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Resveratrol Prevents Cataract Formation by Inhibiting Pro-inflammatory Mediator-induced Dysregulation of Lens Calcium

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Abstract

The effect of resveratrol, a free radical scavenger, during cataract development was evaluated in the Wistar rat pup model. This study investigated the possible free radical scavenging potential of resveratrol at 40 mg/ kg body wt dose in selenite-induced cataract in rat pups. Intraperitoneal injection of sodium selenite (15 µm mol/ kg body wt) in 8 to 10 day old rat pups lead to severe oxidative stress in the tissues evidenced by decreased antioxidants and increased lipid peroxidase, nitric oxide, superoxide anion, hydroxyl radical generation, inducible nitric oxide synthase (iNOS) as well as nuclear factor kappa B (NF-kB) expression levels that probably led to cataract formation. Selenite exposure also caused an increase in total calcium in the eye lens and significantly inhibited the activity of Ca^{2+} ATPase but not Na^{+}/ K^{+} ATPase or Mg^{2+} ATPase. However, both pre - and co-treatments with resveratrol, but not post-treatment, led to an increase in antioxidant levels with a concomitant reduction in oxidative stress and also rescued the selenite-mediated increase in lens Ca^{2+} and inhibition of Ca^{2+} ATPase activity in the eye lens. The results of this study demonstrate antioxidants decrease and increase in free radical generation triggered by selenite causes the inactivation of lens Ca^{2+} ATPase leading to a rise in intracellular Ca^{2+} level. Resveratol treatment was able to prevent selenite-induced oxidative stress and in turn the inhibition of lens opacification. Thus, resveratrol has the potential to function as an anti-cataractogenic agent, possibly by preventing free radical-mediated accumulation of Ca^{2+} in the eye lens.

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Introduction

Cataract is the most common cause of treatable blindness worldwide and develops as a result of progressive loss in lens transparency [1-3]. It has been demonstrated that oxidative stress plays an important role in cataract etiopathogenesis, as with many age-related diseases [4-10]. Oxidative stress occurs when free radical formation exceeds the cellular antioxidant capacity. Cells have evolved to combat these free radicals, utilizing different antioxidant enzymes [2]. The connection of these early effects to subsequent opacification of the lens tissue that is characterized by the presence of disulfide liked proteins of higher molecular weight with lower solubility [11] is quite complex. Such crosslinks are primarily established between lens glutathione and proteins, especially the lens crystallins. In addition, membrane lipids are also peroxidized [12] causing cell damage. Cataract formation may be attributed to oxidative stress triggered by reactive oxygen species (ROS), which include, superoxide anion (O_2^-), nitric oxide (NO), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$-) and hydroxyl radical ($OH^\cdot$). Some of these free radicals may react with each other or other cellular components to yield highly reactive compounds that cause extensive cellular damage [13].

Nitric oxide (NO) is an important signalling molecule that mediates a variety of essential physiological processes, including neurotransmission, vasodilatation and host cell defense. NO is synthesized from L-arginine by NO synthase (NOS). While an inducible nitric oxide synthase (iNOS), one of the three isoforms of NOS, is usually not present under normal conditions, and has been known to be induced by cytokines, advanced glycation end products in addition to the transcription factor, nuclear factor-kappa B (NF-κB) [14, 15] to name a prominent few. Because iNOS promoter has an NF-κB binding site, the activation of NF-κB is important for iNOS induction [16-21]. The induction of iNOS could result in elevated and sustained release of NO, which can be cytotoxic [22, 23]. Of particular relevance is the finding that NO is known to influence apoptosis in a variety of animal models of disease [24]. The over production of NO in response to induction of iNOS is observed in ureitis, vetinins, glaucoma [25, 26] and cataract [27, 28].

