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Protective effect of glutathione and selenium against alloxan induced lipid peroxidation and loss of antioxidant enzymes in erythrocytes

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Abstract. Alloxan is a diabetogenic drug and is known to induce diabetes through generation of free radicals. The toxic oxygen species can be detoxified by antioxidant enzyme system and thus reduce the deleterious effect of lipid peroxidation. Erythrocytes exposed to alloxan induced lipid peroxidation *in vivo* as well as *in vitro*. Although alloxan treatment produced a deleterious effect on antioxidant enzymes, pretreatment with glutathione and selenium led to a recovery of the activities of superoxide dismutase and glutathione peroxidase. However, catalase activity increased on alloxan treatment. Alloxan reduced blood glucose level significantly within 60 min but thereafter a slow and steady rise was observed.

Keywords. Lipid peroxidation; superoxide dismutase; catalase; glutathione peroxidase; alloxan.

1. Introduction

Alloxan is a pharmacological reagent, widely used to induce experimental diabetes in laboratory animals. Increased concentration of alloxan has been reported to attack kidney and liver cells other than (β-cells (Rerup 1970). sembrane damage by alloxan has been suggested to be due to generation of hydroxyl radical (Hekkila *et al* 1976). Membrane lipids are vital for the maintenance and integrity of cell function. Oxidative stress may result from over production of precursors for reactive oxygen species and/or decreased efficiency of inhibitory and scavenging systems. The stress may then be amplified and propagated by an autocatalytic cycle of metabolic stress, tissue damage and cell death, leading to further increase in free radical production and depletion of antioxidants (Baynes 1991). lnitiation of lipid peroxidation (LPO) is a process solely carried out by free radicals such as superoxide, hydroxyl radical and H_2O_2 causing cellular injury (Ashwood–Smith 1975). Glutathione peroxidase (GSH–Px), in concert with the enzymes glutathione reductase, superoxide dismutase (SOD), catalase (CAT) and various low molecular mass antioxidants provides the principal cellular defense system against cytotoxic oxygen species (O^{-}_2, $OH\cdot$, H_2O_2) generated in the course of normal metabolism.

Alloxan inhibits glucokinase activity through interaction with two –SH groups located in the sugar-binding site of the glucokinase, with formation of a disulphide bond and concomitant interaction of the enzyme (Lenzen *et al* 1988). GSH has

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been shown to be protective against autoxidation of dialuric acid and generation of alloxan radicals, it also interferes in the redox cycling between alloxan and dialuric acid (Sakurai and Ogiso 1991). The present study was undertaken to explore in greater detail the action of alloxan on erythrocyte LPO. It also intends to demonstrate the initial effects of the drug on blood glucose and activities of the antioxidant enzymes in erythrocytes. The deleterious effects of alloxan was expected to be counteracted by GSH and selenium pretreatment based on the prior study of these compounds and therefore, the protective roles of GSH and selenium against alloxan-induced LPO have been studied.

2. Materials and methods

Male wistar rats of the age between 6–8 months were chosen for the study. Animals were injected with alloxan of different concentration (20, 40, 60, 80 and 100 mM) through tail vein in 0·2 ml of solution. Alloxan solution was prepared just prior to injection and kept on ice and used as quickly as possible. After the treatment, the animals were provided free access to drinking water and pellet diet. After 3 h of injection the animals were killed by cervical dislocation and blood was collected. Blood was obtained from rats *via* heart puncture. The blood was centrifuged and washed thrice with 1 M phosphate-buffered saline (1 : 9) (PBS), pH 7·4. The washings removed all the buffy coats and plasma. The erythrocytes were then suspended in PBS (1 M), pH 7·4, and adjusted to 5% packed cell volume (PCV).

Another set of animals were given sodium selenite daily ip for 7 days at a dose of 1 ppm in 0·2 ml of solution. A different set of animals were given GSH (5 mg/ml) injections through tail vein in 0·25 ml of solution, 30 min prior to alloxan (100 mM) treatment. Animals treated with 100 mM alloxan were chosen to study the enzyme activities and the effect of pretreatment of selenium and GSH. The animals were killed after 3 h of treatment and blood was collected as described above.

