CHAPTER – 5: DISCUSSION

The main aim of this investigation on the experimental cataract models is to know the sequential changes occurring in the cataractous lens by slit lamp photography and biochemical studies. It is also to know the role and involvement of inducing factors in mechanism of cataractogenesis.

Several studies on different experimental models and on human cataract have now suggested that the level of various antioxidants in lens has important role in cataract development. But, a number of unresolved issues require further investigation. In this chapter the results obtained were described in terms of alterations in the morphology of lens and accordingly changes in the levels of protein, antioxidants and the protective enzymes.

The selenite model was selected for this study because of the rapid and reproducible formation of cataract. The rapid opacification in the selenite model has been associated with oxidation mechanisms, altered membrane transport, elevated intralenticular calcium or selenium levels and proteolysis (Shearer et al., 1987, 1992; Bunce et al., 1984) which are factors associated with modification of normal interactions between proteins and can by themselves or in combination produce lens opacity. While calcium-induced protease activity has been emphasized, cataract Formation in the selenite model appears to be multifactoral (Shearer et al., 1992) and the selenite induced opacities respond to anti-oxidants (Devamanoharan et al., 1991). The rate of opacification in the selenite model is much more rapid, the experimental results of the present study support the use of the selenite cataract as an important multifactoral model for the study of protein aggregation and opacification in cataracts.
As per the design of the study, first the anterior photography and then slit lamp photography was performed. The results of the slit lamp photography showed the significant effect of the aqueous extract of *Andrographis paniculata* which is having a potential effectiveness in restricting the progress of cataract development as well is is also found effective in regaining of the transparency of the cataractous lens.

The slit lamp photography and anterior photography was performed to observe the morphological changes occurring in the lens. The results have showed that there is a significant progress in gaining the transparency of the lens after instillation of the aqueous extract of *Andrographis paniculata*. After instillating the aqueous extract of *Andrographis paniculata* in the experimental animals, the level of aggregation is found decreased due to the changes occurred in the level of various enzymes which ultimately resulted in the regaining of the lens transparency. There are some of the antioxidants which are potentially significant in preventing cataract formation.

The results of the first month after instillation of the aqueous extract of *Andrographis paniculata* is showing that the level of aggregation of the proteins is decreased (Fig. 2.1). This decrease in the aggregation of the proteins is because of the effect of antioxidants present in the aqueous extract of *Andrographis paniculata*. The results show that as the time duration of the instillation of the aqueous extract of *Andrographis paniculata* increases, the lens becomes more and more transparent and the protein aggregation decreases. At the time after three months of instillation, the lens becomes clear and no opacification is found (Fig. 2.3).
5.1 Antioxidant activity:

It is known that Andrographis paniculata is having flavonoids and phenolics which acts as antioxidants and scavenges the free radicals forming in the biological systems. The major constituents of Andrographis paniculata are 14-Deoxy-11-dehydroandrographolide, 14-Deoxy-11-oxoandrographolide, 5-Hydroxy-7,8,2',3'-Tetramethoxyflavone, 5-Hydroxy 7,8,2' Trimethoxyflavone, Andrographine, Neoandrographolide, Panicoline, Paniculide-A, Paniculide-B, Paniculide-C (Basu and Basu, 1978; Chao and Linn, 2010).

The results show that the activity of the antioxidants found in the aqueous extract of Andrographis paniculata has shown significant difference in the level of antioxidants in the lenses of group 3 (Table - 2). The level of antioxidants gradually increases on instillation of the aqueous extract of Andrographis paniculata. The minor increase is found after the first month trial but its level increases after the third month and reaches nearer to the normal level of antioxidants of group 1 (Table - 4). It shows that the increase in the level of the antioxidants in the cataractous lenses are time dependant.

Graph : 1 Antioxidant activity after 1, 2 and 3 months trial

The antioxidants acts on sulphydryls and restricts the protein aggregation. In one of the study, in which various antioxidant agents are used to see its efficacy as an anti cataract agents and one of them was pantethine (T. HIRAOKA et al., 1996). Studies done by some researchers, a possible
mechanism to be considered is that pantethine acts to modify protein sulphydryls and inhibits an unfavourable increase in attractive forces leading to aggregation of lens proteins. Protein modification by aldehydes, succinimide, N-ethylmaleimide (NEM), N-bromoacetyl ethanolamine phosphate (NBAEP) and oxidized glutathione in lens proteins inhibited phase separation and protected against opacification (Clark and Benedek, 1980a; Siezen et al., 1985; Pande et al., 1993, 1994).

