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Sensitivity of Redox Cycle Enzymes in Substantiating the Pathophysiology of Cataract

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Abstract: Oxidative modifications play major role in the formation of cataract. Lens contains several protective mechanisms against oxidizing agents viz. catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, and ascorbic acid. To explore the oxidative damage that might be occurring with 'Invitro' development of cataracts induced by sugars, H_2O_2 and steroids. We examined redox status of cataractous lenses by analysing enzymatic defence meachanisms. Lenses were exposed to glucose (50 mM) (Group – I); galactose (35 mM) (Group – II) and xylose (30 mM) (Group – III) and maintained at 37°C for 72 hours so as to induce sugar cataract. H_2O_2 cataract was produced by adding 50 mM (Group – IV) and 100 mM (Group – V), solution to culture media (AAH). Steroid cataract was generated by adding a freshly prepared l x 10^{-4} M dexamethasone (mw 392.5) (Group – VI) in absolute alcohol to the culture media (AAH) and incubated at 37°C for 72 hours. Subsequent to the development of the cataract, the lenses were homogenized and the specific activity of the enzymes catalase (CAT), glutathione peroxidase and glutathione reductase was assessed. Catalase activity did not show any significant decrease in sugar cataract and steroid cataract but a significant decrease was observed in H_2O_2 cataract. However a significant decrease in GSH-Px and GSH-Rx were found in all the three types of experimental cataract as compared to control lenses.

Keywords: Cataract, Catalase, Glutathione Reductase, Glutathione Peroxidase, Free Radicals

1. Introduction

The study of enzymes is a subject which has a special interest because it lays just on the borderline where the biological and the physical sciences meet. One hand, enzymes are of supreme importance in biology, life depends on a complex network of chemical reactions brought about by special enzymes and any modifications of enzymes pattern may have far reaching consequences for the living organisms. On the other hand, enzymes as catalysts are receiving increasing attention. The lens contains a great number of enzymes, usually at a very low concentration, but very little is known of their nature apart from their metabolic activity, whether they are distinct species existing independently of structural proteins, whether they are in some sort of conjugation with the crystallins or whether any of the crystallins bear enzymatically active sites is not known.

One of the various ancillary complications linked with diabetes mellitus is the formation of cataract. Regrettably, the causes of cataract formation in relation to diabetes mellitus are not well understood. The most probable relationship of the higher incidence of cataract in diabetics [1] was attributed to the sorbitol pathway and the enzyme aldose reductase involved in the glutathione metabolism in the lens.

Numerous oxidative modifications of lens constituents were reported to be involved in senile nuclear cataract. A variety of free radicals like superoxide, hydroxyl; as well as oxidants such as hydrogen peroxide (H_2O_2) could lead to the oxidative modifications in the lens. A series of defense mechanisms are available in the lens like other tissues which can safeguard it from the harmful effects of oxidation. Very

little is known about such defense mechanisms. Glutathione (GSH) plays major role in the defence system against oxidative damage. The defence system largely depends on antioxidant enzymes i.e., glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rx), catalase (CAT) and glucose-6-phosphate dehydrogenase (G-6-PD). Other vitamins including ascorbic acid, alpha tocopherol and betacarotene (precursor of vitamin A) function as intracellular and extracellular antioxidant [2].

Today even though cataract can be cured by surgery [3], any means of preventing it still eludes us. It is in this context, the present study is planned to find out some means of preventing or delaying cataract formation. This can be solely achieved by intense research in this area all over the world.

Oxidative modifications are thought to play a major role in the formation of cataract. These oxidative modifications are caused by various oxidants such as superoxide anions, hydrogen peroxide, singlet oxygen (CO_2), hydroxyl radicals (OH). These oxidants can cause oxidation of sulphydryl groups in membrane protein, crystallins and as those of enzymes. Protein precipitation due to the formation of high molecular weight (HMW) aggregates is as a result of oxidation of crystallins. There are no proven and reputable results showing the sequesnce of different oxidative modifications in the lens. However the lens contains several protective mechanisms against oxidizing agents. These include glutathione reductase glutathione peroxidase, catalase, superoxide dismutase, and ascorbic acid.