2.1 *In vitro alloxan treatment*

Blood was collected *via* heart puncture and processed as described above. The cells were then incubated in a total volume of 5 ml with 10 and 100 mM of alloxan concentration for 180 min at 37°C in PBS (1 M), pH 7·4, in a shaker water bath. Formation of malonyldialdehyde (MDA) was found to be linear up to 3 h of incubation. LPO was determined in the PCV as described below.

LPO was estimated in 5% PCV as MDA formed by thiobarbituric acid reaction (Stocks and Dormandy 1971). The cell suspension (4 ml) was exposed to 20 mM hydrogen peroxide and sodium azide (30 mM) in phosphate buffer (1 M), pH 7·4. The mixture was incubated at 37°C for 1 h followed by the addition of 2 ml TCA (28%). After centrifugation 4 ml of the supernatant was transferred to a boiling tube containing 1 ml TBA (1%) and the contents were boiled for 15 min. The tubes were allowed to cool and then centrifuged at 1000 *g* for 2 min. An aliquote of the supernatant was read at 532 nm in Shimadzu UV 160 A spectrophotometer (Japan).

Enzyme assays were performed in erythrocyte lysate prepared according to the method of McCord and Fridovich (1969). SOD was assayed by the method of Markiund and Marklund (1974) and CAT was determined by the method of Aebi (1983). The GSH–Px was measured using the method of Paglia and Valentine (1967). The method was based on the NADPH coupled reaction, whereby oxidised GSH produced by the activity of GSH–Px is converted to the reduced form by exogenous glutathione reductase and NADPH. The reaction mixture comprised of 0·05 M potassium phosphate buffer (pH 7·0) containing 0·25 mM EDTA, 2 units glutathione reductase (obtained from Sigma Chemicals Co., USA), 2·6 mM GSH and 20 mM NADPH in a total volume of 2·5 ml. To this mixture 0·1 ml of the lysate was added and the reaction was started by the addition of 1·5 mM cumene hydroperoxide. The decrease in absorbance was recorded at 340 nm for 5 min and activity was expressed as nmol of NADPH oxidised per min per mg protein. Each enzyme assay was performed in duplicate in two-fold concentration range. Haemoglobin in blood was determined by the method of Dacie and Lewis (1984). Protein content in haemolysate was determined by the method of Lowry *et al* (1951). Blood glucose was determined by the method as described by Hyvarinen and Nikkila (1962). Statistical analysis was done using Student's *t*-test. Probability levels less than 5% were considered significant.

3. Results

Alloxan treatment of erythrocytes *in vitro* showed increased LPO in a dose dependent manner in a stipulated time period of 180 min (table l). lncreased LPO by alloxan treatment confirms the production of toxic free radical that leads to peroxidation of membrane (Fisher and Hamburger 1980). When LPO was determined in alloxan treated animals, an increased LPO was observed in erythrocytes (figure 1). At 100 ms alloxan concentration the increased LPO was counteracted by both selenium

Table 1. Effect of alloxan on *in vitro* lipid peroxidation in erythrocytes.

Alloxan (mM)	Lipid peroxidation (nmol of MDA formed/h/g Hb)
–	225 ± 35
10	235 ± 28[a]
100	280 ± 16[b]

Values are mean ± SD of 8 experiments.
[a] Not significant.
[b] $P < 0{\cdot}01$ as compared with control.
Erythrocytes were incubated in phosphate buffered saline (1 M), pH 7·4 under O_2 at 37°C for 180 Ain. After incubation, the cells were washed in phosphate buffer thrice and then the cell suspension was readjusted to 5% packed cell volume for the determination of lipid peroxidation.

