

CHAPTER 4

CAPTOPRIL AMELIORATES SODIUM SELENITE INDUCED CATARACTOGENESIS IN RATS: AN IN VITRO AND IN VIVO STUDY

4.1. Introduction

In the first experiment as detailed in the previous chapter, though Lisinopril had shown a bit of protective response in cultured rat lenses against sodium selenite induced cataract but failed to show any effectiveness when given to rats pups. The reason was probably due to the absence of thiol group in its structure which is highly responsible to show antioxidant activity. Therefore, the second experiment was conducted to investigate the effectiveness of Captopril, a thiol containing ACEi in the same experimental settings as described in the previous chapter.

Several previous experimental findings postulated that angiotensin converting enzyme inhibitors (ACEi) may act as a “Magic Bullets” against oxidant stress, especially Captopril exhibits a wide variety of biological activities [261-264]. Captopril is a synthetic 3-mercapto-2-methylpropionyl derivative of L-proline. It acts as a competitive inhibitor of peptidyl dipeptidase, which catalyzes the conversion of angiotensin I to angiotensin II. Thus, it is a potent systemic hypotensive drug that functions by decreasing the peripheral vascular resistance in essential hypertension [129]. It also stimulates the kallikrein-kinin system. Besides these effects, Captopril may decrease intraocular pressure [130]. Captopril is easily absorbed through the mucosa and its bioavailability is about 65%. It has been suggested that absorbed Captopril is carried as disulfides with the endogenous sulfhydryl-containing compounds and it is also a scavenger of oxygen free radicals [131]. Its ability of quenching toxic oxygen free radicals could be an additional therapeutic usefulness in the prevention of diquat induced cataract [132]. The
present study was executed to assess the efficacy of the Captopril in preventing selenite-induced cataractogenesis in different experimental settings.

4.2. Materials and Methods

4.2.1. Materials

Captopril was kindly provided by Wockhardt Ltd (Aurangabad, Maharashtra, India) approximate purity was 99.5%. All other chemicals and reagents used were same as detailed in previous chapter.

4.2.2. In vitro phase of the study

Wistar rats of either sex in the weight range 80 to 100 gm were used for the study. Lenses were extracted through a posterior approach from the eyes of rats under deep anesthesia. Lenses were organ cultured in DMEM medium with HEPES buffer, supplemented with 10% foetal bovine serum (FBS), 100 U/ml Penicillin, 0.1 mg/ml Streptomycin and 0.25 µg/ml Amphotericin was also added to prevent bacterial contamination along with sodium bicarbonate (0.2% w/v). Selenite medium was prepared by adding sodium selenite to the medium to give a final concentration of 100 µM. Lenses were maintained in a 24 well culture plate with 2 ml medium and lenses were incubated for 24 hours under 5% CO$_2$ at 37$^\circ$C in a CO$_2$ incubator. After 2 h of incubation, opaque lenses which are damage during dissection were discarded and transparent lenses were taken for the subsequent experimental studies.

Transparent lenses were divided equally into three different groups to serve as normal, control and test group. The lenses in the normal group were cultured in DMEM alone. The lenses in the control group were cultured in DMEM plus 100 µM sodium selenite and those in the test group were cultured in the control medium plus 5 mM Captopril. The dose of Captopril was determined from previous study of Bhuyan et al., [132]. All lenses were incubated for 24 h at the
conditions described earlier. After incubation, lenses were processed for morphological investigation and estimation of biochemical parameters.

4.2.2.1. Morphological investigation of cultured lenses

The cultured lenses were photographed after 24 h of incubation by same technique as described in previous chapter.

4.2.2.2. Reduced glutathione (GSH) assay

The GSH content was estimated by the method of Moron et al., [250] as described in previous chapter.

4.2.2.3. Estimation of Malondialdehyde (MDA)

The extent of lipid peroxidation was determined by the method of Ohkawa et al., [251] as described in previous chapter.