Glutathione reductase system is another important protective system in the lens. The exact function of reduced glutathione in the lens is not clear. It is considered that development of cataract is closely connected with ageing process [4]. The pathogenesis of senile cataract is based on the disturbance in carbohydrate metabolism. It has long been established in animal models the involvement of sorbitol pathway in diabetic cataractogenesis, whereas in human diabetic cataract formation, its role is not clear as yet.

In view of this, the present study was planned to study the overall picture of protective systems like redox cycle enzymes (i.e. Catalase, glutathione peroxidase and glutathione reductase), so as to assess and identify sensitivity of these enzymes in substantiating the pathophysiology of cataract.

2. Materials and Methods

A total of 480 fresh goat lenses were used to induce cataractogenesis artificially. The eye balls of goats were obtained from the slaughter house and they were brought to the laboratory in an ice bath. Lenses were removed with cataract knife by intracapsular lens extraction method from the eyeballs within one hour of the death of the animal and were preserved at -70°C until further use. The age of the animal and wet weight of lenses were recorded. These lenses were incubated in a culture media used for "Lens organ culture technique" [5].

Cataracts were induced artificially using three different methods viz. Sugar, H_2O_2 and Steroid Cataract. Lenses were placed on a sterile tissue culture dish having a dark blue coloured nylon net and were maintained in above tissue culture media. The culture media was bubbled with 95% oxygen and 5% Carbon dioxide and lenses were incubated in a humidified chamber at 37°C for 72 hours without any additions, which served as a control.

In the experimental groups, lenses were exposed to glucose (50 mM) (Group – I); galactose (35 mM) (Group – II) and xylose (30 mM) (Group – III) and maintained at 37°C for 72 hours so as to induce sugar cataract. In order to produce H_2O_2 cataract, freshly prepared, 30% H_2O_2 solution (mw 34.01) was added to culture media (AAH) in such a way that the final concentration of H_2O_2 attained was 50 mM (Group – IV) and 100 mM (Group – V). Similarly, steroid cataract was generated by adding a freshly prepared l x 10^{-4} M dexamethasone (mw 392.5) (Group – VI) in absolute alcohol to the culture media (AAH) and incubated at 37°C for 72 hours.

Subsequent to the development of the cataract, the lenses were homogenized in a glass homogenizer with a Teflon grinder immersed in an ice bath and 5 µl pentanol was added to the homogenate before homogenization to prevent frothing.

For the determination of enzyme activities 10%(w/v*)* homogenate was prepared in 0.02 m. sodium phosphate buffer (pH 7.4) and for the estimation of ascorbic acid, 10%(w/v*)* homogenate was prepared in double distilled water. The homogenate was centrifuged at 10,000 g for 30 minutes at -4°C in a refrigerated centrifuge. The supernatant fluid was used for the estimation of biochemical analysis. All the assays were performed within a week of collection of lenses.

The specific activity of the enzyme catalase (CAT) was carried out by the method described by Aebi (1967) [6]. The supernatant (from 10% homogenate) as the source of the enzyme was diluted 0.2 ml to 100 ml in chilled 50 mM phosphate buffer just before use. 2.0 ml of this sample solution containing the enzyme was pipetted into a cuvette and the reaction was started by the addition of 1.0 ml of 10 mM H_2O_2. A reference cuvette was prepared containing 2.0 ml of enzyme solution, but no substrate (H_2O_2) and 1.0 ml phosphate buffer. The solutions were mixed well with a plastic paddle, and the decrease in extinction was followed on Perkin Elmer UV Visible spectrophotometer (Model Coleman 55) at the wavelength of 240 nm, light path 1 cm. read at 25 ± 0.1°C for 30 seconds. The readings were taken at 10 seconds interval. The values for E240/15 secs were between 0.10 and 0.02. A unit of catalase activity is the amount of enzyme which liberates half the peroxide oxygen from a H_2O_2 solution of any concentration (Co. 10 mM) in 100 secs at 25°C. The unit being related to half life time λ of a first order.

