

## Glutathione and associated enzymes in toxic cataractogenesis-Selenite model

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(Received: 7 September, 2004 ; Accepted: 4 December, 2004)

**Abstract:** Glutathione,  $\gamma$ -glutamyl cysteine synthetase ( $\gamma$ -GCS) and glutathione reductase (GSH-R) activity were determined biochemically in the lens during various stages after subcutaneous administration of sodium selenite in multiple low dosages and single high dosages. The GSH concentration and  $\gamma$ -GCS and GSH-R activity declined progressively after the selenite administration. The changes observed were discussed in relation to the possible role of selenite interaction with GSH and the enzymes.

**Key words:** Selenite, Cataract, GSH,  $\gamma$ -GCS, GSH-R.

### Introduction

Glutathione (L- $\gamma$ -glutamyl-L-cysteinyl glycine) (GSH) is found in high concentration in the lens, and plays a major role in the protection of the lens by detoxification of drugs, reduction of active oxygen species and peroxides, as well as the disulphides and free radicals. It helps to maintain the thiol-disulphide status of cells, transport of amino acids by the gamma glutamyl cycle, and the removal of xenobiotic electrophiles from cells (Rathbun and Murray, 1991). GSH is present in high concentrations in ocular tissues, particularly in the lens (Reddy, 1971). Current thinking is that GSH plays a key role in reducing oxidative damage in the lens and that low levels of GSH are not compatible with lens clarity, and lead to cataract formation (Reddy, 1990; Rathbun, 1990).

Irreversible cataract has been noted to contain low levels of GSH irrespective of whether the cause was UV light, toxic compounds, a metabolic disorder, ageing, or an inheritable defect (Rathbun, 1990). The link between low levels of lens GSH and galactose induced cataract formation, has been well documented in experimental animal models like rats (Lou *et al.*, 1988), guinea pigs (Mackic *et al.*, 1994), and in selenite induced cataracts in rats (Devamanoharan *et al.*, 1991).  $\gamma$ -GCS is the first enzyme required for the two step synthesis of glutathione and GSH-R reduces the disulphide form of glutathione (GSSG) to the sulfhydryl form (GSH). During the present investigation, the lens GSH concentration and  $\gamma$ -GCS and GSH-R activity were analyzed in various stages after selenite administration of different chronic and acute dosages in wistar rats.

### Materials and Methods

The induction and various stages of selenite cataract were produced in both sexes of wistar rat pups using multiple low dosages of 14.4, 19.2, 23, and 27.8 $\mu$ g. sodium selenite/~20g. rat pups subcutaneously, and single high dosages of 32, 40, 56 and 64 $\mu$ g./~20g. rat pups subcutaneously. For multiple low dosages, five doses were given starting on day eight *post partum* and for single high

dosages; a single dose was given on 10th day *post partum*. The dosage was determined after Ostadalova (1978), Bunce and Hess (1981) and our own experimental studies in wistar rats (Mathew *et al.*, 1997).

Biochemical analysis was carried out in different experimental groups, and their controls. Nuclear stage of cataract produced by low dosages, nuclear, mature and hyper mature stages of cataract produced by high dosages, along with their age related controls were used for the biochemical study. Besides this, a first to fifth day biochemical analysis of the lens with their controls, (on each day) after the administration of 40 $\mu$ g. sodium selenite/rat pup, was also carried out. At the required time, the rat pups were sacrificed and the complete eye lenses were dissected out immediately by the posterior approach. The weight of the lens samples were taken on a balance sensitive to 0.01 mg. Determinations of glutathione concentration in the samples were carried out by the method of Grunert and Philips (1951). The activity of  $\gamma$ -GCS in the lens was carried out by measuring the inorganic phosphate formed from ATP by the method of Fiske and Subha Row (1925), using standard  $\gamma$ -GCS assay procedure (Rathbun, 1967). The GSH-R activity was assayed by the method of Goldberg and Spooner (1983). Statistical analysis was performed using ANOVA and student's t-test.

### Results and Discussion

A dosage dependent reduction in the glutathione concentration was observed after selenite administration with all the experimental groups studied (Table 1, 2 and 3). The observation on each day after selenite administration (Table 1) showed the significant decrease even after 24hr of administration. This is because the selenite has a direct influence on the glutathione content of the lenticular system.

Studies with selenium poisoning in experimental animals reported a loss of glutathione from blood and organs (Moxan and Rhian, 1943). Selenium was reported to involve in the non enzymatic oxidation of glutathione (Tsen and Tappel, 1958) as per the following reaction.