Age-related cataract is a significant problem worldwide. Oxidative stress has been suggested as a common underlying mechanism of cataractogenesis, and augmentation of the antioxidant defenses of the lens has been shown to prevent or delay cataract (Spector, 1995). Different agents with diverse chemical structures have shown antioxidant properties in different systems, and their beneficial effects have been demonstrated in various pathologic conditions including cataract (Gupta SK and Joshi S., 2001). During the past few years, great emphasis has been placed on the possible roles of physiologic and nutritional antioxidants in cataract, because cataract is a slowly progressing disease, and a person might have to be on life-long treatment once the cataract has developed.

Lycopene, which is a major carotenoid, also having antioxidative property. It is available primarily from tomatoes and its products (Gupta et al., 2003). Of all carotenoids, lycopene has been shown to exhibit the highest physical quenching rate constant with singlet oxygen, and its plasma level is slightly higher (Di Mascio, Kaiser, Sies., 1989). Lycopene has a high antioxidative activity and exerts a protective effect in various diseases (Clinton SK., 1998). Pollack and colleagues(Pollack et al., 1996), (Pollack et al., 1999) studied the inhibitory effect of lycopene on cataract development in galactosemic rats, and Gale et al. (Gale et al., 2001) reported a positive association between plasma concentration of lycopene and risk of cortical cataract. However, the
abundance of lycopene in the human food supply and its superior antioxidant function to quench singlet oxygen (Di Mascio, Kaiser, Sies, 1989) and other radical species (Kennedy, Liebler, 1992) have warranted studies to further establish the role of lycopene in the prevention of cataract.

5.2 Protein Fractionation

The results of the present study show that there is a significant difference in the level of different fractions of the lens proteins. As mentioned in the previous chapter, the lens was fractionated into five different fractions.

1. Water soluble protein
2. Urea soluble protein
3. Urea+DTT soluble protein
4. Urea+DTT insoluble protein
5. Insoluble protein

Out of all these fractions, the water soluble fraction of the lens is having various types of crystallins. These proteins are playing an important role in maintaining of the lens transparency. In the clear lens, the amount of water soluble fraction of protein is found more. During the process of cataractogenesis the water soluble proteins are getting modified and converted into insoluble forms which ultimately leads to the opacification of the lens.
Graph 2: Protein content in fractions A: Total Protein; B: Water Soluble Protein; C: Urea Soluble Protein after 1: one month; 2: two month; 3: three month
Graph 3: Protein content in fractions A: Urea DTT soluble Protein; B: Urea DTT insoluble Protein after 1: one month; 2: two month; 3: three month

Lens being rich in structural proteins has long been supposed that ultimate event in cataractogenesis is essentially a disturbance in the state of lens proteins. Most of the changes observed in protein structure are due to ageing of the proteins (Waley, 1969; Bloemendal, 1982) which may lead to the loss of transparency (Carper et al., 1982). The lens yellows with age and this change has been attributed to insoluble fraction in both normal (Spector et al., 1975) and cataractous lenses (Pirie, 1968). Aggregation is stabilized either by disulfide bonds formed by oxidation of sulfhydryl groups (Dische and Zit, 1951) or by
unknown non disulfide cross links (Buckingham, 1972; Kramps et al., 1978). Oxidation of lens proteins is usually associated with cataract formation (Augusteyn, 1981; Harding, 1981; Spector, 1984). With oxidation, three dimensional structure of lens proteins unfolds and thiol groups are revealed to form disulfide cross links resulting in production of high molecular mass protein aggregate (Spector, 1984). Harding (1981), however, suggested that oxidation is a result of conformational changes rather than its cause. He showed that lens proteins undergo conformational changes during cataract development (Harding, 1972-b) exposing thiol groups and buried peptide bonds making them susceptible to oxidation (Harding, 1981). Whatever is the sequence of events, the resulting oxidation of lens proteins causes insolubilization (Satoh, 1972; Garner and Spector, 1979), aggregation (Buckingham, 1972; Roy and Spector, 1976), degradation (van Kleef et al., 1975; Roy and Spector, 1978) and coloration (Pirie, 1968; Bando et al., 1975). Loss of soluble proteins is a well documented feature of human cataracts. Electrophoretic and chromatographic studies revealed that a low molecular mass protein fraction, which has slowest mobility on electrophoresis, was diminished from cataractous lens extracts (Francois and Rabaey, 1957; Mach, 1963,1964; Francois et al., 1965, 1969; Charlton and an Heyningen, 1968). The decrease in soluble fraction has been explained by leakage of low molecular mass fraction through capsule (Mach, 1963; Barber, 1968; Charlton and van Heyningen, 1968), conversion to insoluble proteins (Francois et al., 1965; Pirie, 1968; Clark et al., 1969; Sheridan and Zigman, 1971; Croft, L. R., 1973), cross linking to high molecular mass aggregates (Harding, 1972-b).