The calcium level in lens cells is essential for lens physiology, and maintenance of lens transparency. Calcium increase is associated with cataracts in humans and most animal models [29, 30]. It has been shown that selenite cataractogenesis involves Ca^{2+} mediated activation of calpain II, a Ca^{2+}-dependent protease, and irreversible damage of the lens nuclease [31], but the underlying mechanism could very well involve the progressive deterioration in Ca^{2+} homeostasis [32] that leads to disease. We would like to highlight a few reports that have shown that calpain participates in the regulation of lens Ca^{2+} homeostasis [33-36]. Although the physiological function of calpain is not exactly clear, it has been suggested to play important roles in cellular functions that occur in response to mobilized calcium ions [37]. Thus, it seems likely that calpain is involved not only in physiological events but also in various pathological states, including cataract development [38].

Although cataract surgery is recognized as being safe, there is a significant rate of complications leading to irreversible blindness. Thus, much emphasis is being laid on identification of compounds that will help to prevent cataractogenesis in the first place. Natural and synthetic compounds have been shown to prevent cataract formation induced by selenite and other chemicals [39-41]. In these studies aimed at the developmental stages of cataract, there is a strong scientific basis for therapy using antioxidant and anti-inflammatory properties of specific compounds. Resveratrol (3, 4, 5- trihydroxy-trans-stibene), is a naturally occurring phytoalexin, found in grapes and variety of medicinal plants, is a stilbene derivative with three hydroxyl groups. The richest source of this compound is *Polygonum cuspidatum* a plant that has been used in oriental folk medicine against supportive dermatitis, gonorrhoea, favus, athlete's foot and hyperlipemia [42]. It has many biological and pharmaceutical properties, as evidenced through different experimental studies. For instance, resveratrol has been reported for its anti-tumor effects, increased cyclic adenosine monophosphate (cAMP) formation [43] and the inhibition of protein kinase C [44]. It inhibits lipid peroxidation, angiogenesis, scavenges free radicals and modulates lipid as well as lipoprotein metabolism

[45-49]. Further, resveratrol has also been observed to play a role in the prevention of cardiovascular disease [50, 51]. In this study we have made an attempt to unravel the use of resveratrol as a potential anti-cataract agent by analysing its effect on pro-inflammatory changes in eye lens.

Materials and Methods

Chemicals

Resveratrol and sodium selenite were purchased from Sigma Chemicals (St. Louis, MO, USA). Polyclonal rabbit anti-iNOS and NF-κB p65 antibodies were purchased from BD Biosciences (San Jose, CA, USA). Primers for iNOS and the HRP conjugated IgG secondary antibodies were purchased from Bangalore Genei (Bangalore, India). All other chemicals and reagents used were of the highest analytical grade commercially available.

Animals

Male albino Wistar rat pups 8-10 days of age and weighing 15-20 g were procured from the National Institute of Nutrition (Hyderabad, India). All experiments were approved by the Institutional Animal Ethical Committee (IAEC; No. 360/01/a/CPCSEA), India, guidelines. Rat pups were housed in an air-conditioned room at 22 ± 10°C with a lighting schedule of 12 h light and 12 h dark. Rat pups were fed a balanced commercial rat diet (Hindustan UniLever, Mumbai, India) and water ad *libitum*.

A pilot study was performed to determine the LD_{50} value for sodium selenite in rat pups [52]. We observed the induction of cataract in suckling rat pups administered a single dose of sodium selenite *s.c.* (15 µmol/kg body wt) at around 10-12 days post partum with lens opacity evident in both eyes and this procedure was adopted for subsequent studies.

The rat pups were divided into five groups (of six animals each): group I, control rat pups receiving physiological saline; group II, selenite treated (single dose administered sub-cutaneously at 15 µmol/kg body weight); group III, selenite-induced group *co-treated* with resveratrol (single dose of resveratrol gavaged orally at 40 mg/kg body weight); group IV, selenite-induced animals *post-treated* (after 24 h) with resveratrol at the same dose as for group III; group V, rat pups *pre-treated* with resveratrol (same dose as for group III), 24 h before the administration of selenite.

Tissue Preparation

After treatment, rat pups were sacrificed by means of an overdose of pentobarbital at a dose of 50 mg/kg body weight given intraperitoneally. The lens tissue was immediately harvested, washed in ice-cold saline to remove blood, and frozen at -70°C. This was then homogenized using 10% 0.1 M Tris-HCl buffer (pH 7.2) and centrifuged (12,000 rpm, 30 min, 4°C). The supernatant obtained was estimated by the method of Lowry et al. [53] and used for further analyses.

