IJPSR (2012), Vol. 3, Issue 03 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 19 November, 2011; received in revised form 25 December, 2011; accepted 23 February, 2012

EVALUATION OF ANTIPEROXIDATIVE POTENTIAL OF ASCORBIC ACID ON BUSULFAN-INDUCED LIPID PEROXIDATION USING 4-HYDROXY-2-NONENAL AND NITRIC OXIDE AS MODEL MARKERS

Supratim Ray

Division of Pharmaceutical Chemistry, Dr. B C Roy College of Pharmacy & Allied Health Sciences, Bidhannagar, Durgapur- 713 206, West Bengal, India

Keywords:
Busulfan,
Lipid peroxidation,
Ascorbic acid,
4-hydroxy-2-nonenal,
Nitric oxide

Correspondence to Author:

Supratim Ray

Professor & HOD, Division of Pharmaceutical Chemistry, Dr. B C Roy College of Pharmacy & Allied Health Sciences, Bidhannagar, Durgapur- 713 206, West Bengal, India

ABSTRACT

The study was designed with an aim to evaluate the antiperoxidative potential of ascorbic acid on busulfan-induced lipid peroxidation. The study was performed *in vitro* using goat liver as lipid source. This evaluation was done by measuring the 4-hydroxy-2-nonenal (4-HNE) and nitric oxide (NO) content of liver tissue homogenates as markers of lipid peroxidation. The study reveals the lipid peroxidation induction capacity of busulfan and the antiperoxidative potential of ascorbic acid on busulfan-induced lipid peroxidation.

INTRODUCTION: Free radicals are highly reactive molecules with odd number of electrons. They are constantly being generated in the body through various mechanisms and also being removed by endogenous antioxidant defense mechanism that acts by scavenging free radicals, decomposing peroxides and / or binding with pro-oxidant metal ion [1].

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 (4-HNE) and 4-hydroxy-2-alkenals (HAKs) of different chain length [2].

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.

Busulfan, an alkylating agent used extensively in bone marrow transplantation. But there are evidences that organ toxicity in bone marrow transplantation may in part be due to free radical damage [3].

Ascorbic acid has versatile medicinal properties. It has been now established that besides its own physiological effects it has potential antioxidant property. Ascorbic acid has been reported to have protective role against cadmium induced thyroid dysfunction due to its antioxidant action [4].

The protective effect of various antioxidants on anticancer drug-induced lipid peroxidation had been reported earlier by us [5-7]. In continuation of ongoing search for antioxidants, the present work has been carried out *in vitro* to evaluate the antiperoxidative potential of ascorbic acid on busulfan-induced lipid peroxidation.

Experimental:

Materials: The drug sample (busulfan) was provided by Elder Pharmaceuticals, Mumbai. Goat liver was used as the lipid source. Chemicals of analytical grade were used for the present study.

2, 4-Dinitrophenylhydrazine (DNPH) and trichloroacetic acid (TCA) were procured from SD Fine Chem. Ltd., Mumbai and Merck, Mumbai, respectively. The standard sample of 4-HNE was purchased from ICN Biomedicals Inc., Aurora, Ohio. Sulfanilamide was from SD Fine Chem. Ltd., Mumbai; N-naphthylethylene-diamine dihydrochloride was from Loba Chemie Pvt. Ltd., Mumbai; Ascorbic acid was purchased from Sigma chemicals Co. St. Louis, MO, USA.

Methods:

Preparation of Tissue Homogenate: Goat liver was collected from Drugapur Municipal Corporation (DMC) approved outlet. Goat liver was selected because of its easy availability and close similarity with human liver in its lipid profile [8]. 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 (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

One portion of the homogenate was kept as control (C) while a second portion was treated with the busulfan (D) at a concentration of 0.0013 mg/g tissue homogenate. The third portion was treated with both busulfan at a concentration 0.0013 mg/g tissue homogenate and ascorbic acid at a concentration of 0.166 mg / g tissue homogenate (DA) and the fourth portion was treated only with ascorbic acid at a concentration of 0.166 mg / g tissue homogenate (A). After busulfan and /or ascorbic acid treatment, the liver tissue homogenate samples were shaken for two hours.

Determinations of 4-hydroxy-2-nonenal (4-HNE) level in Tissue Homogenate: The estimation was done only at 2 hours of incubation and repeated in five animal sets. In each case three samples of 2 ml of incubation mixture was 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 hour at room temperature. After that, the samples were extracted with hexane, and the extract was evaporated to dryness under argon at 40^{o}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 [9] using Shimadju UV-1700 double beam spectrophotometer.

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 hour. 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^{o}C and the residue was reconstituted in 1 ml of methanol. The absorbance was measured at 350 nm using the 0 μM 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 Nitric Oxide (NO) level from Tissue Homogenate: The estimation was done at 2 hours of incubation and repeated in five animal sets. 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) [10]. In each case three samples of 4.0 ml of tissue homogenate were treated with 2.5 ml of 10% TCA solution and centrifuged at 3000 rpm for 30 minutes. Then 5 ml of the filtrate were treated with 0.5 ml Griess reagent. After 10 minutes 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) using Shimadju UV-1700 double beam spectrophotometer. 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 540nm 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= 0.0108M, where M= nanomoles of NO, A= absorbance, r = 0.99581, SEE= 0.0064.