No conclusive evidence, however, could be presented for any of these proposals. Various groups confirmed that the amount of water and urea insoluble proteins from human lens was found to be increased in aging and cataract formation along with a decrease in water soluble fraction (Mach, 1963; Pirie, 1968; Clark et al., 1969: Kramps et al., 1976; Bessems et al., 1983).
According to Barber (1973), the changes that effect soluble proteins in human cataracts can be summarized as conformational changes, oxidation of sulphhydryl groups, loss of smaller molecules and photooxidative cross links and pigmentation. None of these changes could be speculated as a direct cause of opacification. Present knowledge implicates a number of possible risk factors but it is more likely that important causes are multifactorial and perhaps synergistic.

5.3 ENZYMES

The lens is equipped with a number of detoxifying enzymes, which is another means of self-protection. Detoxification occurs through a multitude of antioxidants, oxidation defense enzymes and by other means such as through NAD+/H dependent reductases, oxidases and dehydrogenases. Antioxidant enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPX) have the ability to reduce reactive oxygen species. Together with catalase, an important lenticular antioxidant enzyme, contain a heme protein with one molecule of NADPH, which is tightly bound to each of the four subunits of catalase in mammals (157), are important enzymatic antioxidant defense systems in the lens.

Antioxidant enzymes are able to catalytically remove free radicals and other reactive species. A wide array of enzymatic antioxidant defences exists, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (Halliwell & Gutteridge, 1999). Expression of some of these enzymes is controlled by redox-sensitive transcription factors, allowing the antioxidant system to respond to fluctuations in production of oxidizing species caused by photo-oxidative processes, especially during sustained exposure (Rahman, 2000). For example, Dudek et
al. (2001) demonstrated that the oxidant-sensitive transcription factor, nuclear factor-κB (NF-κB), was strongly activated at 1 h and returned to basal levels by 2 h when lens epithelial cells were treated with H2O2. However, it is probably the basal expression of the antioxidant system that determines the degree of acute resistance to oxidative stress (Slaughter et al., 2002) and may be key to protection of ocular tissue. The results of the research done by Jie Lei (2006) indicate that H2O2 at low concentration (1 mM) is able to damage lens cells and cause opacification without affecting the reduced glutathione levels and that the exogenous antioxidants have some ability to protect the lens.

### 5.3.1 Catalase

The presence of CAT in the lens has been well demonstrated (Bhuyan and Bhuyan, 1970). Both CAT and Gpx catalyse the transformation of H2O2 within the cell to harmless by-products. The catalases (CAT) catalyse the two electron dismutation of hydrogen peroxide into ground-state oxygen and water.

$$\text{CAT} \quad 2\text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} 2\text{H}_2\text{O} + \text{O}_2$$

GPX and CAT are present in all parts of the eye. Slaughter et al. (2003) reported that rabbit lens contained the highest CAT activity, at up to approximately 3.5-fold that for marmoset and rat and 40% higher than in dog.

The results show that the level of the catalase enzyme decreased significantly in the cataractous lenses of group-2 animals but after the treatment of the aqueous extract of *Andrographis paniculata*, the level of it increases and after three months treatment, it reaches to the level of the
normal control group (Table - 7). It shows that the extract is uplifting the catalase enzyme level which is one of the important enzyme of the antioxidant defence system of the lens.

