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Exploring Protective Role of Ascorbic Acid on Busulfan-Induced Lipid Peroxidation

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ABSTRACT

The present study deals with exploration of lipid peroxidation induction capacity of busulfan, an anticancer drug, and in vitro evaluation of ascorbic acid as a suppressor of busulfan induced lipid peroxidation. Goat liver homogenate has been used as the lipid source. This evaluation was done by measuring the malondialdehyde and reduced glutathione content of the tissue as markers of lipid peroxidation. The study revealed that ascorbic acid could suppress the drug induced lipid peroxidation to a significant extent.
Keywords: lipid peroxidation, busulfan, ascorbic acid, malondialdehyde, reduced glutathione.

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INTRODUCTION

The polyunsaturated fatty acids of membrane phospholipids are particularly susceptible to peroxidation and undergo significant modifications, including the rearrangement or loss of double bonds and, in some cases, the reductive degradation of lipid acyl side chains [1, 2]. Lipid peroxidation leads to generation of peroxides and hydroperoxide that can decompose to yield a wide range of cytotoxic end products most of which are aldehydes as exemplified by molondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE) etc [3]. 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. 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 [4].

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 [5].

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 [6].

The protective effect of various antioxidants on anticancer drug-induced lipid peroxidation had been reported earlier by us [7-9]. 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.

MATERIALS AND METHODS

Materials

Thiobarbituric acid (TBA) 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. Ascorbic acid and 1,1,3,3, tetraethoxypropane, reduced glutathione were from Sigma chemicals Co. St. Louis, MO, USA. All other reagents were of analytical grade. The drug sample (busulfan) was provided by Elder Pharmaceuticals, Mumbai. Goat liver was used as the lipid source.

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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 [10]. 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.

Estimation of malondialdehyde (MDA) level from tissue homogenate

The extent of lipid peroxidation was measured in terms of malondialdehyde (MDA) content using thiobarbuturic acid (TBA) method [11]. The estimation was done at 2 hours of incubation and repeated in five animal sets. In each case three samples of 2.5 ml of incubation mixture were treated with 2.5 ml of 10% (w/v) trichloroacetic acid (TCA) and centrifuged at room temperature at 3000 rpm for 30 minutes 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 minutes. 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 minutes. The solutions were cooled to a room temperature and their absorbance was 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=0.007086M, where M= nanomoles of MDA, A= absorbance, r = 0.995, SEE= 0.006.

Estimation of reduced glutathione (GSH) level from tissue homogenate:

Reduced glutathione (GSH) was measured in accordance with Ellman's method [12]. The estimation was done at 2 hours of incubation and repeated in five animal sets. In each case three samples of 1 ml of incubation mixture were treated with 1 ml of 5% (w/v) TCA in 1 mM EDTA centrifuged at 2000 g for 10 minutes. 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 absorbance of the solutions was 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= 0.00151C, where C= nanomoles of reduced glutathione, A= absorbance, r =0.997, SEE= 0.008.

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 [13, 14] 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 AND DISCUSSION

The percent changes in MDA and GSH 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 results of the studies on busulfan-induced lipid peroxidation and its inhibition with ascorbic acid were shown in Tables 1-2.

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Table 1: Effects of ascorbic acid on busulfan-induced lipid peroxidation: changes in MDA profile

Name of the antioxidant	Name of the drug	Time of incubation (h)	Animal sets	% Changes in MDA 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	16.82[a]	7.42[a]	2.35[d]	F1=40.54 [df=(2,8)]
			An 2	20.12[a]	6.82[b]	2.12[b]	F2=2.95 [df=(4,8)]
			An 3	8.46[a]	2.36[a]	2.02[b]	Pooled variance (S^2)*=5.02
			An 4	12.18[a]	3.42[b]	2.18[c]	Critical difference (p=0.05)[#]
			An 5	15.26[b]	5.86[a]	3.24[a]	LSD =4.22
							Ranked means[**]
			Av. (±SEM)	**14.57** (±1.99)	**5.18** (±0.98)	**2.38** (±0.22)	(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 MDA 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 [13, 14] **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

From Table 1 it was evident that tissue homogenates treated with busulfan showed an increase in MDA (14.57 %) content in samples with respect to control to a significant extent. The observations suggest that busulfan could significantly induce the lipid peroxidation process. MDA is a highly reactive three-carbon dialdehyde produced as a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism [15]. But the MDA (5.18%) content were significantly reduced in comparison to busulfan-treated group when tissue homogenates were treated with busulfan in combination with ascorbic acid. Again the tissue homogenates were treated only with the ascorbic acid then the MDA (2.38 %) level were reduced in comparison to the busulfan treated group. The increase in MDA content with respect to control when the tissue homogenates were treated with ascorbic acid alone indicates its pro-oxidants effect. It was postulated that ascorbic acid could reduce Fe^{3+} to Fe^{2+} which promotes generation of hydroxyl radicals and other reactive oxygen species through Fenton's reaction [16, 17]. Many known antioxidants like vitamins [18], estrogen [19, 20] superoxide dismutase [21] and flavonoids have been reported to act as pro-oxidant in presence of transition metals [22, 23] or at high concentration.

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Table 2: Effects of ascorbic acid on busulfan--induced lipid peroxidation: changes in GSH profile

Name of the antioxidant	Name of the drug	Time of incubation (h)	Animal sets	% Changes in GSH 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	-13.42[a]	2.46[c]	1.82[a]	F1=122.55 [df=(2,8)]
			An 2	-10.46[a]	4.82[b]	4.86[a]	F2=2.36 [df=(4,8)]
			An 3	-7.86[a]	4.21[a]	5.12[a]	Pooled variance (S^2)*=2.27
			An 4	-6.52[b]	3.78[d]	2.82[a]	Critical difference (p=0.05)[#]
			An 5	-7.66[a]	3.19[c]	4.21[a]	LSD =2.84
							Ranked means[**]
			Av.	**-9.18**	**3.69**	**3.77**	(D) (DA, A)
			(±SEM)	(±1.24)	(±0.41)	(±0.63)	

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 GSH 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 [13, 14] **Two means not included within same parenthesis are statistically significantly different at p=0.05 level.

It was evident from Table 2 that tissue homogenates treated with busulfan caused a decrease in GSH (-9.18%) content with respect control to a significant extent. The decrease in GSH content was associated with an increase in lipid peroxidation. When tissue homogenates were treated both with busulfan and ascorbic acid then the GSH (3.69%) levels increased in comparison to busulfan treated group. Tissue homogenates treated only with ascorbic acid also increase the GSH (3.77%) contents in comparison to the control samples. The increase in GSH level suggests the antiperoxidative potential of ascorbic acid. Glutathione is an important antioxidant and plays a very important role in the defense mechanism for tissue against the reactive oxygen species [24]. 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.

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 MDA / GSH in busulfan -treated group is only statistically significantly different from the busulfan and ascorbic acid-treated group as well as only ascorbic acid -treated group. But

there is no statistically significantly difference among the busulfan and ascorbic acid -treated group and only ascorbic acid -treated group.

CONCLUSION

The results also suggest the antiperoxidative effects of ascorbic acid and demonstrate its potential to reduce busulfan-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. However a detailed study is required to conclude such hypothesis.

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