

GLUTATHIONE REDUCTASE, REDUCED GLUTATHIONE AND CERULOPLASMIN IN DIETHYLDITHIOCARBAMATE TREATED RATS

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ABSTRACT: Intraperitoneal injection of rats with diethyldithiocarbamate (1.2 g/kg body weight) led to a maximum diminution of glutathione reductase at 12 hr by 40% and 56% in liver and red blood cells respectively, with a gradual return to the normal level at 48 hr after administration. Significant inhibition of reduced glutathione was also observed, which returned to normal at 48 hr after administration. However, maximum decline in its content was at 12 hr by 71% and 37% in liver and red blood cells respectively. A similar pattern was exhibited with respect to liver and serum ceruloplasmin in the diethyldithiocarbamate injected rats. It is possible that oxidant stress is developed in the rats by the administration of diethyldithiocarbamate with concomitant decrease in the activity of antioxidant enzymes and the other antioxidants such as reduced glutathione and ceruloplasmin.

KEYWORDS: Glutathione reductase, reduced glutathione, ceruloplasmin, diethyldithiocarbamate.

INTRODUCTION: Intraperitoneal injection of rats with diethyldithiocarbamate (DDC), a powerful copper chelator, causes decline in superoxide dismutase in various tissues of experimental animals.^{1,2} DDC administration to rats significantly decreased the cellular activity of selenium-dependent glutathione peroxidase.³ There are several metabolic processes which generate superoxide anion radical (O_2^-) in the respiring cells.⁴ If these radicals are not effectively removed, they are toxic to the cells due to their ability to promote lipid peroxidation^{4,5} and to provoke the denaturation of certain enzymes.⁶ Superoxide dismutase, which catalyses the dismutation of O_2^- is considered to be the essential enzyme in the defense against the toxic effects of O_2^- . Maintenance of intracellular level of this enzyme is necessary to prevent free radical toxicity. In view of these reports, it was felt necessary to study the effect of DDC administration on the levels of glutathione reductase and reduced glutathione in liver and erythrocytes and serum ceruloplasmin of rats. This study is aimed at establishing the mutually supportive role of the antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione reductase, ceruloplasmin and antioxidant such as reduced glutathione.

MATERIALS AND METHODS: CHEMICALS: Bovine serum albumin (fraction V) and 5, 5 - dithiobis 2- nitrobenzoic acid were purchased from Sigma Chemical Company (St. Louis MO,

USA). Diethyl dithiocarbamate was obtained from BDH Chemicals Ltd. (Poole, England). Oxidised glutathione and NADPH were purchased from CSIR centre for Biochemicals. All other chemicals used were commercial products of highest analytical grade available in India.

ANIMALS: Male albino rats (Wistar strain; 150-180 g) used in the present study were maintained on pellet diet and water *ad libitum* for 7 weeks. A group of 6 rats received a single dose of 0.5 ml normal saline intraperitoneally (i.p.) and were sacrificed exactly 1 hr after the injection. This group of rats was considered as control. A second group of 30 rats were injected (i.p.) with DDC (1.2 g/kg) as has been reported earlier¹. These rats in batches of 6 each were sacrificed successively at an interval of 1, 6, 12, 24 and 48 hr after the injection. The experimental design has approval of Institutional Ethical Committee.

MODE OF SACRIFICE: Rats were sacrificed under ether anesthesia. They were bled by cardiac puncture and blood was collected using heparin rinsed syringes. Liver was quickly excised off, washed in sequence with saline, saline phosphate buffer (10 mM, potassium phosphate buffer containing 0.15 M NaCl; pH 7.4) blotted dry and stored at 4°C. All the estimations were completed within 48 hr of sacrifice.

PREPARATION OF TISSUE HOMOGENATES: Liver homogenates (10% W/V) were prepared in potassium phosphate buffer (10 mM) containing 0.15M NaCl (pH 7.4), using mechanical homogenizer. Unbroken cells and cell debris were removed by centrifugation at 700 g for 10 min using Remi C - 24 refrigerated centrifuge. The supernatant thus obtained was termed as tissue homogenate, was used for assay of reduced glutathione, glutathione reductase and ceruloplasmin.