Quantitative Analysis of Enzyme Activities

Catalase (CAT)

CAT activity was determined by the method of Beers and Sizer [54]. In this test, dichromatic acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2, with the formation of perchloric acid as an unstable intermediate. In the test, green colour development was read at 590 nm against blank in a spectrophotometer. The activity of catalase was expressed as µmol of H_2O_2 consumed/mg protein/min.

Superoxide Dismutase (SOD)

SOD activity was determined by the method of Misra and Fridovich [55]. In this test, the degree of inhibition of pyrogallol auto-oxidation by serum and lens homogenate supernatant was measured. The change in absorbance was read at 470 nm against blank every 3 min on a spectrophotometer and the enzyme activity was expressed as 50% inhibition of adrenaline auto oxidation/min.

Glutathione Peroxidase (GPx)

The GPx activity was determined essentially as described by Rotruck et al. [56]. The rate of glutathione oxidation by H_2O_2, as catalysed by GPx present in the supernatant is determined and colour developed was read against a reagent blank at 412 nm using a spectrophotometer. In the test, the enzyme activity was expressed as µmole of glutathione oxidized/mg protein/ min.

Glutathione-S-transferase (GST)

The GST activity was determined by the method of Habig et al. [57]. The conjugation of GSH with

1-chloro-2, 4-dinitrobenzene (CDNB), a hydrophilic substrate was observed spectrophotometrically at 340 nm to measure the GST activity and result was expressed in conjugate/µmol of CDNB with GSH/min/ mg protein.

Reduced Glutathione (GSH)

The GSH content was estimated by the method of Moron et al. [58]. The serum and lens homogenate was centrifuged at 5000 rpm for 15 min at 4°C. To the resulting supernatant, 0.5 ml of 10% trichloroacetic acid was added and centrifuged. The resulting protein-free supernatant was allowed to react with 4 ml of 0.3 M Na_2HPO_4 (pH 8.0) and 0.5 ml of 0.04% (w/v) 5.5-dithiobis-2-nitrobenzoic acid. The absorbance of the resulting yellow colour was read spectrophotometrically at 412 nm and results were expressed as µmol of NADPH oxidized/min/mg.

Estimation of Superoxide Anion Generation in Eye Lens

The generation of O_2^- in the eye lens of Wistar rat pups was estimated spectrophotometrically using the cytochrome c method [59]. Briefly, clear eye lens obtained from the various experimental groups was incubated with 500 µl phosphate buffer (pH 7.8, 0.1 M EDTA) and 100 µl of cytochrome c (0.002 mM) with or without superoxide dismutase (SOD), for 15 min. At the end of the reaction the absorbance was read at 550 nm in a UV-160 A Shimadzu spectrophotometer against a suitable blank and O_2^- generated was expressed as absorbance at 550 nm/15 min.

Determination of the Lipid Peroxidation Product, Malondialdehyde (MDA)

Lipid peroxidation was determined by the method of Ohkawa et al. [60]. The principle of this method being that malondialdehyde (MDA), an end product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to form a pink chromogen. For this assay, 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid aqueous solution were added in succession in a reaction tube. To this reaction mixture, 0.2 ml of the serum and lens homogenate was added, and 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 2236 x g for 15 min following which the upper layer was separated, and the intensity of the resulting pink color was read at 532 nm. Tetramethoxypropane was used as an external standard and the level of lipid peroxides was expressed as nmol of MDA formed/g wet weight.

