CHAPTER 3

LISINOPRIL ATTENUATES SODIUM SELENITE INDUCED EXPERIMENTAL CATARACT DEVELOPMENT: AN IN VITRO AND IN VIVO STUDY

3.1. Introduction

Several risk factors have been investigated which are responsible for the cataract formation such as aging, diabetes, malnutrition, diarrhoea, sunlight, smoking, hypertension and renal failure [19]. Oxidative stress associated with free radical formation is postulated as a major factor leading to senile cataract formation [11]. This hypothesis is supported by the anti cataract effect of various nutritional and physiological antioxidants in experimental animals [237, 238]. Selenite cataract is a rapidly-induced, convenient model for the study of senile nuclear cataractogenesis. The morphological and biochemical characteristics of this model have been extensively investigated. Moreover, this model shows a number of general similarities to human cataract. The reliability and extensive characterization of selenite cataract makes it a useful rodent model for rapid screening of potential anti cataract agents [239].

Physiologic antioxidant such as Pyruvate and nutritional antioxidant Vitamin E, Ascorbic acid and Carotenoids has been found to delay the experimental cataract. ACE inhibitors (ACEi) have an ability to reduce arterial blood pressure by the inhibition of angiotensin II formation [240-242]. Apart from this, they are found to be useful antioxidants for the attenuation of oxidative stress and fibrosis. The antioxidant potential of ACEi thought to be due to the enhancement of endogenous antioxidant defense system that leads to the protection of cells from oxidant stress [243]. Conflicting results have been documented while
studying the ability of ACEi to scavenge reactive oxygen and nitrogen species [244, 245]. Some studies, mainly carried out in vitro, indicate that both, sulfhydryl-containing (i.e., Captopril) and non-sulfhydryl-containing ACEi (i.e., Lisinopril) can scavenge free radicals [245]. By contrast, other reports show that only sulfhydryl-containing ACEi are effective free radical/oxidant scavengers [246, 247]. Selenite cataract, first described by Ostadalova et al., in 1978, is an excellent model of oxidative stress-induced cataractogenesis in vitro and in vivo [248], hence it was used in the present study to evaluate the efficacy of non-thiol ACE inhibitor, Lisinopril as an anti cataract agent.

3.2. Materials and Methods

3.2.1. Materials

Lisinopril was kindly provided by Torrent Pharmaceutical Ltd. (Ahmedabad, Gujarat, India) approximate purity was 98%. Dulbeco’s Modified Eagles Medium (DMEM) was obtained from Hi Media Laboratories (Mumbai, India). Foetal bovine serum (FBS) and sodium selenite purchased from Sigma Chemical Company, St. Louis, MO. 24 wells Falcon plastic culture plate was acquired from Genei, Bangalore, India. All other chemicals and solvents were procured from SRL, Mumbai, India.

3.2.2. In vitro phase of the study

Wistar rats of either sex in the weight range 80 to100 gm were used for the study. Rats used for the study were obtained from the animal house stock of the Department of Pharmacology, SRM College of Pharmacy, Kattankulathur, India and handled in accordance with the guidelines as per the “Institutional Animal Ethical Committee” and CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) rules. Lenses were extracted through a posterior approach from the eyes of rats under deep anesthesia. Lenses were organ cultured in Dulbeco’s Modified Eagles Medium (DMEM) medium with HEPES
(4-(2-hydroxylethyl)-1-piperazinethanesulfonic acid) buffer, supplemented with 10% fetal 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. 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°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 10 µM Lisinopril. The dose of Lisinopril was determined from previous study of Ghazi-Khansari et al., on liver mitochondrial cells culture study [249]. The stock solution (10 mM) of Lisinopril was prepared in double distilled water by vigorous shaking and was clear to the unaided eye. 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.

### 3.2.2.1. Morphological investigation of cultured lenses

Lenses were photographed using digital camera (Sony Cybershot DSC-F505V) fitted to a stand (Haiser R3XA). A lens from each group was placed on black grid lines with white background light. Images of the anterior epithelium of the lens were taken with an image resolution of 1024 x 768 pixels as a RGB true color JPEG image.
3.2.2.2. Reduced glutathione (GSH) assay

The GSH content was estimated by the method of Moron et al., [250]. Half of the lenses from each group were weighed and homogenized in 1 ml of 5% trichloroacetic acid (TCA) and a clear supernatant was obtained by centrifugation at 5000 rpm for 15 min. To 0.5 ml of this supernatant, 4.0 ml of 0.3 M Na$_2$HPO$_4$ and 0.5 ml of 0.6 mM 5,5'-dithiobis-2-nitrobenzoic acid in 1% trisodium citrate was added in succession. The intensity of the resulting yellow color was read spectrophotometrically at 410 nm. Reduced GSH was used as a standard.

