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

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Summary

The present study sought to assess the efficacy of the captopril in preventing selenite-induced cataractogenesis in an experimental setting. The first, *in vitro* phase of the study was performed on lenses from Wistar rats incubated for 24 h at 37^0C in Dulbecco's Modified Eagle Medium (DMEM) alone (control, Group I), or in DMEM containing 100 µM of selenite (Group II) or in DMEM containing 100 µM of selenite and 5 mM captopril was added (Group III). The second, *in vivo* phase of the study, 9 days old Wistar rat pups divided into control and test groups were injected subcutaneously with 19 µM/kg sodium selenite. One day before the selenite challenge, the pups in the test group were injected intraperitoneally a single dose of 50 mg/kg of captopril 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. In the first phase, the mean activities of reduced glutathione, total protein, water soluble protein, Ca^{2+} ATPase activity and the antioxidant enzymes activity were significantly lower in Group II than in Group I or Group III lenses, while malondialdehyde concentration (an indicator of lipid peroxidation) and lens calcium ion concentration was significantly higher in Group II lenses than that in Group I or Group III lenses. The second, *in vivo* phase of the study revealed dense opacification (cataract formation) in 100% of Wistar rat pups receiving sodium selenite alone (19 µM/kg s.c) but in only 37.5% of those receiving subcutaneous selenite and captopril (50 mg/kg i.p). The study on the evaluation of the anticataract potential of captopril in experimental animals indicated that it modulates antioxidant parameters in the enucleated eye lenses. It also attenuates selenite-induced cataract both *in vitro* and *in vivo*, so it may be useful for cataract therapy.

Keywords: Cataractogenesis; ACE inhibitors; Captopril; Lipid peroxidation.

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Introduction

Cataract is a multifactorial disease associated with several risk factors such as aging, diabetes, malnutrition, diarrhoea, sunlight, smoking, hypertension and renal failure (1). Free radical-induced oxidative stress is postulated to be perhaps the major factor leading to senile cataract formation (2). This hypothesis is supported by the anticataractogenic effect of various nutritional and physiological (3,4) antioxidants in experimental animals. 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 anticataract agents (5). Physiologic antioxidant such as pyruvate and nutritional antioxidant vitamin E, ascorbic acid and carotenoids were found to delay the experimental cataract. Several previous experimental findings postulated that angiotensin converting enzyme inhibitors (ACEi) may act as a "Magic bullets" against oxidant stress especially captopril which exhibits a wide variety of biological activities (6-9). Buyan et al in 1992, has evaluated the anticataractogenic effect of topical captopril in diquat-induced cataract in rabbits (10). Selenite cataract, first described by Ostadalova et al in 1978, is an excellent model of oxidative stress-induced cataractogenesis *in vitro* and *in vivo* (11), which is resemblance to human cataract; hence it was used in the present study to evaluate the efficacy of captopril as an anticataractogenic agent to make a study more complimentary with respect to previous study.

Material and Methods

Chemicals

Captopril was kindly provided by Wockhardt Ltd (Aurangabad, Maharashtra, India) approximate purity was 98%. Dulbeco's modified Eagles medium (DMEM) was obtained from Hi Media Laboratories (Mumbai, India). Fetal bovine serum (FBS), sodium selenite and chemicals required for enzyme assay were 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.

In vitro phase of the study

Wistar rats of either sex in the weight range 80 to100gm 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 handle 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 wistar strain rats under deep anesthesia. Lenses were organ cultured in DMEM medium with HEPES buffer, supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 µg/ml amphotericin was also added to prevent bacterial contamination which contained sodium bicarbonate (0.2% w/v) but did not contain calcium. 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 groups. 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 (10). All lenses were incubated for 24 h at the conditions described earlier. After incubation, lenses were processed for the estimation of biochemical parameters.

Reduced glutathione (GSH)

The GSH content was estimated by the method of Moron et al (12). 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_2HPO_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.