Estimation of Nitric Oxide Generation in Eye Lens

Generation of NO in the eye lens of Wistar rat pups was determined by the method of Ozbek et al. [61] with slight modifications. For this assay, 100 µl of clear eye lens homogenate supernatant obtained from the various experimental groups was mixed with 150 µl Tris-HCl buffer (pH 7.4). It was incubated with 5 µl of 0.01 U nitrate reductase and 10 µl of 2 mM β-NADH for 20 min at 22°C in the dark with constant shaking. After this, 50 µl of 1% sulfanilamide followed by 50 µl of 0.1% naphthylethylenediamine dihydrochloride was added and incubated for 10 min at room temperature. At the end of incubation, the samples were centrifuged (12,000 rpm, 15 min, 4°C) to pellet any precipitate that may have formed and the absorbance of the clear supernatant was read at 540 nm in a Shimadzu (UV-160 A) spectrophotometer against a reagent blank consisting of buffer and Griess reagent. The nitrite (=NO) generated in the eye lens was determined against sodium nitrite in a standard curve and the amount of nitrite was expressed as µM nitrite.

Estimation of Hydroxyl Radical Generation in the Eye Lens

The generation of $OH^{\cdot}$ in the eye lens of Wistar rat pups was detected spectrophotometrically by Halliwell and Gutteridge [62]. Briefly, eye lens obtained from the various experimental groups was incubated in 700 µl phosphate buffer (pH 7.8, 0.1 M EDTA), 2 mM sodium salicylate, and 40 µl 10 N HCl and to this 0.25 g of NaCl was added. To this mixture an equal volume of chilled diethyl ether was added and incubated for 30 min at 25°C. The absorbance was read at 510 nm in a UV-160 A Shimadzu spectrophotometer against a suitable reagent blank and the $OH^{\cdot}$ generation was expressed as absorbance at 510 nm/30 min.

Intracellular Calcium Analysis

Total lens calcium was estimated by the method of Inomata et al. [63]. The dry weight of lenses was measured after heating at 100°C for 20 h. The lenses

were extracted with 0.2 ml of concentrated HCl at room temperature overnight and made up to 1.0 ml with deionized water. The mixtures were then centrifuged at 10,000 rpm for 10 min to remove insoluble material and calcium concentration in the supernatant fraction was measured by an atomic absorption spectrophotometer.

Estimation of Lens ATPase: Ca^{2+}, Na^{+}/K^{+}, and Mg^{2+} ATPases

Ca^{2+} ATPase activity was assayed according to the method of Hjerten and Pan [64]. This involves 10 mM ATP as substrate in the presence of Tris-HCl buffer (125 mM, pH 8.0) containing 50 mM calcium chloride. Lens Na^{+}/K^{+} ATPase activity was estimated by the method of Bonting [65]. The reaction mixture contained 40 mM ATP as substrate and Tris–HCl buffer (90 mM, pH 7.5) containing 50 mM magnesium sulfate, 50 mM potassium chloride, 600 mM sodium chloride and 1 mM EDTA. Mg^{2+} ATPase activity in eye lens was estimated by the method of Ohnishi et al. [66], in a reaction containing 0.01 M ATP and Tris-HCl buffer (375 mM, pH 7.6) containing 60 mM magnesium chloride.

Western Blot Analysis

The whole lenses from each group were homogenized in 10 volumes (v/w) of 20 mM Tris-HCl (pH 7.4) containing 5 mM EDTA and 10 mM mercaptoethanol using a glass–glass Dounce homogenizer. The homogenates were centrifuged at 12,000 rpm, 30 min, 4°C and the recovered supernatants were used as lens protein. The lens proteins prepared from normal and treated rat pups were electrophoresed by the method of Laemmli, [67] in 12% SDS-PAGE slab gels. After electrophoretic separation, the proteins were transferred onto Immobilon nitrocellulose membranes. Immunoblotting was performed with rabbit and rat polyclonal primary antibodies, i.e., anti- p65 subunit of NF-κB, anti-iNOS and anti- αA-, αB-crystallin respectively (at a dilution of 1:500 each), after blocking with non-fat dry milk powder. This was later incubated with peroxidase-tagged goat anti-rabbit IgG secondary antibody, and immune complexes were detected using DAB (0.01%) as well as H_2O_2.