3.2.2.3. Estimation of Malondialdehyde (MDA)

The extent of lipid peroxidation was determined by the method of Ohkawa et al., [251]. Briefly, 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.81% thiobarbituric acid aqueous solution was added in succession. To this reaction mixture, 0.2 ml of the tissue sample (lens homogenate prepared in 0.15 M potassium chloride) was added. The mixture was then heated in boiling water for 60 min. After cooling to room temperature, 5 ml of butanol: pyridine (15:1 v/v) solution was added. The mixture was then centrifuged at 5000 rpm for 15 min. The upper organic layer was separated and the intensity of the resulting pink color was read at 532 nm. Tetramethoxypropane was used as an external standard. The level of lipid peroxide was expressed as MDA formed in μmol/g wet weight for lenses.

3.2.2.4. Estimation of levels of lens Na$^+$, K$^+$ and Ca$^{2+}$

Electrolyte (Na$^+$, K$^+$ and Ca$^{2+}$) estimation was done by flame photometry and the results were expressed as %weight wet tissue. Standard stock solutions of cations were prepared by individually dissolving sodium chloride and potassium chloride in deionised water. Calcium carbonate was dissolved in 1% nitric acid for the stock calcium standard solution.
3.2.2.5. Estimation of lens protein

To estimate total protein, the lens homogenate was prepared in 5% trichloroacetic acid and the resultant precipitated protein was dissolved in sodium hydroxide and used as aliquots for the estimation of total proteins. Soluble and insoluble fractions of the protein were estimated by preparing homogenate in double distilled water. The water soluble supernatant was used for estimation of soluble protein and the residue was dissolved in sodium hydroxide and used for the estimation of insoluble protein. The protein content of the samples was determined by the modified method of Lowry et al., [252] using bovine serum albumin as a standard. The detail of the procedure is given in Appendix 2.

3.2.3. In vivo phase of the study

Nine days old rat pups (Wistar strain) were used in this study. The pups were housed with parents in large spacious cages and the parents were given food and water ad libitum. The animal room was well-ventilated and had a regular 12:12 h light/dark cycle throughout the experimental period. The control and test groups had 10 pups in each group. Pups in both groups were injected subcutaneously with 19 μM/kg body weight of sodium selenite. One day before the selenite challenge, the pups in the test group were injected intraperitoneally a single dose of 5 mg/kg of Lisinopril and was repeated once daily for five consecutive days thereafter. When the pups first opened their eyes (when the pups were 15 days old), their eyes were examined by slit-lamp biomicroscopy (Topcon, Japan) and were also photographed. Cataracts were observed in both groups on postnatal day 16, when the eyes of the pups first opened. Mydriasis was achieved by using a topical ophthalmic solution containing Tropicamide with Phenylephrine (Maxdil Plus, Hi-Care Pharma, Chennai, India).
3.2.4. 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 μM group as control and the chi-square test were applied wherever relevant.

3.3. Results

3.3.1. *In vitro* phase of the study

3.3.1.1. Effect on lens morphology

After 24 h of incubation in selenite 100 μM, lenses in control group became completely opaque as against lenses incubated in DMEM alone. Incubation of lenses with Lisinopril 10 μM, seem to retard the progression of lens opacification, compared with control group. This is because the background grids are certainly visible in Lisinopril treated group than in selenite treated group as shown in Fig 3.1.

![Fig. 3.1 Digital image of the rat lenses in the culture media under various conditions. Rat lenses were cultured in (A) normal DMEM, (B) 100 μM sodium selenite and (C) 100 μM sodium selenite + 10 μM Lisinopril exposure. Photographs were taken after 24 h.](image)

3.3.1.2. Effect on biochemical parameters

Incubation of lenses with selenite 100 μM 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. Control lenses showed significantly higher Na⁺ (P<0.01) and Ca²⁺
Lisinopril treated lenses showed significantly high $K^+$ (P<0.01), while Na$^+$ and Ca$^{2+}$ concentration was significantly lower (P<0.01) compared with control group (Table 3.1). Lenses in control group also showed significantly low concentrations of proteins (total and water soluble proteins) in the lens homogenate (P<0.01) and very high insoluble protein (P<0.01) compared with normal group having normal lenses (Table 3.1). Lisinopril treated group had significantly higher concentrations of total lens proteins and water soluble protein (P<0.01), compared with control group. At the same time, they had lower water-insoluble proteins (P<0.01) compared with control group. Statistically significant difference (P<0.01) was found when compared with group II values. The mean GSH value in the normal lenses was 2.40±0.05 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 Lisinopril, 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 1.36 ± 0.01 and 2.19 ± 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 (0.74 ± 0.02 μmol/g of fresh weight of lens; P<0.01). Lisinopril supplementation significantly protected (P<0.01) the test group lenses from lipid peroxidation; the MDA content was 0.067 ± 0.001 μmol/g of wet weight of lens (Table 3.1).