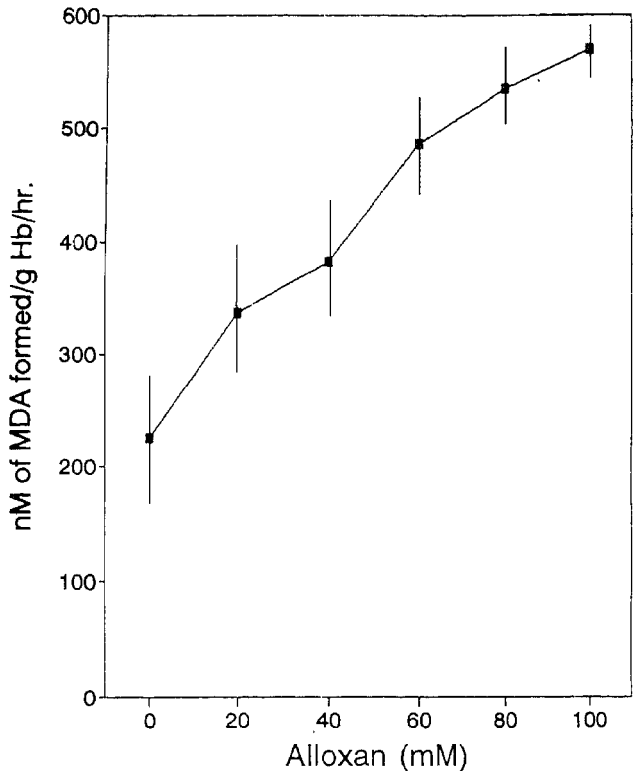

Figure 1. Lipid peroxidation determined in erythrocytes in rats treated with different concentrations of alloxan (20, 40, 60, 80 and 100 mmol) in 0·2 ml of solution. Values presented in the figure represents amount of MDA formed after 180 min of the treatment and expressed as nmol of MDA formed/hfg Hb. Each value represents mean ± SE of 8 animals.

and GSH pretreatment (table 2). Alloxan administration also decreased the activity of the antioxidant enzymes except CAT (table 3). The activity of SOD and GSH–Px decreased significantly as compared to control values while CAT activity increased to significant level. GSH administration increased SOD and GSH–Px activity. Selenium pretreatment could not revert the hazardous effect of alloxan on SOD although GSH–Px was significantly increased as compared to alloxan treatment (table 3).

An important finding that was pinpointed in the following experiments was of an initial decrease in blood glucose level, while after 60 min, a slow and steady rise in blood glucose level was observed at all the concentrations of alloxan tested (table 4).

4. Discussion

In the *in vitro* study, LPO was measured at different concentrations of alloxan (table 1). In this dose dependent experiment, there was a significant increase in LPO. lt has been reported that dialuric acid can react *in vitro* with oxygen to form

Table 2. Effect of alloxan, selenium and glutathione on lipid peroxidation.

	Lipid peroxidation (nmol of MDA/h/g Hb)	
Treatment	Control	+ Alloxan
–	211 ± 14	569 ± 38
Selenium		431 ± 49[a]
GSH		301 ± 37[a]

Values are mean ± SD of 8 experiments in each group. Alloxan (100 mM) treated group was compared with control, while alloxan + selenium (1 ppm daily) and alloxan + GSH (5 mg/ml) group was compared with alloxan treated animals.
[a]$p < 0{\cdot}001$.

Table 3. Effect of selenium and glutathione on antioxidant enzymes after alloxan treatment.

	Enzyme activities		
Groups	SOD[a]	CAT[b]	GSH–Px[c]
Control	301 ± 13	208 ± 23	211 ± 14
Alloxan	108 ± 16*	293 ± 16*	134 ± 31*
Alloxan + selenium	106 ± 20	297 ± 17	240 ± 71[†]
Alloxan + GSH	253 ± 19*	398 ± 11*	278 ± 71*

Values are mean ±SD of 8 experiments in each group. Alloxan (100 mM) treated group was compared with control, while rest of the groups were compared with alloxan group. All enzymes were assayed in erythrocyte lysate.
[a]Units/mg protein;[b]mmol of H_2O_2 decomposed/mg protein/min; [c]nmol of NADPH oxidised/mg protein/min.
[†]$P < 0{\cdot}01$; *$P < 0{\cdot}001$.

Table 4. Effect of alloxan treatment on the level of blood glucose.

	Alloxan (mM)		
Time (min)	20	60	100
0	87·79 ± 9·88	91·24 ± 11·74	94·43 ± 6·78
60	64·54 ± 5·0[a]	67·68 ± 7·84[a]	60·21 ± 6·74[a]
120	72·59 ± 7·57[a]	72·46 ± 5·66[a]	76·90 ± 7·54[a]
180	93·38 ± 8·63	107·18 ± 13·20[b]	112·44 ± 7·35[a]

Blood glucose expressed in mg/100 ml blood.
Values are mean ± SD.
$n = 4$.
All values were compared with 0 time.
[a]$P < 0{\cdot}01$; [b]$p < 0{\cdot}001$.