Reversed Transcription-polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from normal and cataract lenses using the acid guanidium thiocyanate-phenol-chloroform extraction method by Chomcynski and Sacchi, [68] with Trizol reagent (Sigma) according to the manufacturer's instructions. Oligo(dT)-primed first-strand cDNA was prepared from total lens RNA using AMV reverse transcriptase at 37°C for 60 min. PCR was performed with gene-specific primers using Taq DNA polymerase (Bangalore Genei). The primers used for iNOS were based on the rat sequence of Inomata et al. [27], 5′-GCCTCCCTCTGGAAGA-3′ (sense) and 5′-TCCATGCAGACAACCTT-3′ (antisense). The primers used for αA-, αB-crystallin and Hsp 70 were also based on the rat sequences 5′-CACCGTGAAGGTACTGGAAG-3′ (sense), 5′- TCAGGAAGGCAGACTCTTG-3′ (antisense), 5′ -AGA GCA CCT GTT GGA GTC TG-3′(sense), 5′-TTC CTT GGT CCA TTC ACA GT-3′ (antisense), 5′-ATG AAG GAG ATC GCC GAG G-3′ (sense), and 5′- GTC GAA GAT GAG CAC GTT G-3′ (antisense) respectively. The primers for β -actin as the reference control were 5′-GTGGCCGCTCTAGGCACCA-3′ and 5′ CGGTTGGCCTTAGGGTTCAGGGGGG-3′. The following cycling conditions were used: 120 s of initial denaturation at 94°C followed by 30 cycles of 90 s at 94°C, 60 s at 60°C, and 60 s at 72°C, followed by 5 min at 72°C. The amplification products were electrophoresed on an agarose gel (2%) in Tris-acetate EDTA buffer (pH 8.2). Bands, stained by ethidium bromide, were visualized by a UV-trans illuminator (Bio-Rad Gel Doc system).

Cytochemical Localization of Nitroblue Tetrazolium (NBT)-Reducing Substances in the Eye Lens

The generation of O_2^{-} in the eye lens of Wistar rat pups was detected cytochemically using NBT reduction method by Manikandan et al. [69]. Briefly, the whole eye lens was incubated with 100 µl of 0.3% NBT for 1 h at 22°C. After incubation, the eye lenses obtained from various experimental groups were washed with Tris-HCl buffer (pH 7.4, 0.1M Tris). The eye lenses were then examined for blue formazan deposits under bright-field optics (4×) using Carl Zeiss Axiolab microscope.

Immunohistochemical Analysis for iNOS and αA- & αB– Crystallin Expression

Immunohistochemistry was carried out by the method of Yao et al. [70] on 5-µm paraffin-embedded tissue sections on poly-L-lysine-coated glass slides. The tissue sections were deparaffinized by placing the slides in an oven at 60°C for 10 min and then rinsed twice in xylene for 10 min each. The slides were then hydrated in a graded ethanol series (100, 90, 70, 50, 30% for 10 min each) and then finally in double-distilled water for 10 min. The sections were incubated with 1% H_2O_2 in double-distilled water for 15 min at 22°C, to quench endogenous peroxidase activity. The sections were rinsed with Tris-HCl containing 150 mM NaCl (pH 7.4) and blocked in blocking buffer (tris-buffered saline (TBS), 0.05% Tween, 5% non-fat dry milk (NFDM)) for 1 h at 22°C. After being washed with TBS containing 0.05% Tween 20, the sections were incubated with primary antibodies, anti- αA- and αB-crystallin, as well as anti-iNOS polyclonal rabbit antibody, at a dilution of 1:500 each overnight at 4°C. After incubation the eye lens sections were rinsed with TBS containing 0.05% Tween 20 twice and incubated with secondary antibody, goat anti-rabbit IgG-HRP conjugate, at a dilution of 1:3000, for 1 h at 4°C. After another wash with TBS containing 0.05% Tween 20, the immunoreactivity was developed with 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2 for 1-3 min. The eye lens sections were observed (4×) for brown color formation under bright field using a Carl Zeiss Axioscope microscope.