4.2.2.4. Assay of Ca^{2+} ATPase activity

The activity of Ca^{2+} ATPase in the lens samples was determined by the method of Rorive and Kleinzeller [265]. To the reaction tube, 0.25 ml of substrate (40 mM ATP in 0.4 M Tris-HCl buffer, pH 7.4) and 0.1 ml of lens homogenate was added. A tube devoid of the homogenate served as a control. All the tubes were incubated for 30 min in a water bath at 37°C. The incubation was stopped by adding 2 ml of 10% trichloroacetic acid (TCA) then 0.2 ml ATP was added to control tubes and these tubes were subsequently kept in ice for 20 min. All the tubes were then centrifuged at 13,000 rpm for 10 min and the supernatant was collected. The protein free supernatant was analyzed for inorganic phosphate. For this analysis, 3 ml of the supernatant was treated with 1 ml of ammonium molybdate and 0.4 ml of 2,4 dianimonaphthol sulphonic acid. The color developed was read at 680 nm after 20 min.
4.2.2.5. Estimation of lens Ca$^{2+}$

Individual lenses were weighed and digested in concentrated nitric acid:perchloric acid (5:1). After complete digestion, the samples were dried, diluted with 1% nitric acid and made up to 50 ml in a standard flask. The samples were analyzed by flame photometry and the results were expressed as % weight wet tissue. Calcium carbonate was used as a standard and was prepared by dissolving in 1 per cent nitric acid.

4.2.2.6. Estimation of lens protein

Lens total protein and water soluble protein level were determined by the modified method of Lowry et al., [252] using bovine serum albumin as the standard as described in Appendix 2.

4.2.2.7. Lens antioxidant enzymes assay

A separate set of experiment was conducted under the same conditions as described above. After 24 h of incubation, 10% (w/v) homogenate of lenses from each group was prepared in 50 mM phosphate buffer (pH 7.0). The enzyme activities such as Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx) and Glutathione-S-transferase (GST) were measured in the supernatant obtained by the centrifugation of the homogenate at 5000 rpm for 15 min at 4°C. Monitoring spectrophotometrically at 550 nm, the ability of the enzyme to inhibit the reduction of cytochrome c was used to assess the activity of superoxide dismutase (SOD) [266]. SOD activity was defined as one unit inhibited the rate of reduction of cytochrome c by 50% in a coupled system, using xanthine and xanthine oxidase at pH 7.8. The enzyme activity of catalase (CAT) was measured spectrophotometrically at 240 nm by following the decomposition of H$_2$O$_2$ [267]. One unit of CAT activity represented the amount of enzyme required to decompose 1 μM of H$_2$O$_2$/min. Glutathione peroxidase (GPx) activity was monitored at 340 nm [268]. Enzyme activity was defined as one unit catalyzed the
oxidation by \( \text{H}_2\text{O}_2 \) of 1.0 \( \mu \text{M} \) of reduced glutathione to oxidized glutathione per minute at \( \text{pH} \) 7.0 at 25\(^\circ\)C. The conjugation of GSH with 1-chloro, 2-4 dinitrobenzene (CDNB), a hydrophilic substrate, was examined spectrophotometrically at 310 nm to measure glutathione S-transferase (GST) activity [269]. One unit of GST was defined as the amount of enzyme required to conjugate 1 \( \mu \text{M} \) of CDNB with GSH per minute. The details of the procedures for the estimation of SOD, CAT, GPx and GST are given in Appendix 3, 4, 5 and 6 respectively.

4.2.3. In vivo phase of the study

The in vivo phase of the study was carried out by using nine days old Wistar strain rat pups. Captopril (50 mg/kg i.p) was given to test group unless and otherwise all the experimental protocols were same as described in previous chapter.

4.2.4. Statistical analysis

All data were expressed as mean ± standard deviation (SD). The groups were compared using one-way ANOVA with post-hoc Dunnett’s test using selenite 100 \( \mu \text{M} \) group as control and the chi-square test were applied wherever relevant.