Malondialdehyde (MDA)

The extent of lipid peroxidation was determined by the method of Ohkawa et al (13). 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 colour was read at 532 nm. Tetramethoxypropane was used as an external standard. The level of lipid peroxide was expressed as nmoles of MDA formed in μmol/g wet weight for lenses.

Assay of Ca^{2+} ATPase activity

The activity of Ca^{2+} ATPase in the lens samples was measured by the method of Rorive and Kleinzeller (14). 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 were added to control tubes and these tubes were subsequently kept in ice for 20 min. All the tubes were then centrifuged at 2500x g for 10 min and the supernatant was collected. The protein-free supernatant was analyzed for inorganic phosphate. For this, 3 ml of the supernatant were treated with 1 ml of ammonium molybdate and 0.4 ml of 2,4 aminonapthol sulphonic acid (ANSA). The color developed was read at 680 nm after 20 min.

Estimation of levels of Ca^{2+}

The levels of calcium ions in the lenses were estimated as follows. 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 that was prepared by dissolving in 1 per cent nitric acid.

Estimation of Protein values

For total protein estimation the lens homogenate was prepared in 5% trichloroacetic acid. The precipitated protein was dissolved in Sodium hydroxide and used as aliquots for the estimation of total proteins. Soluble fractions of the protein were estimated by preparing homogenate in double distilled water. The water-soluble supernatant was used for estimation of soluble protein. The protein content of the samples was determined by the method of Lowry et al (15) using bovine serum albumin as the standard.

Enzyme assays

A separate set of experiments 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 480 nm, the ability of the enzyme to inhibit the oxidation of epinephrine (16) was used to assess the activity of superoxide dismutase (SOD). One unit of SOD activity was defined as the amount of enzyme inhibiting 50% of the rate of auto-oxidation of epinephrine under the defined assay conditions. The enzyme activity of catalase (CAT) was measured spectrophotometrically at 240 nm by following the decomposition of H_2O_2 (17). One unit of CAT activity represented the amount of enzyme required to decompose 1 μmole of H_2O_2/min. Glutathione peroxidase (GPx) activity was monitored at 340 nm (18). One unit of enzyme activity was defined as the amount of GPx required to use 1 nmole of nicotinomide adenosine dinucleolide phosphate per minute. The conjugation of GSH with 1-chloro,2-4 dinitrobenzene (CDNB), a hydrophilic substrate, was examined spectrophotometrically at 340 nm (19) to measure glutathione S-transferase (GST) activity. One unit of GST was defined as the amount of enzyme required to conjugate 1 μmole of CDNB with GSH per minute.

***In vivo* phase of the study**

Cataract was induced in 9-d-old Wistar rat pups. The control and test groups had equal numbers of pups. 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 50 mg/kg of captopril 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 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).

Statistical analysis

All data were expressed as mean±SD. The groups were compared using one-way ANOVA with post-hoc Dunnett's test using Selenite 100μM group as control and the chi-square test were applied wherever relevant.

Results and Discussion

Incubation of lenses with Selenite 100µM 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 2.40±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 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 1.36 ± 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 (1.128±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 0.067 ± 001 µmol/g of wet weight of lens (Table 1).

Table 1. Levels of reduced glutathione and malondialdehyde in Group I, Group II and Group III lenses

Parameter	Group I	Group II	Group III
GSH (µg/mg wt.)	2.40 ± 0.05*	1.36 ± 0.01	2.19 ± 0.01*
MDA (µmol/g)	0.061 ± 0.001*	1.128 ± 0.02	0.067 ± 0.001*

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. GSH:glutathione; MDA:malondialdehyde.

Selenite 100µM 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 2). Captopril group had significantly higher concentrations of total lens proteins and water soluble protein ($P<0.01$), compared with Selenite 100µM group. At the same time, they had lower Ca^{2+} conc. ($P<0.01$) compared with Selenite 100µM 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 levels (Fig. 1).