Immunofluorescence Expression of p65/NF-κB

Deparaffinized tissue sections were rehydrated and blocked in 5% BSA in TBS with 0.05% Tween 20. Sections were then incubated overnight with anti-p65/ NF-κB (at a dilution of 1: 500) and then with secondary antibody conjugated to FTC (at 1: 40 dilution) in the dark at room temperature for about 2 h. The sections were then counter-stained with either PI or DAPI and visualized under a laser scanning confocal microscope (LSM 10).

Statistical Analysis

All results are expressed as mean ± SD. Independent sample student's *t* test was used to assess the data. A $p > 0.05$ was considered statistically significant.

Results

Resveratol Lowers Selenite Induced MDA Levels in Both Lens and Serum of Treated Rat Pups

Malondialdehyde (MDA) is a major reactive carbonyl product that is well known to cause cell damage arising from oxidative stress. We observed significant elevation in the level of MDA (Fig. 1) in group II treated rat pups that are significantly down-regulated in reseveratol treated groups III and V.

Resveratrol Treatment Down-Regulates Nitric Oxide and Hydroxyl Radical Generation Induced by Sodium Selenite

The generation of free radicals is a characteristic feature in mounting cellular oxidative insult. Two major mediators in this regard, nitric oxide and hydroxyl radical, have been known to play vital roles in promoting cataract formation. In this regard, the inhibition in both these mediators upon selenite treatment (Figs. 2 and 3) was observed to be suppressed by resveratrol (groups III and V) and this was found to be statistically significant.

Selenite Perturbs Total Lens calcium Level and Intracellular Ca^{2+} ATPase Activity that is Regulated by Resveratrol Treatment

In further corroboration to the purported effects of resveratrol on intracellular calcium regulation, we observed a significant elevation in intracellular calcium levels in lens tissue in group II that was lowered in groups III and V when compared to untreated rats (Fig. 4). This can be attributed to the aberration in intracellular calcium levels in selenite treated pups including disruption in Ca^{2+} ATPase activity as shown in Fig. 5. However, resveratrol did not show any significant modulation to the activities of cellular Na^+/K^+ and Mg^{2+} ATPases (Data not shown). This implies that it is lens Ca^{2+} that is probably dysregulated during the pathophysiology of cataract formation.

Resveratrol Replenishes Cellular Enzymic and Non-enzymic Antioxidants

The levels of cellular enzymatic and non-enzymatic antioxidants were assayed among the different experimental groups. Upon analysis, it was revealed that sodium selenite exerted significant inhibition on all cellular antioxidants assayed when compared to controls (Tables 1 and 2). Interestingly,

