparison
	Samples			
	D	DA	A	
An 1	24.58[a]	11.48[a]	-4.93[a]	F1=33.03 [df=(2,8)]
An 2	25.49[a]	10.80[a]	-8.00[a]	F2=1.599 [df=(4,8)]
An 3	33.48[a]	12.68[a]	-13.27[a]	Φ =15.10
An 4	11.32[a]	2.68[c]	-6.11[a]	Pooled variance (S^2)*=33.61
An 5	14.12[a]	1.18[c]	-7.63[a]	Critical difference (p=0.05)[#]
				LSD =10.91
Av.	**21.80**	**7.76**	**-7.99**	Ranked means[**]
(±SEM)	(±4.04)	(±2.41)	(±1.43)	(D) (DA) (A)

Percent changes with respect to control groups of corresponding hours are shown. D, DA & A indicate only flutamide-treated, flutamide & reduced glutathione-treated and only reduced glutathione-treated samples respectively; Av. = Averages of five animal sets; SEM = Standard error of mean (df=4); Significant of '*t*' values of the changes of Total Cholesterol content (df=2) are shown as: a>99%; b=97.5-99%; c=95-97.5%; 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; Φ = Power of ANOVA at p=0.05 level * Error mean square, # Critical difference according to least significant procedure[18, 19] **Two means not included within the same paranthesis are statistically significantly different at p=0.05 level.

reduced glutathione produce a decrease / increase in total cholesterol / HDL-cholesterol content (7.76 / -8.87%) respectively in comparison to flutamide-treated group. Rabbits treated with reduced glutathione also show a tendency of decrease / increase in total cholesterol / HDL-cholesterol content (-7.99 / 10.53%) respectively in comparison tocontrol / flutamide-treated group. These results suggest that reduced glutathione could inhibit flutamide-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 the control group of corresponding hours. It is seen that there are significant differences among various groups (F1) such as flutamide-treated, flutamide and reduced glutathione-treated and only reduced glutathione-treated ones. But within a particular group, differences (F2) are insignificant, which shows that there is no statistical difference in animals in a particular group (Tables 1-6). The high *t* values deduced from paired sample design indicate less variability among samples in a particular group. The differences among objects within a treatment is a measure of the variability of the observation. If the F test is significant and more than two treatments are included in the experiments, it may not immediately be obvious which treatments are different. To solve the problem, multiple comparisons in ANOVA have been done. The tables also indicate that the level of MDA / GSH / NO / Total Cholesterol / HDL Cholesterol in flutamide-treated group, flutamide and reduced glutathione-treated group and only reduced glutathione-treated groups are statistically significantly different from each other. However, the 4-HNE content in flutamide-treated group is only statistically significantly different from the flutamide and reduced glutathione-treated group as well as only reduced glutathione-treated group. But there is no statistically significant difference among the flutamide and reduced glutathione-treated group and only reduced glutathione-treated group.

Table 6. Effects of reduced glutathione on flutamide -induced changes in cholesterol content: changes in HDL Cholesterol profile

Animal sets	% Changes in HDL Cholesterol content (with respect to corresponding control) due to treatment with flutamide and or reduced glutathione			Analysis of variance and multiple comparison
	Samples			
	D	DA	A	
An 1	-22.15[a]	-10.68[b]	7.20[a]	F1=144.77 [df=(2,8)]
An 2	-22.32[a]	-7.70[a]	9.31[a]	F2=3.68 [df=(4,8)]
An 3	-17.17[a]	-2.80[a]	11.28[a]	Φ =67.74
An 4	-21.20[a]	-17.33[a]	8.44[a]	Pooled variance (S^2)*=8.49
An 5	-19.66[a]	-5.82[a]	16.41[a]	Critical difference (p=0.05)[#]
				LSD =5.49
Av.	**-20.50**	**-8.87**	**10.53**	Ranked means[**]
(±SEM)	(±0.96)	(±2.47)	(±1.61)	(D) (DA) (A)

Percent changes with respect to control groups of corresponding hours are shown. D, DA & A indicate only flutamide-treated, flutamide & reduced glutathione-treated and only reduced glutathione-treated samples respectively; Av. = Averages of five animal sets; SEM = Standard error of mean (df=4); Significant of 't' values of the changes of HDL-Cholesterol content (df=2) are shown as: a>99%; 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; Φ = Power of ANOVA at p=0.05 level * Error mean square, # Critical difference according to least significant procedure18, 19 **Two means not included within the same paranthesis are statistically significantly different at p=0.05 level.