PREPARATION OF HEMOLYSATE FOR GLUTATHIONE REDUCTASE: To 0.2 ml of packed, saline washed erythrocytes, 4.8 ml of distilled water was added and mixed. The supernatant obtained after centrifugation at 4°C was used immediately.

ASSAY OF GLUTATHIONE REDUCTASE ACTIVITY: The activity of glutathione reductase was determined by the procedure of Horn and Burns as given by Bergmeyer⁷. The cuvette contained 1.6 ml of 0.067 M potassium phosphate buffer (pH 6.6), 0.12 ml of 0.06% NADPH, 0.12 ml of 1.15% G-S-S-G, 0.1 ml of the tissue homogenate or hemolysate and water in a final volume of 2 ml. The decrease in O.D. was recorded at 340 nm for 3-5 min in Spectronic 21 spectrophotometer. For each series of measurements, controls were done containing water instead of G-S-S-G. Under the conditions of the assay, NADPH dependent glutathione reductase activity was linear with time. The enzyme activity was expressed as nmoles of NADPH oxidized/min/mg protein.

ESTIMATION OF REDUCED GLUTATHIONE (GSH): The GSH content of erythrocytes and tissue homogenates was determined by the improved method of Beulteret *al*⁸. To 0.2 ml of the packed erythrocytes or tissue homogenate, was added 1.8 ml of distilled water and 3 ml of the precipitating solution (1.67 gm of glacial metaphosphoric acid, 0.2 gm of EDTA and 30 gm of NaCl per 100 ml of distilled water). After mixing, the solution was allowed to stand for 5 min and filtered. Two ml of the filtrate was added to 8 ml of phosphate solution (0.3M Na₂HP0₄ in distilled water) followed by 1 ml of DTNB solution (0.04% 5, 5' diothiobis 2-nitrobenzoic acid). The O.D. was measured at 412 nm. Blank was prepared by substituting the sample with water and following the entire procedure as for test. A standard curve was calibrated with freshly

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prepared GSH solution from which the sample values were calculated and expressed as mgs of GSH/gm of tissue protein or as mgs of GSH/dl of erythrocytes.

DETERMINATION OF SERUM CERULOPASMIN: The method followed was essentially that of Henry *et al*⁹. To the blank, 1 ml of sodium azide reagent (0.1% in 0.1 M acetate buffer, pH 6.0), 1 ml of acetate buffer (0.1 M, pH 6.0) and 1 ml of PPD reagent (0.25% p-phenylene diamine in 0.1 M acetate buffer) were added. To the test, 2 ml of the acetate buffer and 1 ml of PPD reagent were added. The tubes were incubated at 37°C for 5 min in the dark. Then 0.1 ml serum (or tissue homogenate 10% W/V in 10 mM Potassium Phosphate buffer, pH 7.4) were added to both test as well as blank and mixed. The absorbance of the test against blank were read 10 min and again 40 min after the addition of the sample. The formula used for the calculation of the results is given below.

Mgs of ceruloplasmin /dl serum = $A_{40 \text{ min}} - A_{10 \text{ min}} \times 1000 \times 0.06$. In the case of liver homogenates, the values were expressed as mgs of ceruloplasmin /gm protein.

ESTIMATION OF PROTEIN: Protein estimation in the tissue homogenates was done by the method of Lowry *et al*¹⁰. Data were expressed as Mean \pm SE and the significance of difference was assessed by the student's t test.

RESULTS: Table 1 summarizes the effect of i.p. administration of DDC 1.2 gm/kg body wt on glutathione reductase activity in liver and RBC's of rats at different intervals. The maximum decrease was about 40% and 56% respectively, in liver and erythrocytes at 12 hrs.

The GSH content of liver and erythrocytes of DDC treated rats was markedly lowered at 6 and 12 hrs after the injection of DDC with a stimulatory effect at 24th hr in liver but not in erythrocytes (Table 2). Highest decrease (about 71%) was found both at 6 and 12 hrs in liver after the administration of an equivalent dose of DDC to the rats.