The activity of the enzyme glutathione peroxidase was assayed using the method of Mills and Randall (1958) [7] with a few modifications. The reaction was initiated by blowing rapidly 1.0 ml of 0.0018 M H_2O_2 into the assay

system {containing 0.6 ml of sample (supernatant at 15,000 r. p. m), 0.6 ml of buffered saline, 0.6 ml of reduced glutathione (2 mg/ ml) and 3.0 ml of 0.002 M EDTA + 0.005 M Sodium azide} at zero time. The reaction was stopped exactly at 2 and 3 minutes intervals. This was accomplished by transferring 2.0 ml aliquots into tubes containing 5.0 ml of 0.7% metaphosphoric acid. To this was then added 2.0 ml of 7.5% metaphosphoric acid and 3.0 g sodium chloride. This was followed by a vigorous shaking of the solution for about 8 minutes. The solutions were filtered and the filtrate was used for GSH determination by the method of Beutler (1963) [8] i.e.2.0 ml of the filtrate was added to 2.5 ml tris buffer and 0.2 ml DTNB reagent. Optical density was read at 412 nm on Carl Zeiss PMQ II spectrophotometer. A reagent blank was prepared by substituting 2.0 ml of distilled water for 2.0 ml of the filtrate.

Specific activity of Glutathione Reductase was assessed by the method of Racker (1955) [9] as described by Carlber and Mennervik (1975) [10]. Briefly, the reactionwas initiated by addition of 0.2 ml of 10% (W /V) homogenate to the reaction mixture (2.8 ml) consisting of 2 ml of 250 µM of Tris-Hcl buffer (pH 8.0), 0.2 ml of 1.5 µM of EDTA, 0.2 ml of 9.9 µM of GSSG and 0.2 ml of 0.3 µM of NADPH. The decrease in optical density at 340 nm was noted for 6-9 minutes continuously and the enzyme activity was expressed as, micro mole of NADPH oxidized per lens per minute using its extinction coefficient value of 6.1 m^{-1} cm^{-1}. During the period of assay the activity of the enzyme studied were found to be linear.

3. Results

Catalase activity in goat lenses and experimental cataractous lenses is presented in table – 1 and figure -1. Group I and Group II lenses showed decrease of 30% and 45% in catalase activity as compared to control lenses. This decrease was not statistically significant. Group III lenses showed 56% decrease in catalase activity and was statistically not significant (P > 0.1). Group IV lenses showed 73% decrease in catalase activity as compared to control lenses. This decrease was statistically significant. (P < 0.5) The decrease of 64% was observed in Group V lenses and was statistically significant (P < 0.05). Group VI lenses showed 32.8% decrease in catalase activity as compared to control lenses and it was statistically insignificant (P > 0.l).

Table 1. *Catalase activity in goat lenses and experimental cataractous lenses.*

Groups	Type of Lenses	No. of Lenses	GSH-PX Specific activity U/mg Protein Mean ± S. D.
Control		60	1.25 ± 0.44
Experimental Cataracts			
A. Sugar Cataract			
Group - I	Glucose induced cataract	10	0.87 ± 1.01***
Group - II	Galactose induced cataract	10	0.68 ± 0.92***
Group - III	Xylose induced cataract	10	0.55 ± 0.11***
B. H_2O_2 Cataract			
Group - IV	50 mm H_2O_2 induced cataract	10	0.33 ± 0.16*
Group - V	100 mm H_2O_2 induced cataract	10	0.45 ± 0.21**
C. Steroid Cataract			
Group - VI	Steroid Cataract	10	0.84 ± 0.17***