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 [11-12] were also performed on the percent changes data of various groups such as busulfan-treated (D), busulfan and ascorbic acid (DA) and only ascorbic acid-treated (A) with respect to control group of corresponding time.

RESULTS & DISCUSSION: The percent changes in 4-HNE and NO content of different samples at two hours 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. The 4-HNE content of different animal sets and their averages are shown along with statistical analysis in **Table 1**.

Incubation of tissue homogenates with busulfan results an increase in 4-HNE content (13.91%) with respect to corresponding control. This observation suggests lipid peroxidation induction potential of the drug. Lipid peroxidation leads to the generation of variety of cytotoxic products. More over it causes disruption of membrane structure and change in fluidity [13]. When tissue homogenates were treated combines with busulfan and ascorbic acid, 4-HNE content **(**-0.288%) was significantly reduced in comparison to control as well as busulfan-treated group. 4-HNE as well as related aldehydes display strong cytotoxicity [14, 15] and their effective removal could play an important role in a general defense system of the liver *in* vivo against damaging effects of lipid peroxidation [16-18].

Incubation of tissue homogenates only with ascorbic acid also reduces the 4-HNE levels (-5.76%). This implies that ascorbic acid could inhibit busulfan-induced lipid peroxidation to a significant extent.

TABLE 1: EFFECTS OF ASCORBIC ACID ON BUSULFAN-INDUCED LIPID PEROXIDATION: CHANGES IN 4-HNE PROFILE

Name of the antioxidant	Name of the drug	Time of incubation (h)	Animal sets	% Changes in 4-HNE content (with respect to corresponding control) due to treatment with drug and or antioxidant			Analysis of variance and multiple comparison
				Samples			
				D	DA	A	
Ascorbic acid	Busulfan	2	An 1	12.82[a]	-1.26[a]	-5.66[d]	F1=886.7 [df=(2,8)]
			An 2	14.64[b]	1.24[b]	-6.24[b]	F2=5.78 [df=(4,8)]
			An 3	14.66[a]	-1.12[a]	-5.42[b]	Pooled variance
			An 4	12.18[a]	-1.20[b]	-7.24[b]	(S^2)*=0.581
			An 5	15.26[b]	0.90[a]	-4.26[a]	Critical difference (p=0.05)[#]
			Av.	**13.91**	**-0.288**	**-5.76**	LSD =0.828
			(±SEM)	(±0.596)	(±0.56)	(±0.49)	Ranked means[**] (D) (DA) (A)

Percent changes with respect to controls of corresponding hours are shown. C, D, DA & A indicate control (not treated with busulfan or ascorbic acid), only busulfan -treated, busulfan and ascorbic acid -treated and only ascorbic acid-treated samples respectively; Av. = Averages of five animal sets; SEM = Standard error of estimate (df=4); Significant of '*t*' values of the changes of 4-HNE content (df=2) are shown as: a>99%; b=97.5-99%; c=95-97.5%; d=90-95%; e=80-90%; f=70-80%; g=60-70%; h<60%; 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 [11, 12] **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

The nitric oxide (NO) content of different animal sets and their averages are shown along with statistical analysis in **Table 2**. Incubation of tissue homogenates with busulfan reduced the NO content (-10.95%) with respect to corresponding controls. Nitric oxide is a prooxidant [19], potential antioxidant [20, 21] and plays a

very important role in host defense [22]. It was further found that incubation of tissue homogenates combines with busulfan and ascorbic acid result an increase in NO content (-0.252%) with respect to control group. Incubation of tissue homogenates only with ascorbic acid also enhances NO level (5.27%) with respect to corresponding controls. These results suggests that NO could inhibit lipid peroxidation to a significant extent. 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 [20-22].

TABLE 2: EFFECTS OF ASCORBIC ACID ON BUSULFAN--INDUCED LIPID PEROXIDATION: CHANGES IN NO PROFILE

Name of the antioxidant	Name of the drug	Time of incubation (h)	Animal sets	% Changes in NO content (with respect to corresponding control) due to treatment with drug and or antioxidant			Analysis of variance and multiple comparison
				Samples			
				D	DA	A	
Ascorbic acid	Busulfan	2	An 1	-10.12[a]	1.12[c]	5.42[a]	F1=488.99 [df=(2,8)]
			An 2	-10.46[a]	-1.12[b]	5.22[a]	F2=5.56 [df=(4,8)]
			An 3	-9.82[a]	1.1[a]	6.24[a]	Pooled variance
			An 4	-14.12[b]	-1.46[d]	4.28[a]	(S^2)*=0.6959
			An 5	-10.24[a]	-0.9[c]	5.22[a]	Critical difference (p=0.05)[#]
			Av.	**-10.95**	**-0.252**	**5.27**	LSD =1.57
			(±SEM)	(±0.79)	(±0.56)	(±0.31)	Ranked means[**] (D) (DA) (A)