Graph 4: Catalase 1: one month; 2: two month; 3: three month

The study performed by Geraldine et al. (2006) shows that the mean activity of catalase in lenses incubated in DMEM containing 100 mM sodium selenite was significantly lower than that in normal control lenses incubated in DMEM alone and was also significantly lower where ALCAR was added to the DMEM medium at the same time that selenite was added. The mean catalase activity in the lenses of this was significantly lower than that in normal control lenses. The similar kind of study was performed by Sakthivel et al. (2008). In his study, he has confirmed that the mean activity of catalase in lenses of rats of cataractous control group was significantly lower than that in lenses of normal control group and was also significantly lower than that in treated animal lenses. However, the mean catalase activity in the lenses of treated group of animals was also significantly lower than that in normal control group lenses. The catalase activity gradually decreased with increase in the stage of opacity i.e., with increasing opacification. Other workers have also observed the similar thing in their study (Bhuyan and Bhuyan, 1977; Varma et al., 1982, 1984; Fecondo and Augusteyn, 1983; Dwivedi and Pratap, 1987; Cekic et al., 1999; Gupta et al., 2002). Hong Yan et al. (2008) have worked on the effect of carnosine, aminoguanidine and aspirin drops to prevent cataract. In their study they found that carnosine eye drops produced a
better inhibitory effect than the other two drugs. They also found the similar results for catalase activity. In one of the study on curcumin shows different results. The study performed by Manikandana et al. (2009) shows that though Catalase is a specific scavenger of hydrogen peroxide and CAT activity were significantly reduced in selenium treated cultured lenses, interestingly, catalase level of treated groups lenses remained unchanged as that of control as they were treated with curcumin along with selenium. However, we observed that the mean activities of catalase in Group III lenses (treated with normal extract) were significantly higher than the values in Group II lenses although still lower than the values in Group I lenses. The animals of group IV and V has also increased the level of catalase but is is found lesser than the results of group III. Thus, the more enhanced level of catalase was found in group III which is nearer to the level of group I.

5.3.2 Glutathione peroxidise (GPx)

Glutathione peroxidase (EC 1.11.1.9, GPX) is a kind of well-characterized mammalian selenoprotein. It distributes extensively in cells, blood, and tissues and its activity decreased when organism suffered from diseases such as diabetes, Keshan disease, angioardiopathy and cataract, etc. Therefore, GPX is one of the most important antioxidant enzymes in living organisms and it is involved in many pathological conditions. Many reports have demonstrated its efficacy of antioxidants and free radicals scavengers (Ye Sun et al., 2005).

Gpx is required to check lipid peroxidation initiated by superoxide in the phospholipid bilayer, for maintenance of membrane integrity. Peroxidase enzymes such as GPX remove H2O2 by using it to oxidize another substrate, reduced sulphur:

\[
\text{SH}_2 + \text{H}_2\text{O}_2 \quad \rightarrow \quad \text{Sulphur} + 2\text{H}_2\text{O}
\]
GPX is the predominant GSH-consuming enzyme and the GPX family uses GSH as a cofactor to destroy hydrogen peroxide and lipoperoxides. Reddy & Giblin (1984) confirmed that there was considerable evidence that GPX was involved in the breakdown of H2O2. Furthermore, the major detoxification of H2O2 occurred in the epithelium of the lens (Giblin et al., 1982). During their experiment, various concentrations of H2O2 were maintained in the culture medium. They found that during a 3 h exposure of lens epithelial cells (from 4-6-day old rabbits) to a 0.03 mM H2O2, there was nearly a 10-fold stimulation in hexose monophosphate shunt activity and the cells remained undamaged. The maximum shunt activity of the epithelial cells was twice that of the same number of rabbit skin fibroblasts and, in contrast to the lens epithelial cells, the fibroblasts were severely damaged by 0.03mM-H2O2. The results indicate the importance of glutathione metabolism and the epithelium in protecting the whole lens against H2O2 –induced oxidative damage.

\[
\text{GPx} \\
\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]

Therefore, the cooperation of glutathione peroxidase and catalase contributes to removal of hydrogen peroxide \textit{in vivo}.

The results of this study shows that the level of GPx is significantly increases in the lenses of the treated animals of Group 3, 4, and 5 but the highest significant difference is in Group III which was treated with the normal extract of \textit{Andrographis paniculata}.
The study performed by Geraldine et al. (2006) shows that the mean activity of GPx enzyme in the cataractous control group was lower than the normal control group but the same was found higher in the treated groups of animals. Similar results are found in another study done by Sakthivel (2008). In contrast with the other study performed by Manikandana (2008), though GPx is important for scavenging hydrogen peroxide and along with CAT which is a potent barrier against lipid peroxidation in the eye lens, The GPx level in cultured lenses of cataractous control group were significantly decreased ($P < 0.05$). Such decrease of GPx activity was also observed in sodium selenite and curcumin treated lenses of treated group. Interestingly, GPx levels in treated groups of cultured lenses in the presence of curcumin produced no change in GPx activity which was equal to that observed with control lenses. However in this study, the results suggested that the normal extract of *Andrographis paniculata* is having antioxidative property which elevates the GPx levels in the cataractous lenses when treated with it.