The effect of DDC administration on ceruloplasmin content of rat liver and serum is presented in Table 3. The level of ceruloplasmin was significantly lowered at the various periods with a gradual return to the control level in 48 hrs. DDC had an almost immediate effect on liver ceruloplasmin with a decrease to about 35% in 1 hr whereas the decrease at this period was not so apparent in the sera of DDC treated rats as compared to the controls. Significant diminution of ceruloplasmin level was seen in the liver and sera, even 48 hrs after the injection of DDC.

Table-1: Glutathione reductase activity of tissues from rats injected with diethyldithiocarbamate (1.2 gm/kg, in saline, i.p.). Rats were sacrificed at different intervals of time as indicated in column 1. (Results are Mean \pm SD of 6 rats from each group).

Hrs after DDC injection	Glutathione reductase activity			
	Liver		Red blood cells	
	Activity	% decrease	Activity	% decrease
Control ^a	59.1 \pm 5.8	-	61.8 \pm 7.4	-
1	47.8 \pm 5.1	19.1**	51.6 \pm 6.3	16.5*
6	44.5 \pm 5.0	24.7***	34.3 \pm 5.1	44.5***
12	35.7 \pm 4.3	39.6***	27.4 \pm 3.8	55.7***
24	39.0 \pm 4.6	34.0***	38.7 \pm 4.0	37.4***
48	52.5 \pm 5.1	11.2 #	56.6 \pm 4.7	8.4#

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Control ^a rats received injections of the saline vehicle. An enzyme unit is defined as n moles of NADPH oxidized per min per mg protein. ****P* < 0.001, ***P* < 0.01, **P* < 0.05, #NS (Not Significant).

Table-2: Reduced glutathione content of tissues from rats injected with diethyldithiocarbamate (1.2 gm/kg, in saline, i.p.). Rats were sacrificed at various intervals of time after the injection as given in column 1 (Mean ± SD of 6 rats from each group)

Hrs after DDC injection	GSH Content			
	Liver		Red blood cells	
	mgs/gm protein	% change	mgs/100 ml	% decrease
Control ^a	4.5 ± 0.7	-	60.1 ± 8.6	-
1	3.6 ± 0.7	-20.0#	53.5 ± 5.1	11.0#
6	1.3 ± 0.7	-71.1***	47.1 ± 6.4	21.6*
12	1.3 ± 0.5	- 71.1***	37.3 ± 6.6	37.4***
24	5.4 ± 0.7	+ 20.0*	57.3 ± 6.9	4.7#
48	4.9 ± 1.0	+ 8.9#	59.9 ± 7.3	0.3#

^a Control rats received injections of saline vehicle. ****P* < 0.001, ***P* < 0.01, **P* < 0.05, #NS (Not significant)

Table-3: Ceruloplasmin content of tissues from rats injected with diethyldithiocarbamate (1.2 gm/kg, in saline, i.p.). Rats were sacrificed at different intervals as given in column 1. The results are the Mean ± SD of 6 rats from each group.

Hrs after DDC injection	Ceruloplasmin Content			
	Liver		Red blood cells	
	mgs/gm protein	% change	mgs/100 ml	% decrease
Control ^a	9.9 ± 2.0	-	24.9 ± 4.7	-
1	6.4 ± 1.2	35.4**	20.2 ± 4.2	18.9#
6	5.7 ± 1.5	42.4***	47.1 ± 3.2	21.6*
12	4.3 ± 1.5	56.6***	11.4 ± 3.2	54.2***
24	6.9 ± 1.0	30.3**	18.4 ± 2.4	26.1*
48	7.2 ± 1.0	27.3*	20.0 ± 2.4	19.7*

^a Control rats received injections of saline vehicle. ****P* < 0.001, ***P* < 0.01,

**P* < 0.05, #NS (Not Significant).

DISCUSSION: It is believed that GSH is one of the important protective factors against the oxidative damage. It may act not only as a scavenger by itself but also through the GSH dependent enzymes¹¹. GSH depletion or inhibition of GSH-GSSG redox cycle has been found to sensitize the cells to an imposed oxidative stress¹². The decreased level of GSH in the tissues and erythrocytes of DDC treated rats might be related to the level of glutathione reductase, since the function of this enzyme is mainly to keep the glutathione in the reduced state¹³. The results of our present study confirm the earlier reports of Goldstein *et al*¹⁴.