4.3. Results

4.3.1. In vitro phase of the study

4.3.1.1. Effect on lens morphology

After 24 h of incubation in selenite 100 \( \mu \text{M} \), lenses became completely opaque as against lenses incubated in DMEM alone. Incubation of lenses with Captopril 5 mM, seem to retard the progression of lens opacification, compared with control group. This is because the background grids are clearly visible in Captopril treated group than in selenite treated group as shown in Fig 4.1.
4.3.1.2. Effect on biochemical parameters

Incubation of lenses with Sodium selenite showed opacification starting after 2 hrs at the periphery, on the posterior surface of the lenses. This progressively increased towards the centre, with complete opacification at the end of 24 hrs. The mean GSH value in the normal lenses was 2.59±0.03 μg/mg of fresh weight of lens. A significant decrease in GSH level was observed in the presence of sodium selenite in the control as opposed to the normal group (P<0.01). In the presence of Captopril, there was a significant restoration of GSH level in the treated lenses (P<0.01) as opposed to the control lenses. The mean GSH values in the control and test groups were 1.19± 0.01 and 2.36± 0.01 μg/mg of fresh weight of lens, respectively. A significant increase in MDA level was found in the control opposed to the normal lenses (1.12±0.02 μmol/g of fresh weight of lens; P<0.01). Captopril supplementation significantly protected (P<0.01) the test group lenses from lipid peroxidation; the MDA content was 0.096± 001 μmol/g of wet weight of lens (Table 4.1).

Selenite treated lenses also showed significantly low concentrations of proteins (total and water soluble proteins) in the lens homogenate (P<0.01) and very high Ca^{2+} conc. (P<0.01) compared with normal group having normal lenses (Table 4.2). Captopril supplemented group had significantly higher concentrations of total lens proteins and water soluble proteins (P<0.01), compared to selenite
treated group. At the same time, they had lower Ca$^{2+}$ conc. (P<0.01) compared to selenite treated group. Activity of the membrane ionic pump, Ca$^{2+}$ ATPase, was found to be decreased significantly following selenite induction whereas, treatment with Captopril was found to maintain activity close to the normal level (Fig. 4.2).

The effect of 5 mM Captopril on different antioxidant enzymes (SOD, CAT, GPx and GST) is presented in Table 4.2. It was observed that, in presence of selenite stress in group II lenses, antioxidant enzymes were significantly reduced as compared with the normal group. In the presence of Captopril, there was a significant positive modulation of enzyme activities observed in group III lenses.

Table 4.1 Levels of various biochemical parameters in Group I, Group II and Group III lenses

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (μg/mg wt.)</td>
<td>2.59 ± 0.03*</td>
<td>1.19 ± 0.01</td>
<td>2.36 ± 0.01*</td>
</tr>
<tr>
<td>MDA (μmol/g wt.)</td>
<td>0.061 ± 0.001*</td>
<td>1.12 ± 0.02</td>
<td>0.096 ± 0.001*</td>
</tr>
<tr>
<td>Calcium (Ca$^{2+}$) (%wt)</td>
<td>0.012 ± 0.004*</td>
<td>0.044 ± 0.002</td>
<td>0.016 ± 0.001*</td>
</tr>
<tr>
<td>Total Protein (mg/mg wt.)</td>
<td>0.319 ± 0.003*</td>
<td>0.246 ± 0.012</td>
<td>0.298 ± 0.014*</td>
</tr>
<tr>
<td>Water Soluble Protein (mg/mg wt.)</td>
<td>0.272 ± 0.004*</td>
<td>0.171 ± 0.001</td>
<td>0.239 ± 0.002*</td>
</tr>
</tbody>
</table>

All values are expressed as mean±SD of five determinations. Group I: Normal, Group II: lenses exposed to Sodium selenite only. Group III: lenses exposed to Sodium selenite and Captopril. Statistically significant difference (*P < 0.01) when compared with group II values.
Fig. 4.2 Activity of Ca\(^{2+}\) ATPase in lens. All values are expressed as mean±SD of five determinations. Group I: Normal, Group II: lenses exposed to selenite only. Group III: lenses exposed to selenite and Captopril. Statistically significant difference (\(^#P < 0.05\)) (\(^*P < 0.01\)) when compared with group II values.