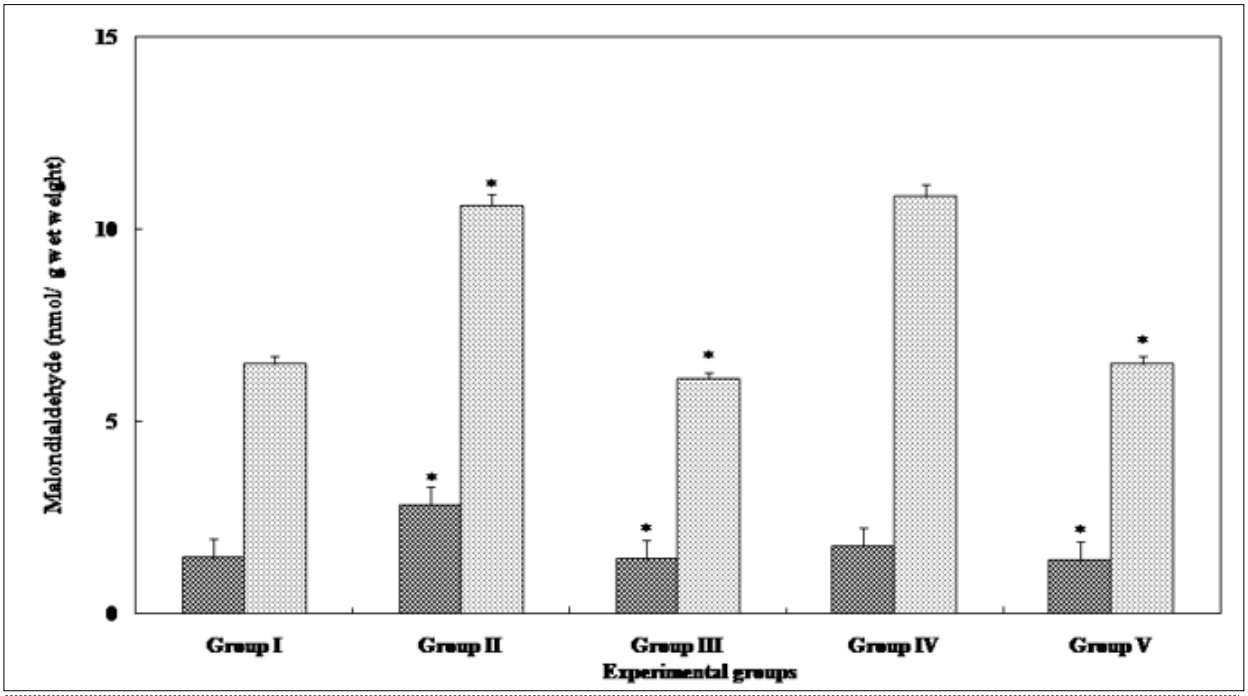

Figure 1. Malondialdehyde level in serum (dark shaded bars) and eye lens (light shaded bars) from different experimental group animals. Each value represents mean ± SD of 4 determinations using samples from different preparations. The difference in MDA levels observed between groups I & II and groups II & III-V animals were statistically significant at * $p < 0.05$.