**Table – 1:** Glutathione concentration,  $\gamma$ -glutamyl cysteine synthetase ( $\gamma$ -GCS) and glutathione reductase (GSH-R) activities in the lens samples of a 40 $\mu$ g sodium selenite dosage from first to fifth day after administration (n=8).

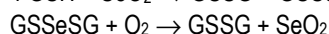
Days after administration	Glutathione $\mu$ g/mg wet wt.	$\gamma$ -GCS $\mu$ M.ip formed/g wet wt/h	GSH-R units/mg wet wt/h
Control	2.34 $\pm$ 0.01	1.39 $\pm$ 0.02	0.38 $\pm$ 0.01
1st Day	1.49** $\pm$ 0.01	1.36 <sup>NS</sup> $\pm$ 0.01	0.38 <sup>NS</sup> $\pm$ 0.02
Control	2.35 $\pm$ 0.02	1.41 $\pm$ 0.02	0.35 $\pm$ 0.04
2nd Day	1.47** $\pm$ 0.02	1.31** $\pm$ 0.04	0.30** $\pm$ 0.02
Control	2.37 $\pm$ 0.01	1.51 $\pm$ 0.04	0.34 $\pm$ 0.01
3rd Day	1.45** $\pm$ 0.01	1.24** $\pm$ 0.03	0.23 $\pm$ 0.02
Control	2.41 $\pm$ 0.01	1.56 $\pm$ 0.02	0.36 $\pm$ 0.03
4th Day	1.43** $\pm$ 0.02	1.25** $\pm$ 0.04	0.19** $\pm$ 0.02
Control	2.43 $\pm$ 0.02	1.59 $\pm$ 0.05	0.27 $\pm$ 0.02
5th Day	1.41** $\pm$ 0.01	1.25** $\pm$ 0.03	0.14** $\pm$ 0.03

\*\* p<0.01, NS - Not significant, All values are Mean  $\pm$  S.D.

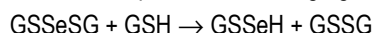
**Table – 2:** Glutathione concentration,  $\gamma$ -glutamyl cysteine synthetase ( $\gamma$ -GCS) and glutathione reductase (GSH-R) activities in the lens samples of selenite cataract with multiple low (chronic) dosage of sodium selenite (n=8).

Parameters	Nuclear stage Dosage in $\mu$ g/~20 mg rat pup				
	Control	9.6	14.4	23	27.8
Glutathione $\mu$ g/mg wet wt.	2.77 $\pm$ 0.10	1.92** $\pm$ 0.05	1.80** $\pm$ 0.08	1.67** $\pm$ 0.07	1.47** $\pm$ 0.07
$\gamma$ -GCS $\mu$ M. ip formed/g wet wt./h	1.66 $\pm$ 0.05	1.35** $\pm$ 0.05	1.27** $\pm$ 0.05	1.21** $\pm$ 0.06	1.18** $\pm$ 0.05
GSH-R Units/mg wet wt.	0.25 $\pm$ 0.02	0.22* $\pm$ 0.03	0.19** $\pm$ 0.03	0.18** $\pm$ 0.05	0.12** $\pm$ 0.03

\* p<0.05, \*\* p<0.01, All values are Mean  $\pm$  S.D.



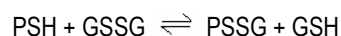
Selenodiglutathione (GSSeSG) is converted to glutathione selenopersulfide in the presence of enough glutathione



The different oxidised forms of glutathione have serious actions within the body. In order to regenerate the GSSG formed, a satisfactory amount of NADPH is required as the source to reduce GSSG to GSH. Bunce and Hess (1981) observed an abrupt decrease at 24 hr after the selenite administration in NADPH pool, and a recovery by 72 hr, would mean that the HMS activity initially was unable to meet the increased demand. It is clear that the progressive loss of glutathione observed in the various experimental groups, was due to the extensive oxidation of glutathione by selenite. The dosage dependent loss point towards the fact that an increased selenite stress further catalyses the process.