H_2O_2, superoxide radical (O_2^- and the hydroxyl radical ($OH^·$) (Cohen and Heikkila 1974). H_2O_2, the two electron reduction product of oxygen has been detected *in vivo* in mice after administration of alloxan (Heikkila *et al* 1974). H_2O_2, O_2^- and OH are very reactive with known cytotoxicities. ln an attempt to investigate these factors it was observed that RBC treated *in vitro* with alloxan showed increased LPO. lt is suggested that similar reactive mechanisms *in vivo* might cause an enhancement of peroxidation of membrane as is evident from the result depicted in figure 1 and table 1. Effect of alloxan *in vitro* (table 1) is a direct effect of the drug on erythrocyte membrane and peroxidation is a consequence of such an interaction. However, the peroxidation induced *in vivo* may be due to other activities of the drug. ln this regard, earlier work suggests of production of reactive oxygen species like superoxide anion and the diabetogenic action of the drug was counteracted by administration of SOD (Grankvist *et al* 1981).

Reaction of alloxan that produced toxic ions *in vivo* resulted in decrease of antioxidant system accompanied by increased LPO. This finding provides a perfect correlation between LPO and decreased activity of SOD and GPx in erythrocytes. Administration of GASH or selenium prior to the treatment of alloxan decreased LPO (table 2). GSH is a potent antioxidant and reacts with peroxides to form glutathione disulphide (GSSG). Glutathione protection effectively indicates that alloxan disturbs its status in the cell by affecting the pathway of its generation. Decrease in LPO by GSH shows that GSH status in the cell is an important factor to reduce peroxidation. Selenium through GSH-Px activity combats the risk of H_2O_2 which may be formed due to peroxidation. Decrease in antioxidant enzymes is an indication that there might be generation of an active radical/a factor which is a key to alteration of these enzymes. Decrease in SOD activity on alloxan treatment can be attributed to two factors. First there may be a drug-enzyme interaction resulting in the deactivation of the enzyme (Crouch *et al* 1981). Secondly H_2O_2 has been found to act as an inducer of tissue SOD (Matkovics 1977). Since CAT activity in our experiments increased significantly on alloxan treatment, H_2O_2 might not be available to trigger the SOD activity. A significant increase in CAT activity was observed which supports the earlier hypothesis that CAT function increases as the rate of H_2O_2 production is enhanced (Freeman and Crapo 1982). The increase in CAT activity indicates production of H_2O_2 by alloxan in cells and the threat can be counteracted by increased activity of CAT.

Antagonistic role between CAT and GSH-Px has been observed by alloxan treatment. It is suggested that free radical damage by the drug may be site specific in the cell. Animals pretreated with selenium restored the enzyme activity suggesting that metabolic status of selenium may be altered by alloxan or it might interfere in the synthesis of GSH–Px. GSH at higher concentration suppresses the generation of OH· through the inhibition of the redox cycling between alloxan and dialuric acid (Sakurai and Ogiso 1991). The observation reflects that GSH can alter the peroxidative effect of alloxan and effectively could restore the antioxidant enzymes.

Decrease in blood glucose level on administration of alloxan is reported here for the first time. A significant decrease in blood glucose suggests that a rapid utilization of the cellular glucose occurs after alloxan administration. lncreased production of hydrogen peroxide due to alloxan mimics a number of insulin effects like receptor kinase activation (Kadota *et al* 1986), which may therefore be the reason for decrease in blood glucose during the early hours. Hydrogen peroxide

generated during alloxan treatment may increased insulin release resulting in early decrease in blood glucose.

Alloxan induced LPO in rat erythrocytes through the generation of hydrogen peroxide. The extent of peroxidation deactivates the antioxidant enzymes SOD and GSH–Px suggesting overproduction of reactive oxygen species. Protection by GSH and selenium reflects these as the targets of alloxan in the cell, signifying toxic effects of the drug on GSH metabolism and selenium status.

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