Table 2. Levels of total protein, water soluble protein and Ca^{2+} conc. in Group I, Group II and Group III lenses

Parameter	Group I	Group II	Group III
Total Protein (mg/mg wt.)	0.389 ± 0.003*	0.336 ± 0.012	0.368 ± 0.014*
Water Soluble Protein (mg/mg wt.)	0.282 ± 0.005*	0.180 ± 0.001	0.248 ± 0.004*
Calcium (Ca^{2+}) (%wt)	0.014 ± 0.004*	0.044 ± 0.002	0.016 ± 0.001*

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.

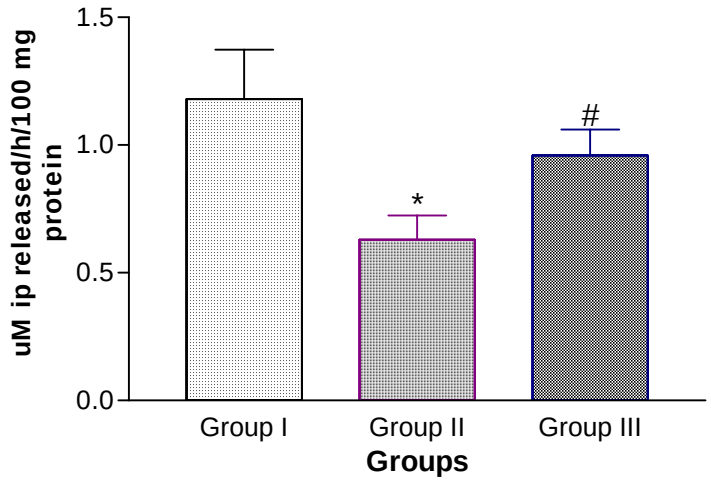


Fig. 1. Activity of Ca^{2+} ATPase in lens. All values are expressed as mean±SD of five determinations.
Group I: Normal, Group II: lenses exposed to selenite only. Group III: lenses exposed to selenite and captopril.
Statistically significant difference ($^*P < 0.01$) when compared with group II values.

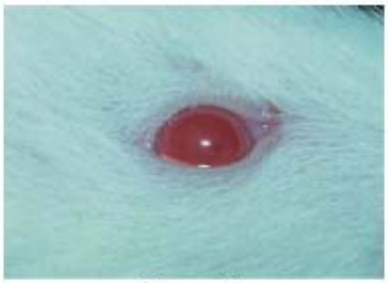
The effect of 5 mM captopril on different enzymes (SOD, CAT, GPx and GST) is presented in Table 3. It was observed that in presence of selenite stress in group II lenses, antioxidant enzymes were significantly reduced as compared with the normal group. In presence of captopril, there was a significant positive modulation of enzyme activities observed in group III lenses.

Table 3. Levels of antioxidant enzymes in Group I, Group II and Group III lenses

Parameter (IU/mg protein)	Group I	Group II	Group III
SOD	2.46 ± 0.14*	0.28 ± 0.03	1.08 ± 0.01*
CAT	1.19 ± 0.001*	0.01 ± 0.01	0.45 ± 0.02*
GPx	10.73 ± 0.91*	1.07 ± 0.10	6.49 ± 0.17*
GST	2.06 ± 0.19*	0.16 ± 0.01	1.06 ± 0.13*

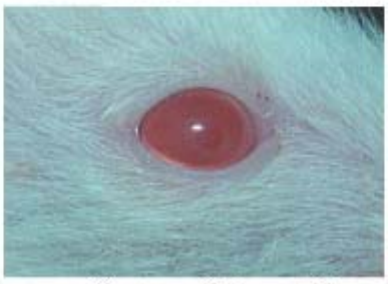
All values are expressed as mean±SD of five determinations.
Group I: Normal, Group II: lenses exposed to selenite only. Group III: lenses exposed to selenite and captopril.
Statistically significant difference ($^{*}P < 0.01$) when compared with group II values.