* P < 0.05; ** P < 0.001*** P > 0.1

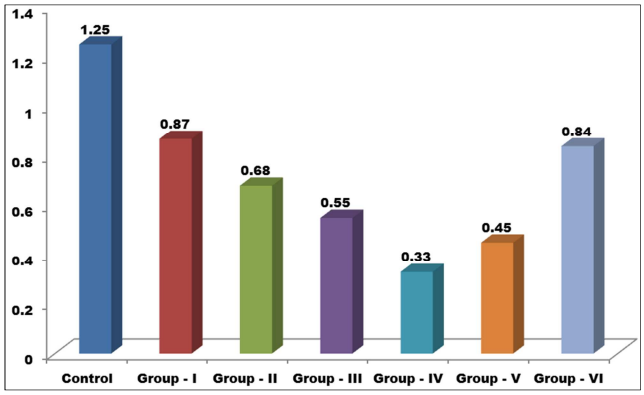

Figure 1. *Catalase activity in goat lenses and experimental cataractous lenses.*

Glutathione peroxidase (GSH-PX) activity in goat lenses and experimental cataractous lenses is presented in table – 2 and figure -2. Group-I and group-II lenses indicated a decrease of 28% and 43% in the enzyme activity. This decrease was statistically significant. A lowest decrease of 6% was observed in group-III lenses as compared to control lenses. This decrease was not significant statistically. Group-IV and group-V lenses showed the highest decrease of 74% and 78% in enzyme activity respectively, as compared to control lenses. This decrease was very much significant statistically (P 0.001). A decrease of 29% was observed in group-VI and it was statistically significant.

Table 2. *Glutathione peroxidase (gsh-px) activity in goat lenses and experimental cataractous lenses.*

Groups	Type of Lenses	No. of Lenses	GSH-PX Specific activity U/mgProtein Mean ± S. D.
Control		60	7.93 ± 2.7
Experimental Cataracts			
A. Sugar Cataract			
Group - I	Glucose induced cataract	10	5.63 ± 0.83 *
Group - II	Galactose induced cataract	10	4.50 ± 2.40*
Group - III	Xylose induced cataract	10	7.42 ± 3.02
B. H_2O_2 Cataract			
Group - IV	50 mm H_2O_2 induced cataract	10	2.04 ± 0.47**
Group - V	100 mm H_2O_2induced cataract	10	1.70 ± 0.70**
C. Steroid Cataract			
Group - VI	Steroid Cataract	10	5.64 ± 0.43*

* P <0.05 ** P< 0.001

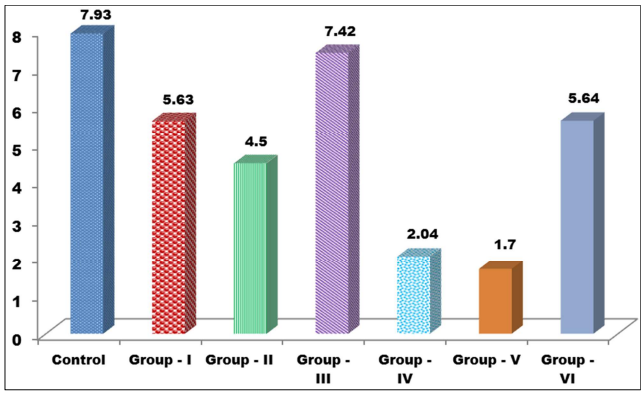

Figure 2. *Glutathione peroxidase (GSH-PX) activity in goat lenses and experimental cataractous lenses.*

Glutathione reductase (GSH-RX) activity in goat lenses and experimental cataractous lenses is presented in table – 3 and figure -3. Group I and Group III lenses showed a decrease of 51% and 50% in the enzyme activity as compared to control lenses. This decrease was statistically significant. The lowest enzyme activity was observed in Group II lenses of sugar cataract. This decrease was 58% when compared with control lenses which was statistically highly significant ($P < 0.001$). A decrease of 36% and 45% in the enzyme activity was observed in Group IV and Group V lenses as compared to control lenses. This decrease was statistically significant. ($P < 0.05$). Group VI lenses showed the lowest enzyme activity as compared to control lenses. This decrease was 61% and was statistically highly significant.