Out of the many biological functions ascribed to the GSH in the lens, its role in the protection of SH groups of protein, is possibly the most important. Oxidised glutathione (GSSG) produced by oxidation of GSH, can react with SH

groups of lens crystallins, to produce protein glutathione mixed disulphides (PSSG) (Mostafapour and Reddy, 1982/83; Shearer et al., 1992). This result is consistent with the hypothesis that bovine  $\gamma^I$  crystallins possess accessible cysteine residues that are available for reaction with oxidised glutathione by a disulphide exchange mechanism (Torehinsky, 1981). The PSSG so formed functions as potential reservoir for GSH and/or act to protect the lens against protein aggregation caused by protein-protein disulphide (PSSP) linking. Harding (1970) has postulated that PSSG is probably in equilibrium with free glutathione.



Normally, the free GSH keeps the protein bound GSH at a low concentration, and the GSSG formed is reduced to GSH by NADPH, and glutathione reductase. Since in cataractous lenses, as the level of GSH decreased the above reaction would tend towards an increase in PSSG formation. In fact, measurements of Harding (1970) showed that the level of PSSG increased in cataract. GSSG can penetrate, from its site of formation in the cortex, into the inner parts of the lens, to perform the above mentioned role, since it is chemically less

**Table – 3:** Glutathione concentration,  $\gamma$ -glutamyl cysteine synthetase and glutathione reductase activities in the lens samples of selenite cataract with various high (acute) dosages of sodium selenite (n=8).

Nuclear stage		Dosage in $\mu\text{g}/\sim 20$ mg rat pup				
Parameters	Control	32	40	56	64	
Glutathione $\mu\text{g}/\text{mg}$ wet wt.	2.40 $\pm 0.05$	1.40** $\pm 0.07$	1.32** $\pm 0.05$	1.21** $\pm 0.06$	1.11** $\pm 0.04$	
$\gamma$ -Glutamyl cysteine synthetase ( $\gamma$ -GCS) $\mu\text{M}$ ip formed/g wet wt./h	1.69 $\pm 0.08$	1.41** $\pm 0.13$	1.34** $\pm 0.08$	1.17** $\pm 0.08$	1.14** $\pm 0.08$	
Glutathione reductase (GSH-R) Units/mg wet wt.	0.29 $\pm 0.04$	0.18** $\pm 0.04$	0.15** $\pm 0.03$	0.13** $\pm 0.02$	0.12** $\pm 0.03$	
Mature stage		Dosage in $\mu\text{g}/\sim 20$ mg rat pup				
Parameters	Control	32	40	56	64	
Glutathione $\mu\text{g}/\text{mg}$ wet wt.	2.65 $\pm 0.05$	1.11** $\pm 0.07$	1.06** $\pm 0.04$	0.88** $\pm 0.04$	0.80** $\pm 0.04$	
$\gamma$ -Glutamyl cysteine synthetase ( $\gamma$ -GCS) $\mu\text{M}$ ip formed/g wet wt./h	1.80 $\pm 0.03$	0.86** $\pm 0.08$	0.79** $\pm 0.08$	0.68** $\pm 0.06$	0.63** $\pm 0.07$	
Glutathione reductase (GSH-R) Units/mg wet wt.	0.23 $\pm 0.02$	0.12** $\pm 0.02$	0.11** $\pm 0.02$	0.10** $\pm 0.02$	0.08** $\pm 0.03$	
Hyper mature stage		Dosage in $\mu\text{g}/\sim 20$ mg rat pup				
Parameters	Control	32	40	56	64	
Glutathione $\mu\text{g}/\text{mg}$ wet wt.	2.82 $\pm 0.05$	0.82** $\pm 0.03$	0.73** $\pm 0.03$	0.55** $\pm 0.03$	0.40** $\pm 0.04$	
$\gamma$ -Glutamyl cysteine synthetase ( $\gamma$ -GCS) $\mu\text{M}$ ip formed/g wet wt./h	1.85 $\pm 0.03$	0.69** $\pm 0.02$	0.61** $\pm 0.02$	0.57** $\pm 0.02$	0.50** $\pm 0.03$	
Glutathione reductase (GSH-R) Units/mg wet wt.	0.21 $\pm 0.02$	0.09** $\pm 0.01$	0.08** $\pm 0.02$	0.05** $\pm 0.02$	0.02** $\pm 0.01$	

\*\*  $p < 0.01$ , All values are Mean  $\pm$  S.D.

polar than GSH (Mostafapour and Reddy, 1982/83). The presence of high levels of protein-glutathione mixed disulfides in the inner parts of the lens (Harding, 1970; Reddy and Han, 1976) lends support to this notion about the role of GSSG. Furthermore, GSH can reduce the disulphide bond of PSSG to regenerate PSH (Mostafapour and Reddy, 1982/83; Augusteyn, 1979). Shearer *et al.* (1992) suggested that  $\gamma$ -crystallins, in particular  $\gamma^{\text{I}}$  crystallins, can readily react with reduced glutathione (GSH) resulting in its oxidation and the removal of cysteine residues from the cluster in the N-terminal domain of  $\gamma$ -crystallin is associated with the decrease in the sites available for reaction with GSH.