Table 4.2 Levels of antioxidant enzymes in Group I, Group II and Group III lenses

<table>
<thead>
<tr>
<th>Parameter (IU/mg protein)</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD</td>
<td>2.59 ± 0.03*</td>
<td>1.19 ± 0.01</td>
<td>2.36 ± 0.01*</td>
</tr>
<tr>
<td>CAT</td>
<td>0.061 ± 0.001*</td>
<td>1.12 ± 0.02</td>
<td>0.096 ± 0.001*</td>
</tr>
<tr>
<td>GPx</td>
<td>0.012 ± 0.004*</td>
<td>0.044 ± 0.002</td>
<td>0.016 ± 0.001*</td>
</tr>
<tr>
<td>GST</td>
<td>0.319 ± 0.003*</td>
<td>0.246 ± 0.012</td>
<td>0.298 ± 0.014*</td>
</tr>
</tbody>
</table>

All values are expressed as mean±SD of five determinations. Group I: Normal, Group II: lenses exposed to selenite only, Group III: lenses exposed to selenite and Captopril. Statistically significant difference (*\(P<0.01\)) when compared with group II values. SOD, Superoxide dismutase; CAT, Catalase; GPx, Glutathione peroxidase; GST, Glutathione S-transferase.

4.3.2. In vivo phase of the study

As shown in Fig 4.3, the lenses of rat pups that had received selenite alone (group A), a mature dense opacity involving the entire lens was observed in all (100%) 10 animals. In contrast, 5 out of 10 (50%) rat pups in group B that had received selenite along with Captopril exhibited clear lenses and other 5 animals had a minimal nuclear opacity with swollen fibers or posterior subcapsular
scatterings lenses. This difference was statistically significant ($x^2 \ [df =1] = 4.27; P<0.05$).

![Image](image.png)

Fig. 4.3 Digital image of the eyes of rat pups in sodium selenite treated and Captopril treated group.

### 4.4. Discussion

In cataractogenesis, oxidative stress is postulated to be the major factor due to the formation of free radicals. Reduction of free radical load by means of reducing oxidative stress has been shown to prevent or delay cataract [25]. Numerous chemical agents with diverse chemical structures having antioxidant properties have shown beneficial effects in various pathologic conditions including cataract. Captopril was found to be one of them [132]. In the present study, sodium selenite-induced cataract model was selected because of its rapid, effective and reproducible experimental properties with regards to cataract formation. This experimental model shares many general similarities to human cataract. However, the rate of opacification in the selenite model is much more rapid than in human cataract. Cataract formation associated with the single dose of selenite administration is due to the impairment of endogenous antioxidative defense system, membrane damage and opacification [239]. Reactive oxygen species damage lens components, as evidenced by loss of transparency and decreased active transport of cations, GSH and ATP as well as protein insolubilization and generation of lipid peroxides [78, 96]. The biochemical analysis carried out in the present study clearly demonstrated a significant depletion of GSH and increased membrane damage as indicated by the levels of MDA, the product of membrane
lipid peroxidation and decrease water soluble protein content in selenite treated lenses. Lens GSH depletion and increased MDA level in presence of selenite have been reported [117]. Captopril treated lenses could restored GSH and MDA levels, protected against aggregation and insolubilization of lens proteins and maintained lens clarity without doubt confirms its protective ability. Lens cells differentiation and other essential cellular activities are governed in presence of calcium [111]. The level of divalent cation Ca\(^{2+}\) in the lens is maintained at submicromolar range and is lower than that of the aqueous humor [270]. Alterations of lenticular Ca\(^{2+}\) homeostasis have been implicated in cataractogenesis [271]. In this study, activity of Ca\(^{2+}\) ATPase was significantly decreased in selenite treated lenses with a corresponding increase in Ca\(^{2+}\) concentration. Ca\(^{2+}\) ATPase activity and level of Ca\(^{2+}\) was found to be normal Captopril treated lenses. The inward passive diffusion of Ca\(^{2+}\) from lens to the aqueous humor is counteracted by Ca\(^{2+}\) ATPase pump that leads to the maintenance of normal Ca\(^{2+}\) level within lens [272]. Ca\(^{2+}\) ATPase is a major factor involved in maintaining lenticular Ca\(^{2+}\) levels and loss of its activity could explain the rise in Ca\(^{2+}\). The development of lens opacity is due to the oxidation of the critical sulfhydryl groups of Ca\(^{2+}\)-ATPase on lens epithelial membrane, influx of calcium from the aqueous humor, activation of calpain, cleavage of N-terminal extensions of β-crystallins of the lens, interaction between exposed charged groups and the formation of insoluble protein aggregates. The same culprits have been documented with regard to the in vitro study of selenite-induced opacification of the lens [273]. Our observations are in agreement with the aforesaid findings that the selenite-induced oxidative stress resulted in higher levels of lipid peroxidation, loss of activity of Ca\(^{2+}\) ATPase and accumulation of Ca\(^{2+}\) in the lens. Conversely, Captopril treated lenses were associated with lower levels of Ca\(^{2+}\), higher activity of Ca\(^{2+}\) ATPase and decreased levels of lipid peroxidation that could be attributed to its antioxidant protection against selenite induced oxidative stress. SOD, CAT, GPx and GST are important components of the innate enzymatic defenses of the lens. SOD, a chain-breaking antioxidant, was first described by McCord and Fridovich [274] in red blood cells. Varma et al., [275] first described its occurrence in the lenses of different species. SOD converts
superoxide to \( \text{H}_2\text{O}_2 \). The enzyme exists in two forms, one containing \( \text{Mn}^{2+} \), restricted to the mitochondria and a cytosolic form containing \( \text{Zn}^{2+} \) and \( \text{Cu}^{2+} \). The occurrence of GPx in the lens was first shown by Pirie [276].