### 5.3.3 Glutathione Reductase (GR)

Reduced glutathione is an important defense against free radical mediated damage and it is found in high concentrations in the eye and is known to protect lens from damage by ROS. There is a
direct link between the rate of formation of oxidized glutathione (GSSG) and the stimulation of the hexose monophosphate shunt through the generation of NADPH.

\[
\text{GR} \\
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

Graph 6: Glutathione Reductase 1: one month; 2: two months; 3: three months

The study performed by Hong Yan et al. (2008) shows that Glutathione reductase (GR) did not decrease in the lens of the untreated diabetic rats 13 weeks after injection of streptozotocin whereas carnosine seemed to have raised GR activity when normal control group was compared with untreated diabetic group. This shows that the part of the mechanism of the protective effect of carnosine against diabetic cataract is by protecting GR, which in turn generally protect against peroxide and oxidation. Another study performed by Sakthivel (2008) shows that The mean activity of GR (expressed as nmoles of NADPH oxidized/min/mg protein) was significantly lower in cataractous control group rat lenses than that in normal control group rat lenses. However, no significant differences were observed between the mean activity of GR in lenses of cataractous control Group and treated Group rats, and also between that of treated Group and normal control Group rat lenses. The activity of GR decreased gradually with increase in the stage of opacity
(i.e., with increasing opacification) in the lenses of cataractous control group and treated group rats.

In this study, the level of GR was found interestingly higher in Normal extract group 3 than the normal control group 1 but after the 2 months and 3 months trial it was found lesser than normal control group. Though in the first month, it was found higher, in the subsequent months trial the level was significantly higher than the cataractous control group 2. The group 4 and 5 was also found significantly higher than cataractous control group 2. The level of GR was found lower in fraction 2 of Aqueous extract of *Andrographis paniculata* (group 5) but gradually after the instillation of the extract in subsequent months trials it was found elevated in comparison to the cataractous control group 2 of the animals. At the three months trial, the level of GR in Group 4 and 5 was found almost similar and lower than group 3 but higher than group 2.

5.3.4 Superoxide dismutase (SOD)

The chemical definition of ‘dismutation’ is a reaction in which the same species is both oxidized and reduced. Superoxide dismutases are present in all eye tissues (Halliwell & Gutteridge, 1999) and are specific for catalytic removal of superoxide. SOD activity was measured in the eye lens as it is a specific scavenger of superoxide anion and selenium was earlier shown to induce O$_2^-$ generation in eye lens. SOD is a chain-breaking antioxidant enzyme that occurs in lenses of different species (Verma et al., 1977) The enzymes GPx and CAT in the lens (Pirie, 1965, Bhuyan & Bhuyan, 1970), maintains integrity of the phospholipid bilayer of membranes by putting a break on the lipid peroxidation initiated by superoxide. Both SOD and CAT defends against oxidative stress by decomposing O$_2^-$ and H$_2$O$_2$, respectively, and this mechanism against lipid peroxidation depend heavily on glutathione.
In a study by Manikandana et al. (2009), the mean SOD activity in cultured lenses of cataractous group was significantly reduced. Such reduction of SOD activity was also observed in culture lenses of group, which were treated with 100_M sodium selenite and 100_M curcumin. By contrast, in cultured lenses belonging to groups IV, V and VI presence of curcumin (group IV; 200_M) or AG along with selenium produced no change in SOD activity when compared to cataractous group lenses and the levels were equal to that observed with control lenses. In one of the study by Javadzadeh et al. (2009) shows that onion juice, as a flavonoid rich source, can provide an additional support to the antioxidant agents, leads to the elevation of total antioxidant levels and GPx and SOD activities in the rat lens. They have summarized that instillation of onion juice into rat eyes can effectively prevent selenite induced cataract formation. The present study shows that there is a significant variation in the level of SOD between the group 1 and 3. The SOD level is found increased in the group 3 compared to the group 2 which shows that the enzyme activity increases by administering the aqueous extract of *Andrographis paniculata* in the cataractous eyes. The SOD activity gradually decreased with increase in the stage of opacity (i.e., with increasing opacification) in the lenses of group 2. Similar results were found in one of the study by Sakthivel et al. (2008).