In rat pups that had received selenite alone (Group A), dense opacification of the lens (Grade +++ opacity) was observed in all (100%) 8 animals. In contrast, only 3 of 8 (37.5%) rat pups in Group B (received selenite and captopril) exhibited opacification (Grade + or ++) (Fig. 2). This difference was statistically significant (x^2 [df =1] = 9.6; $P < 0.01$).



Normal

Selenite Induced



Captopril Treated

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 (20). 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. Captopril was found to be one of them (10, 21).

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. A single dose of selenite administration leads to impaired oxidative defense, membrane damage and cataract formation (5). The lens *in vitro* is highly susceptible to damage by ROS, 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 (22, 23).

In the present study, 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 and decrease water soluble protein content. Such changes in GSH and MDA levels in presence of selenite have been reported (24). 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 captopril.

Calcium is essential for various lens fiber cell processes including its differentiation (25). The levels of the divalent cation Ca^{2+} in the lens is maintained at submicromolar range and is lower than that of the aqueous humor (26). It has also been found that alterations in the homeostasis of lenticular Ca^{2+} have been implicated in cataractogenesis (27). In this study, activity of Ca^{2+} ATPase was significantly decreased in selenite treated lenses with a corresponding increase in Ca^{2+} concentration. Treatment with captopril was observed to maintain the activity of Ca^{2+} ATPase and level of Ca^{2+} close to normal range. The level of Ca^{2+} is maintained by Ca^{2+} ATPase, which counteracts the inward passive diffusion of Ca^{2+} (28). 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+}. 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 are some of the steps leading to the development of opacification. Selenite-induced opacification of the lens *in vitro* also has been demonstrated (29). These findings are in agreement with our observation that 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. The lower levels of Ca^{2+}, higher activity of Ca^{2+} ATPase, and decreased levels of lipid peroxidation in the lens of the captopril-treated group could be attributed to its antioxidant protection against selenite- induced oxidative stress.

SOD, CAT, GPx and GST are important components of the innate enzymatic defences of the lens. SOD, a chain-breaking antioxidant, was first described by McCord and Fridovich (30) in red blood cells. Varma et al. (31) first described its occurrence in the lenses of different species. SOD converts superoxide to H_2O_2. The enzyme exists in two forms, one containing Mn^{2+}, restricted to the mitochondria, and a cytosolic form containing Zn^{2+} and Cu^{2+}. The occurrence of GPx in the lens was first shown by Pirie (32).

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 (33). The presence of CAT in the lens has been well demonstrated (34). Both CAT and Gpx catalyse the transformation of H_2O_2 within the cell to harmless by-products, thereby curtailing the quantity of cellular destruction inflicted by products of lipid peroxidation (35, 36). GST is important for detoxification process. The level of these enzymes were significantly hampered with selenite and positively modulated in the presence of captopril. The data clearly demonstrated that captopril significantly improves the antioxidant defense mechanisms of the normal lens.

Based on our findings of *in vitro* studies that captopril acts as an antioxidant, captopril was evaluated against selenite-induced cataracts in young rats. Captopril significantly protected the lens morphology and clarity: 37.5% of the eyes had grade + or ++ opacity; in contrast, 100% of the control eyes developed dense opacity or grade +++ opacity. From the current study, it is evident that captopril protects the lens against oxidative stress. Our results on selenite-induced cataracts *in vitro* and *in vivo* not only demonstrate the protective effect of captopril but also indicate that captopril prevents cataractogenesis by virtue of its antioxidant properties. Captopril, therefore, may be useful for prophylaxis or therapy against cataracts.

In conclusion, the study on the evaluation of the anticataract potential of captopril in experimental animals indicated that it modulates antioxidant parameters in the enucleated eye lenses. It also attenuates selenite-induced cataract both *in vitro* and *in vivo*, so it may be useful for cataract therapy.

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