Percent changes with respect to controls of corresponding hours are shown. C, D, DA & A indicate control (not treated with busulfan or ascorbic acid), only busulfan -treated, busulfan and ascorbic acid -treated and only ascorbic acid-treated samples respectively; Av. = Averages of five animal sets; SEM = Standard error of estimate (df=4); Significant of '*t*' values of the changes of NO content (df=2) are shown as: a>99%; b=97.5-99%; c=95-97.5%; d=90-95%; e=80-90%; f=70-80%; g=60-70%; h<60%; 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 [11, 12] **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

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 control of corresponding hours. It is seen that there is significant differences among various groups (F1) such as busulfan-treated, busulfan and ascorbic acid -treated and only ascorbic acid -treated.

But within a particular group, differences (F2) are insignificant which shows that there is no statistical difference in animals in a particular group (Tables 1-2). The Tables also indicate that the level of 4-HNE / NO in busulfan -treated group, busulfan and ascorbic acid -treated and only ascorbic acid –treated groups are statistically significantly different from the each other.

CONCLUSION: The data presented in this work demonstrate the lipid peroxidation induction potential of busulfan, which may be related to its toxic potential. The results also suggest the antiperoxidative effects of ascorbic acid and demonstrate its potential to reduce busulfan induced toxic effects. However, further extensive study is required to draw any final conclusion.

REFERENCES:

1. Gutteridge JMC, Halliwell B: Free radicals and antioxidants in the year 2000. A historical look to the future. Ann. N. Y. Acad. Sci. 2000; 899: 136-147.
2. Halliwell B: Drug antioxidant effects-A basis for drug selection? Drugs 1991; 42: 569-605.
3. Dürken M, Agbenu J, Finckh B, Hübner C, Pichlmeier U, Zeller W, Winkler K, Zander A, Kohlschütter A: Deteriorating free radical-trapping capacity and antioxidant status in plasma during bone marrow transplantation. Bone Marrow Transplant1995; 15: 757-762.
4. Gupta P, Kar A: Role of ascorbic acid in cadmium-induced thyroid dysfunction and lipid peroxidation. J. Appl. Toxicol. 1998; 18: 317-320.
5. Ray S, Roy K, Sengupta C: Cisplatin-induced lipid peroxidation and its inhibition with ascorbic acid. Indian J. Pharm. Sci. 2006; 68: 199-204.
6. 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. 2007; 64: 335-344.
7. 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: 217-218.
8. Hilditch TP, Williams PN: The Chemical Constituents of Fats. Chapman & Hall, London, 1964.
9. Kinter M: Free Radicals- A Practical Approach. Oxford University Press, Oxford 1996: 133-145.

10. 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.
11. Snedecor GW, Cochran WG: Statistical Methods. Oxford & IBH Publishing Co. Pvt. Ltd., New Delhi, 1967.
12. Bolton S: Statistics. In Gennaro AR: Remington: The Science and Practice of Pharmacy. Lippincott Williams & Wilkins, Philadelphia. 2000; 124-158.
13. 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. Biochem. Biophys. Acta. 1986; 876: 154-166.
14. Benedetti A, Comporti M, Esterbauer H: Identification of 4-hydroxynonenal as a cytotoxic product originating from the peroxidation of liver microsomal lipids. Biochem. Biophys. Acta. 1980; 620: 281-296.
15. Krokan K, Grafstrom R, Sundquist K, Esterbauer H, Harris C: Cytotoxicity, thiol depletion and inhibition of O6-methylguanine-DNA methyltransferase by various aldehydes in cultured human bronchial fibroblasts. Carcinogenesis 1985; 6: 1755-1759.
16. Esterbauer H, Zollner H, Lang J : Metabolism of the lipid peroxidation product 4-hydroxynonenal by isolated hepatocytes and by liver cytosolic fractions. Biochem. J. 1985; 228: 363-373.
17. Jensson H, Guthenberg C, Alin P, Mannervik B: Rat glutathione transferase 8-8, an enzyme efficiently detoxifying 4-hydroxyalk-2-enals. FEBS Lett. 1986; 203: 207-209.
18. Ishikawa T, Esterbauer H, Sies H: Role of cardiac glutathione transferase and of the glutathione S-conjugate export system in biotransformation of 4-hydroxynonenal in the heart. J. Biol. Chem. 1986; 261: 1576-1581.
19. Beckman JS, Beckman TW, Chen J, Marshal 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: 1620-1624.
20. 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: 170-174.
21. Rubho H, Parthasarathy S, Barnes S, Kirk M, Kalyanaraman B, Freeman BA: Nitric oxide inhibition of lipoxygenase-dependent liposome 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.
22. Nathan C: Nitric oxide as a secretory product of mammalian cells. Faseb J. 1992; 6: 3051-3064.

****