Thus by the above mentioned process, the protein sulfhydryl groups are preserved, and this prevents the formation of high molecular weight protein aggregates which cause the light scattering and opacification. The significant loss of GSH observed with the maturation of selenite cataract is an accelerating factor leading to complete opacity. It is reported

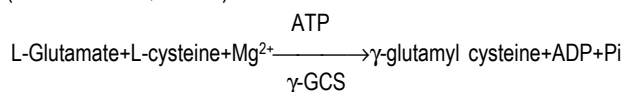
that decreased level of hexokinase and G-6-PDH activity and a reduction in the level of GSH itself would cause a marked decrease in GSH regeneration rate (Ogawa *et al.*, 1996). A decrease in G-6-PDH activity was also observed during our studies (Mathew *et al.*, 1999).

GSH stabilises the membrane permeability and ion homeostasis by protecting the membrane SH groups of the epithelial cells and fibre membranes in the reduced state. The oxidation of critical membrane SH group is the primary cause observed in selenite cataract (Rogers and Augusteyn, 1978). The critical membrane SH groups are involved in the regulation of cation transport besides those transport enzymes like ATPases. A significant loss of GSH in the selenite treated lens will accelerate the oxidation of critical SH group involved in the regulation of ion homeostasis.

Other functions attributed to the glutathione include the reduction of disulphides, free radicals and peroxides, in which GSH gets itself oxidised to GSSG. The role of glutathione

as a shuttle for reducing equivalents in coupled redox reactions with glutathione reductase and glutathione peroxidase, would not deplete the high concentrations. It follows that glutathione takes part in reactions that involve either degradation or its loss from lens cells (Rathbun *et al.*, 1983). An inter organ transport system is functioning whereby a synthesis of glutathione in different tissues of the body, is followed by its inputs into the plasma circulation and uptake elsewhere as required (Rankin *et al.*, 1983). However, there is also a recycling of GSH within a cell through the activity of the enzymes of the  $\gamma$ -glutamyl cycle. The rate of synthesis of glutathione is high in the lens, and it has been estimated that half the glutathione is replaced every 28 hours (Reddy *et al.*, 1966). A loss of decreased enzyme activity of  $\gamma$ -glutamyl cycle in cataract (Rathbun and Murray, 1991; Rathbun *et al.*, 1993) results in further depletion of glutathione concentration in the cataract lens. Other studies have demonstrated that transport systems in the lens become deficient under pathological conditions, such as the GSH depletion (Reddy and Han, 1976). Zlokovic *et al.* (1992) have reported that the GSH transport from blood to lens is abolished in guinea pigs with galactose induced cataract. In selenite cataract, a decrease observed in glutathione concentration during the present study would further weaken the defensive system against the lens oxidative damage.

A significant decrease in  $\gamma$ -GCS activity was observed with the low- and high-dosage group (Table 2 and 3). The day to day monitoring of  $\gamma$ -GCS activity revealed a progressive decline on each day after selenite administration (Table 1). All the observed decrease was found to be dosage-dependent. The above findings were supportive of the fact that a significant decrease was observed with the GSH concentration in the advanced cataract stages. A high decrease in the  $\gamma$ -GCS activity retards the glutathione synthesis.  $\gamma$ -GCS, which combines L-glutamate and L-cysteine in a peptide bond, is the rate limiting enzyme, while L-cysteine is the rate limiting substrate of lenticular glutathione synthesis (Rathbun *et al.*, 1986a).



The second step involves the combination of glycine to  $\gamma$ -glutamyl cysteine in the presence of glutathione synthetase to yield GSH. Normally, the glutathione synthesis in the lens is very high, since about 12% of ATP derived from glycolysis in the lens, is used for the process (Rathbun, 1982). Glutathione is the primary antioxidative agent present in the lens, to defend the various oxidative insults. A significant reduction in the  $\gamma$ -GCS activity during the advanced cataract stages further weakens the system, due to decreased production of GSH, and increased oxidation of glutathione.

The rate of glutathione synthesis was shown proportional to the uptake of L-cysteine from the medium (Rathbun and Murray, 1991). Damage to this vulnerable transport system by selenite enhances the cataractogenesis.