Gpx is required to check lipid peroxidation initiated by superoxide in the phospholipid bilayer, for maintenance of membrane integrity. CAT is a hemoprotein that requires NADPH for regeneration to its active form [277]. The presence of CAT in the lens has been well demonstrated [278]. The transformation of \( \text{H}_2\text{O}_2 \) to harmless by-products within the cell is governed by CAT and Gpx, thereby protecting the cells against destruction by products of lipid peroxidation [279]. GST is important for detoxification process. The level of these enzymes were significantly depleted due to selenite and positively modulated in the presence of Captopril. The data clearly demonstrated that Captopril significantly improves the antioxidant defense mechanisms of the lens.

Based on the findings of \textit{in vitro} studies, Captopril was evaluated against selenite-induced cataracts in young rats. Captopril significantly protected the lens morphology and clarity: 50\% of the eyes had almost clear lenses; in contrast, 100\% of the control eyes developed dense nuclear opacity. From the current study, it is evident that Captopril protects the lens against oxidative stress. These results in selenite-induced cataracts \textit{in vitro} and \textit{in vivo} studies not only demonstrate the protective effect of Captopril but also indicate that it prevents cataractogenesis by virtue of its antioxidant properties. Captopril, therefore, may be useful for prophylaxis or therapy against cataracts.

4.5. Conclusion

In conclusion, the study on the evaluation of the anti cataract potential of Captopril in experimental animals indicated that it modulates antioxidant parameters in the enucleated eye lenses. It also attenuates selenite-induced cataract both \textit{in vitro} and \textit{in vivo}, so it may be useful for cataract therapy. Captopril was
found to be effective in both the experimental settings unlike Lisinopril. The thiol group in the Captopril might be responsible to prove its superiority over Lisinopril.

The study indicated that the antioxidant potential is merely important to attenuate oxidant stress induced cataract. The next study was planned to search the research based evidence product which would be more potent than Captopril. Published reports showed that Allylmercaptopraptopril which is the combined form of Captopril and Allicin would be more effective than Captopril alone. Therefore, a third experiment was designed to evaluate anti cataract potential of Allylmercaptopraptopril in the same experimental model as described in this chapter.