Table 3. *Glutathione reductasease (gsh-rx) activity in goat lenses and experimental cataractous lenses.*

Groups	Type of Lenses	No. of Lenses	GSH-PX Specific activity U/mgProtein Mean ± S. D.
Control		60	0.13 ± 0.01
Experimental Cataracts			
A. Sugar Cataract			
Group - I	Glucose induced cataract	10	0.063 ± 0.013*
Group - II	Galactose induced cataract	10	0.054 ± 0.010**
Group - III	Xylose induced cataract	10	0.065 ± 0.012*
B. H_2O_2 Cataract			
Group - IV	50 mm H_2O_2 induced cataract	10	0.071 ± 0.01*
Group - V	100 mm H_2O_2induced cataract	10	0.083 ± 0.012*
C. Steroid Cataract			
Group - VI	Steroid Cataract	10	0.05 ± 0.014**

* P <0.05 ** P < 0.001

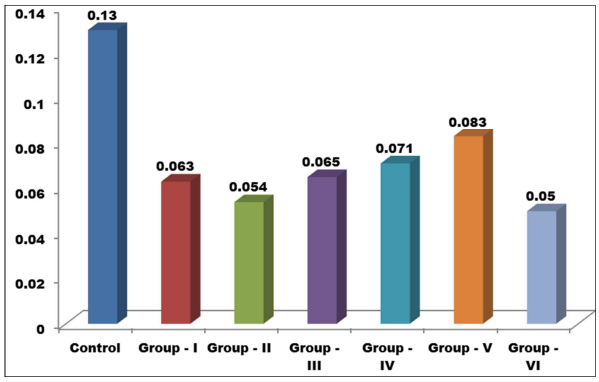

Figure 3. *Glutathione reductase (GSH-RX) activity in control goat lenses and experimental cataractous lenses.*

4. Discussion

Every important advance in the understanding of the disease often stems from basic research and has significant impact on medical practice. However, the process of dissemination of such information to the clinician is quite incomplete. Nowhere is this phenomenon better illustrated than in case of cataract. Experimental investigations of cataracts however have still limited value in our efforts to understand the etiology of most human senile cataracts. Experimentally it is possible, by selecting suitable conditions, to implicate individual factors as the causes of opacities. Clinical cataracts can only in certain instances be traced to such a simple mechanism. It is usually very

difficult to determine the precise locus of the major commotion within the multi-complex system of lens metabolism. Because of the large proportion of structural proteins (Crystallins and Albuminoid) in the lens and their importance in determining optical properties of the lens, much research has centered on their fractionation and characterization. The study of enzymes, as proteins is consequently languished.

The biochemical and molecular mechanisms underlying cataract formation are not clearly understood yet. Oxidative modification of lenticular constituents, occurring primarily as a result of oxygen derived active species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$)and hydroxyl radical (OH^-)either generated in the cell during normal metabolic reactions or induced due to interplay of external factors (UV light exposure and subsequent photo-oxidation) probably have a role in protein aggregation and lens opacification.

The objectives of the present study were to explore the possibilities of oxidative damage that might be occurring with 'Invitro' development of cataracts induced by sugars (glucose, galactose & xylose), H_2O_2 and steroids. We examined redox status of cataractous lenses by analysing enzymatic defence mechanisms.

We found that catalase activity did not show any significant decrease in sugar cataract and steroid cataract but a significant decrease was observed in H_2O_2 cataract. However a significant decrease in GSH-Px and GSH-Rx were found in all the three types of experimental cataract as compared to control lenses. (Table Nos. 1, 2, 3). Several oxidative modifications of lens constituents were reported to be involved in senile nuclear cataract. Some of these are oxidation of glutathione, oxidation of cysteine and methionine residues in a protein, protein-protein disulfide bond formation and protein thiol compound, disulfide exchange of disulfide bonds, exposure of sulfydryl groups due to protein unfolding with protein insolubilization and protein aggregation [11,12]. As the age prolongs most of the changes mentioned will become prominent but at a minimal pace [13]. Oxidation is the principal commencing element finally culminating in the lens opacification.

Diverse free radicals like peroxides, hydroxyl ions and hydrogen peroxide (H_2O_2) can lead to oxidative modifications in the lens. The ultraviolet reduction of oxygen, ionizing oxidation or photo-oxidation can give rise to these oxidizing agents in the lens [11]. Different protective enzyme mechanisms, like catalase, superoxide dismutase, and glutathione peroxidase maintain these oxidants at very low nontoxic levels in the normal lenses.