3.3.2. In vivo phase of the study

Rat pups (group II) treated with sodium selenite showed dense nuclear cataract in all 10 (100%) animals at the end of study. While, in Lisinopril treated group (group III) 7 out of 10 animals (70%) had dense nuclear cataract on 16th postpartum day. This difference was statistically insignificant ($x^2$ [df =1] = 1.57; P>0.05).
Table 3.1 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>Sodium (Na⁺) (%wt)</td>
<td>0.196 ± 0.001*</td>
<td>0.315 ± 0.002</td>
<td>0.263 ± 0.004*</td>
</tr>
<tr>
<td>Potassium (K⁺) (%wt)</td>
<td>0.954 ± 0.002*</td>
<td>0.565 ± 0.002</td>
<td>0.896 ± 0.002*</td>
</tr>
<tr>
<td>Calcium (Ca²⁺) (%wt)</td>
<td>0.014 ± 0.004*</td>
<td>0.025 ± 0.002</td>
<td>0.016 ± 0.001*</td>
</tr>
<tr>
<td>Total Protein (mg/mg wt.)</td>
<td>0.389 ± 0.003*</td>
<td>0.336 ± 0.012</td>
<td>0.368 ± 0.014*</td>
</tr>
<tr>
<td>Water Soluble Protein (mg/mg wt.)</td>
<td>0.282 ± 0.005*</td>
<td>0.180 ± 0.001</td>
<td>0.248 ± 0.004*</td>
</tr>
<tr>
<td>Water Insoluble Protein (mg/mg wt.)</td>
<td>0.070 ± 0.002*</td>
<td>0.136 ± 0.001</td>
<td>0.102 ± 0.002*</td>
</tr>
<tr>
<td>GSH (μg/mg wt.)</td>
<td>2.40 ± 0.05*</td>
<td>1.36 ± 0.01</td>
<td>2.19 ± 0.01*</td>
</tr>
<tr>
<td>MDA (μmol/g wt.)</td>
<td>0.061 ± 0.001*</td>
<td>0.74 ± 0.02</td>
<td>0.067 ± 0.001*</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 Lisinopril. Statistically significant difference (*P<0.01) when compared with group II values.

3.4. Discussion

The biochemical parameters usually associated with lens are electrolytes (Na⁺, Ca²⁺ and K⁺), malondialdehyde (MDA), reduced glutathione (GSH) and proteins (total proteins and water soluble proteins) which can be measured for to study cataractogenesis. With regard to cataract, the selenite model was selected because of the rapid, effective and reproducible cataract formation. Although the rate of opacification in the selenite model is much more rapid than in human cataract, it has many general similarities to human cataract like increased calcium, protein aggregation, decreased water soluble proteins and level of reduced glutathione [239, 253]. This study is in agreement with the previous finding that the Na⁺-K⁺-ATPase pump is important to maintain the ionic equilibrium in the lens and its impairment leads to deposition of Na⁺ and loss of K⁺ with hydration and
swelling of the lens fibers leading to cataractogenesis [254]. This alteration in the Na\(^+\)-K\(^+\) ratio alters the protein content of the lens, leading to a decrease in water soluble proteins content and increase in insoluble proteins. This causes lens opacification [255]. This study showed higher total and water-soluble proteins and K\(^+\) ions whereas lower water insoluble protein and Na\(^+\) as well as Ca\(^{2+}\) ions concentration in Lisinopril treated group. Therefore, Lisinopril prevented Na\(^+\) and K\(^+\) imbalance, which may be due to a direct effect on lens membrane Na\(^+\)-K\(^+\)-ATPase or indirect effect through their free radical scavenging activity. Chemical analysis of selenite treated lenses clearly demonstrated a significant depletion of GSH and increased membrane damage as indicated by the levels of MDA, the product of membrane lipid peroxidation. Such changes in GSH and MDA levels in presence of selenite have been reported [111]. Restoration of GSH and MDA levels, protection against aggregation and insolubilization of lens proteins and maintenance of lens clarity without doubt establish the protective action of Lisinopril. Noda Y et al., [256] reported the nitric oxide scavenging activity of Lisinopril. The water-soluble proteins level has been increased by Lisinopril that retards the process of cataractogenesis initiated by oxidative stress by sodium selenite.

Conversely, the result of the in vivo phase of the study shows that Lisinopril is unable to show protective response against oxidant stress generated rat pups. This might be due to the absence of intrinsic ability of Lisinopril for the binding with lens specific enzymes or proteins. Because, Lisinopril is a non-thiol ACE inhibitor and there are always controversies between the effectiveness of non-thiol and thiol ACE inhibitors, as thiol group is important for the quenching of free radicals [257-260].

3.5. Conclusion

In conclusion, Lisinopril significantly protected cultured rat lenses against sodium selenite induced cataract. On the other hand, it failed to produce any significant effect in an in vivo experimental condition due to lacking of its intrinsic
ability to quench the free radicals generated by sodium selenite and that might be due to the absence of thiol group. These preliminary results are encouraging to evaluate the protective ability of other thiol containing ACE inhibitors against oxidant stress induced cataract development. A thiol group containing ACE inhibitor could possibly afford the protection of lenses against oxidant stress which thus led to the planning and execution of the second experiment (in the next chapter) using Captopril under same experimental settings.