The major factor contributing to the diminished serum ceruloplasmin levels appears to be the copper chelating property of DDC. Al-Timimi and Dormandy¹⁵ have demonstrated that

copper is essential for the antioxidant action of ceruloplasmin and that the copper-free apoprotein is ineffective.

The decrease in the level of SOD in the tissue of rats treated with DDC occur almost immediately after the administration of DDC¹ and the decrease of other antioxidant enzymes such as se-dependent Glutathione peroxidase³ and glutathione reductase was seen at a later stage, possibly due to the inefficient elimination of O₂⁻ leading to the inactivation of GSHpx and Glutathione reductase and hence decreased level of GSH.

The present study concludes that glutathione reductase, reduced glutathione and ceruloplasmin are decreased when rats are subjected to oxidative stress on administration of diethyldithiocarbamate.

REFERENCES:

1. Heikkila RE, Cabbat FS, Cohen G. *In vivo* inhibition of superoxide dismutase in mice by diethyldithiocarbamate. *J Biol Chem.* 1976; 251: 2182-2185.
2. Goldstein BD, Rozen MG, Quintavalla JC, Amoruso MA. A decrease in mouse lung and liver glutathione peroxidase activity and potentiation of the lethal effects of ozone and paraquat by superoxide dismutase inhibitor diethyldithiocarbamate. *Biochem Pharmacol.* 1974; 28: 27-30.
3. Ramachandra Prabhu H. *In vivo* inhibition of selenium dependent glutathione peroxidase and superoxide dismutase in rats by diethyldithiocarbamate. *Ind J of Exp Biology.* 2002; 40:258-261.
4. Fridovich I. Superoxide radical and superoxide dismutases. *Annu Rev Biochem.* 1995; 64: 97-112.
5. Tyler DD. Role of superoxide radicals in lipid peroxidation of intracellular membranes. *FEBS Lett.* 1975; 79: 180-183.
6. Bartosz G, Fried R, Grzelinska E, Leyko W. Effect of hyperoxide radicals on bovine erythrocyte membrane. *Eur J Biochem.* 1977; 73: 261-264.
7. Horn H D, Burns FH. *Methods of enzymatic analysis.* (ed. Bergmeyer HV) Academic Press, N Y. 1978 pp 875-879.
8. Beutler E, Duron O, Kelly B M. Improved method for the determination of blood glutathione. *J Lab Clin Med.* 1963; 51: 882-888.
9. Henry RJ, Chiamori N, Jacobs SL, Segalor N. Determination of ceruloplasmin oxidase in serum. *Proc Soc Exp Biol.* 1960; 104: 620-624.
10. Lowry OH, Rosebrough NJ, Farr AL, Randall R. J. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951; 193: 265-275.
11. Nagasaka Y, Fujii S, Kanekot. Microsomal glutathione-dependent protection against lipid peroxidation acts through a factor other than glutathione peroxidase and glutathione S transferase in rat liver. *Arch Biochem Biophys.* 1989; 274: 82-86.
12. Miccadei S, Kyle M E, Gilfor D, Farber J L. Toxic consequences of the abrupt depletion of glutathione in cultured rat hepatocytes. *Arch Biochem Biophys.* 1988; 265: 311-320.
13. Williams C H Jr. *The Enzymes*, 3rd ed. Boyer PD(editors) Academic Press, New York: 1976. p 89-172.
14. Goldstein B D, Rozen M G, Quintavalla JC, Amoruso MA. Decrease in mouse lung and liver glutathione peroxidase activity and potentiation of the lethal effects of ozone and

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paraquat by the superoxide dismutase inhibitor diethyldithiocarbamate. *Biochem Pharmac.*1979; 28: 27-30.

15. Al-Timini D J, Dormandy T.L. The inhibition of lipid autoxidation by human ceruloplasmin. *Biochem J.* 1977; 168: 283-288.