Fecondo and Augusteyn (1983) [14] suggested that failure of one of these mechanisms could result in an increase of oxidative stress in the lens. The present study is related to the chief protection systems that shield the lens from oxidative insult. The enzymes of the anti-oxidant defence system include superoxide dismutase, catalase, glutathione peroxidase glutathione reductase and glucose, 6-phosphate dehydrogenase. The activities of catalase, glutathione peroxidase and glutathione reductase were measured in sugar, H_2O_2 and steroid induced cataract.

Our results are in good agreement with the reports of Fecondo and Augusteyn (1983) [14], Dwivedi and Pratap (1986) [15] Giblinet al (1987) [16], Reddan et al (1988) [17] Bhat et al (1989) [18]. The comparative efficacy of GSH-Px and catalase in detoxifying H_2O_2 has been of great debate in the scientific works. However in one of the studies, the primary defense mechanism against H_2O_2 in the lens was suggested to be the enzyme catalase [19]. This was established upon their statement that in cataracts stimulated using a catalase inhibitor viz. 3-amino-1H, 1, 2, 4-triazole (3-AT,), there was two to three times upsurge in the levels of H_2O_2 in the vitreous and aquous humours. They opined that in the lens also there could be a similar increase.

It is known that catalase regulates the intracellalar peroxide concentration to a nontoxic level. It reacts catalytically at a high concentration of H_2O_2 and peroxidatically at low concentration of H_2O_2 in the presence of suitable hydrogen donor [20, 21]. The evidence is available in the literature demonstrating an endogenous source of H_2O_2 generated by light catalyzed auto-oxidation of ascorbic acid in aqueous humor [22] and the presence of high catalase activity in lens capsule epithelium. It was observed that amizol in vivo produces cataract with a simultaneous fallin lens catalase, therefore indicates that catalase may be one of the important factors involved in cataractogenesis [23, 24].

Reduced activities of enzymes catalase, GSHPX, GSHRX in experimental cataracts in our study could be the result of intermolecular or intramolecular cross-linking of proteins induced by oxidation or other unknown factors. A contemplation of the comparative activities of the lens enzymes GSHPx and catalase and their varying kinetic behavior suggests that catalase become more important when the concentration far exceeds the physiological level whereas GSH-Px is more important when there are low levels of H_2O_2 in the tissues. Therefore in cataracts the swift decrease in GSH-Px activity shows that there may be noticeable reduction in the ability of this tissue to detoxify internal H_2O_2. The enzymes GSH-Px, GSH-Rx and G6PD (by generating NADPH) take part in the defence against oxygen species that are accountable for enhanced oxidative stress in the ocular tissues.

Giblin, et al., [25] in their 'in vitro' studies with lens have detected a connotation between increased oxidative stress and stimulation of HMP shunt pathway. In yet another study it has been shown that the activity of GSH-Px is lowered in animals deficient in selenium as its activity is dependent upon selenium [26]. Hence there is a benefit of doubt that selenium deficiency can be a cause in the development of senile cataract. Decreased activities of _GSH-Px and GSH-Rx in cataract and GSH-Rx and catalase could be a causative factor. Our laboratory reports are in confirmation of the previous findings [27, 28, 29].

5. What Was Known

- The biochemical and molecular mechanisms underlying cataract formation are not clearly understood yet.
- Oxidative modification of lenticular constituents, occurring primarily as a result of oxygen derived active species such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (OH^-) either generated in the cell during normal metabolic reactions or induced due to interplay of external factors (UV light exposure and subsequent photo-oxidation) probably have a role in protein aggregation and lens opacification.

6. What This Paper Adds

- Sugars, Hydrogen peroxide and steroids does produce cataract.
- The enzymes GSH-Px, GSH-Rx and G6PD (by generating NADPH) take part in the defence against oxygen species that are accountable for enhanced oxidative stress in the ocular tissues.

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