The transport of L-cysteine was shown to diminish 70% with ageing in cultured human lenses (Rathbun *et al.*, 1993). The GSH depletion caused by the treatment with buthionine sulfoximine, a specific inhibitor of  $\gamma$ -GCS (Griffith and Meister, 1979), induces the formation of cataracts in mice (Calvin, 1986). The inhibition of glutathione synthesis enhanced a pathway which competed with  $\gamma$ -GCS for the use of L-cysteine, which most likely was the pathway leading to the formation of hypotaurine and taurine (Rathbun and Murray, 1991). A reduction in the  $\gamma$ -GCS activity observed in the present study, increases the chance for decreased GSH production. Since glutathione plays a major role in the metabolism and maintenance of the transparency of the lens, a decreased activity of  $\gamma$ -GCS and glutathione biosynthesis is the possible explanation given, to the maturation of selenite cataractogenesis.

A dosage dependent decrease in glutathione reductase (GSH-R) activity was observed with the low and high dosage group studied (Tables 2 and 3). A progressive reduction was observed with 40  $\mu\text{g}$ . dosage with a high decrease noted by the 3rd day after selenite administration (Table 1). The results revealed that the initial attack of selenite is not on the enzyme system. This is further proved by the fact that a highly significant drop in the enzyme activity was only observed with the advanced stages of the cataract.

One of the major mechanisms believed to protect the lens from oxidative stress involves the enzyme glutathione reductase (GSH-R). It uses NADPH to reduce the disulphide form of glutathione (GSSG) to the sulfhydryl form (GSH) that can protect the lens constituents from damage by various oxidants, including  $\text{H}_2\text{O}_2$  and free radicals (Reddy, 1990). It has been demonstrated that oxidative stress damages lens enzymes (Jedziniak *et al.*, 1987). A decrease has been found in the level of GSH-R in cataractous lenses (Ohrloff *et al.*, 1984); supporting the view that cataract arises as a result of oxidative damage due to reduction in the antioxidative systems.

The hexose monophosphate shunt pathway (HMP) provides NADPH, for the reduction of oxidised glutathione, through the glutathione reductase system. When cells are subjected to oxidative stress, this pathway is markedly stimulated due to an increase in the availability of NADP, resulting from the activity of the glutathione reductase system. Evidence has been presented that the availability of GSSG is the rate limiting step in HMP metabolism (Paniker *et al.*, 1970), and that under excess GSSG, glutathione reductase is the rate controlling enzyme of the glutathione redox cycle (Rathbun *et al.*, 1986a). Since selenite cataractogenesis is primarily an oxidative event, a decreased activity of glutathione reductase further enhances the process of cataractogenesis. Glutathione concentration was observed to decrease significantly after selenite administration in the present study. Huang *et al.* (1992) reported a decrease in the GSH-R activity in selenite cataract. Since a significant decrease in the GSH-R activity was observed with the advanced stages of selenite cataract, it seems probable

that the observed decrease was due to the cataract formation rather than the cause of selenite cataract.

The GSH-R activity was found to decrease in the control lens with the increasing age, in the present study. A similar decrease was reported in the human, bovine, and Rhesus monkeys (Rathbun *et al.*, 1986b; Ohrloff *et al.*, 1984). This decrease is not due to ageing, since the total activity represents an average for the whole tissue as the lens consists of new and older cells in the cortex and nucleus which have different activities. Zhang and Augusteyn (1994) claimed that the decrease in activity is not due to ageing but it is related rather to the interaction of the fibre cells. One possibility is that GSH-R is rapidly turned over or inactivated in the maturing fibre cells, and that its replacement decreases as the cell matures. In the absence of further GSH-R synthesis, this would reduce the specific activity of the enzyme (Zhang and Augusteyn, 1994). Since the only function of the fibre cell is to provide a medium of high refractive index in the lens, there would be no requirement for any metabolic activity, and the synthesis of unwanted enzymes would be eliminated. Thus the observed decrease in the GSH-R activity is a part of the fibre-cell maturation. The mature cells would then totally depend on the overlying epithelial and new fibre cells, to protect them from various external stresses.

#### Acknowledgments

The work was supported by financial assistance from Mahatma Gandhi University, Kottayam. We are deeply indebted to former Principal, Rev. Dr. W. T. Philips, and former Bursar, Fr. Jose Virupel, St. Berchmans' College for providing all the required facilities in the laboratory.

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