Conclusion

The finding from *in vivo* model indicates the lipid peroxidation induction potential of flutamide, which may be related to its toxic potential. The results also suggest the antiperoxidative effects of reduced glutathione and demonstrate its potential to reduce flutamide-induced lipid peroxidation and thus to increase therapeutic index of the drug by reducing toxicity that may be mediated through free radical mechanisms. In addition it is observed that flutamide 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.

ACKNOWLEDGEMENT

This work was supported in part 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).

REFERENCES

1. Dianzani MU. Free radical in physiology and pathology. *Boll Soc Ital Biol Sper* 68: 491-511, 1992.
2. Gutteridge JMC, Halliwell B. Free radicals and antioxidants in the year 2000. A historical look to the future. *Ann N Y Acad Sci* 899: 136-147, 2000.
3. Stohs SJ. The role of free radicals in toxicity and disease. *J Basic Clin Physiol Pharmacol* 6: 205-228, 1995.
4. Romero RJ, BoschMorell F, Romero MJ, Jareno EJ, Romero B, Marin N, Roma J. Lipid peroxidation products and antioxidants in human disease. *Environ Health Perspect* 106: 1229-1234, 1998.
5. Parola M, Belloma G, Robino G, Barrera G, Dianzani MU. 4-Hydroxynonenal as a biological signal: molecular basis and pathophysiological implications. *Antioxid Redox Signal* 1: 255-284, 1999.
6. Halliwell B. Drug antioxidant effects-A basis for drug selection. *Drugs* 42: 569-605, 1991.
7. 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* 1360: 45-52, 1997.

8. Vargas F, Rivas C, Méndez H, Fuentes A, Fraile G, Velásquez M. Photochemistry and phototoxicity studies of flutamide, a phototoxic anti-cancer drug. *J Photochem Photobiol_B* 58: 108-114, 2000.
9. Ray S, Roy K, Sengupta C. Cisplatin-induced lipid peroxidation and its inhibition with ascorbic acid. *Indian J Pharm Sci* 68: 199-204, 2006.
10. 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* 69: 378-383, 2007.
11. Ray S, Roy K, Sengupta C. *In vitro* evaluation of protective effects of ascorbic acid and water extract of *Spirulina plentesis* (blue green algae) on 5-fluorouracil-induced lipid peroxidation. *Acta Pol Pharm Drug Res* 64: 335-344, 2007.
12. 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* 3: 217-218, 2007.
13. Ohkawa H, Ohishi N, Yagi K. Assay of lipid peroxidation in animal tissue by thiobarbituric acid reaction. *Anal Biochem* 95: 351-358, 1979.
14. Kinter M. Quantitative analysis of 4-hydroxy-2-nonenal. In Punchard NA, Kelly GJ, editors. *Free Radicals- A Practical Approach*. Oxford: Oxford University Press; 1996. p. 130-36.
15. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys* 82: 70-77, 1959.
16. Sastry KVH, Moudgal RP, Mohan J, Tyagi JS, Rao GS. Spectrophotometric determination of serum nitrite and nitrate by copper-cadmium alloy. *Anal Biochem* 306: 79-82, 2002.
17. Wybenga DR, Pileqqi VJ, Dirstine PH, Di Giorquio J. Direct manual determination of serum total cholesterol with a single stable reagent. *Clin Chem* 16: 980-984, 1970.
18. Snedecor GW, Cochran WG. Statistical Methods. New Delhi: Oxford & IBH Publishing Co Pvt Ltd; 1967. p. 375-85.
19. Bolton S. Statistics. In: Gennaro AR, editor. Remington: The Science and Practice of Pharmacy. 20th ed. Volume I. Philadelphia: Lippincott Williams & Wilkins; 2001. p. 124-58.
20. Winrow VR, Winyard PG, Morris CJ, Black DR. Free radicals in inflammation: second messengers and mediators of tissue destruction. *Br Med Bull* 49: 506-522, 1993.
21. 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* 876: 154-166, 1986.
22. Kosower EM, Kosower NS. Glutathione Metabolism and Function. New York: Raven Press; 1976. p. 133-39.
23. Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 6: 3051-3064, 1992.