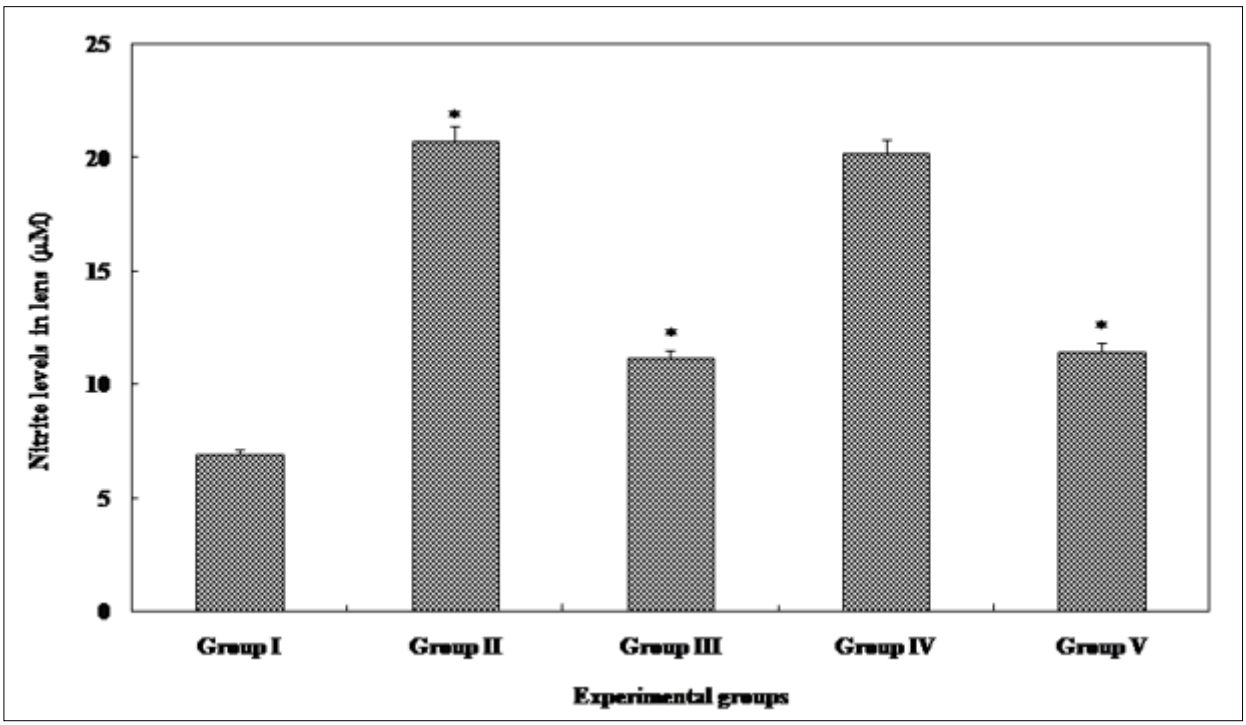

Figure 2. Nitric oxide level in the eye lens from different experimental group animals. Each value represents mean ± SD of 4 determinations using samples from different preparations. The difference in nitric oxide levels observed between groups I & II and groups II & III-V animals were statistically significant at * $p < 0.05$.

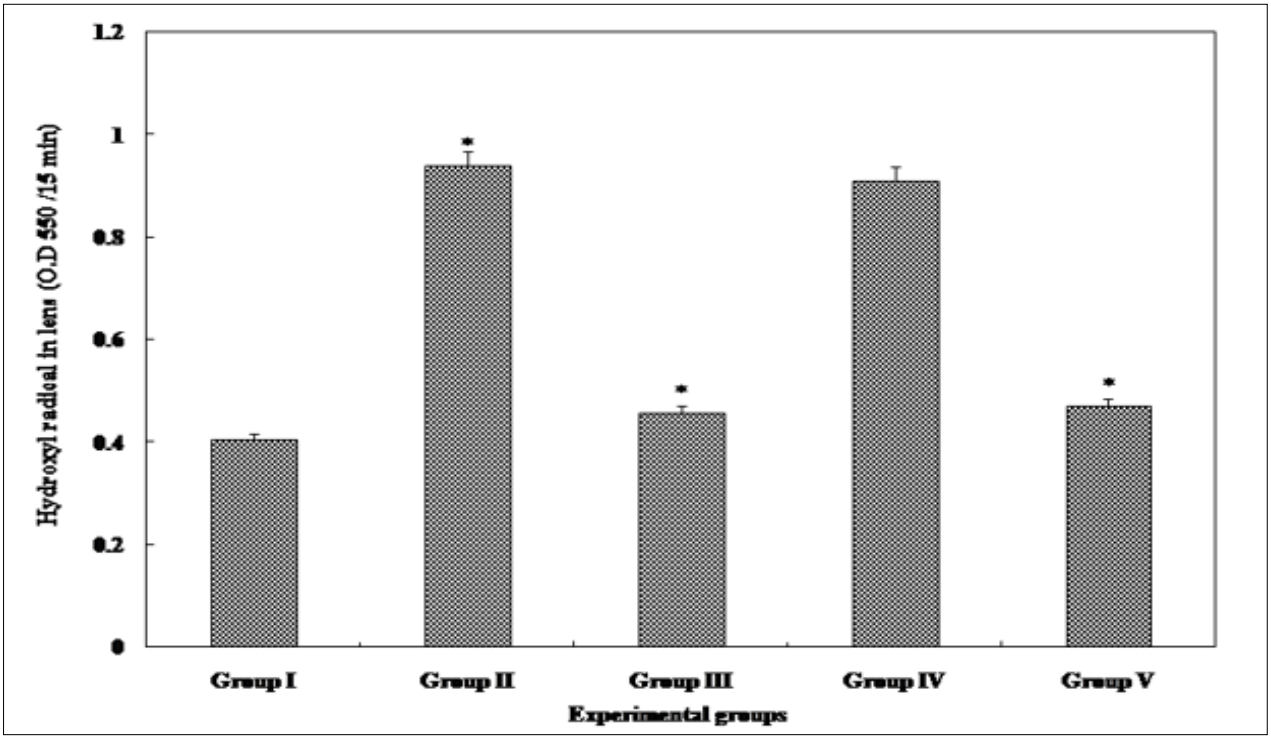

Figure 3. Hydroxyl radical level in from different experimental group animals. Each value represents mean ± SD of 4 determinations using samples from different preparations. The difference in hydroxyl radical levels observed between groups I & II and groups II & III-V animals were statistically significant at * $p < 0.05$.

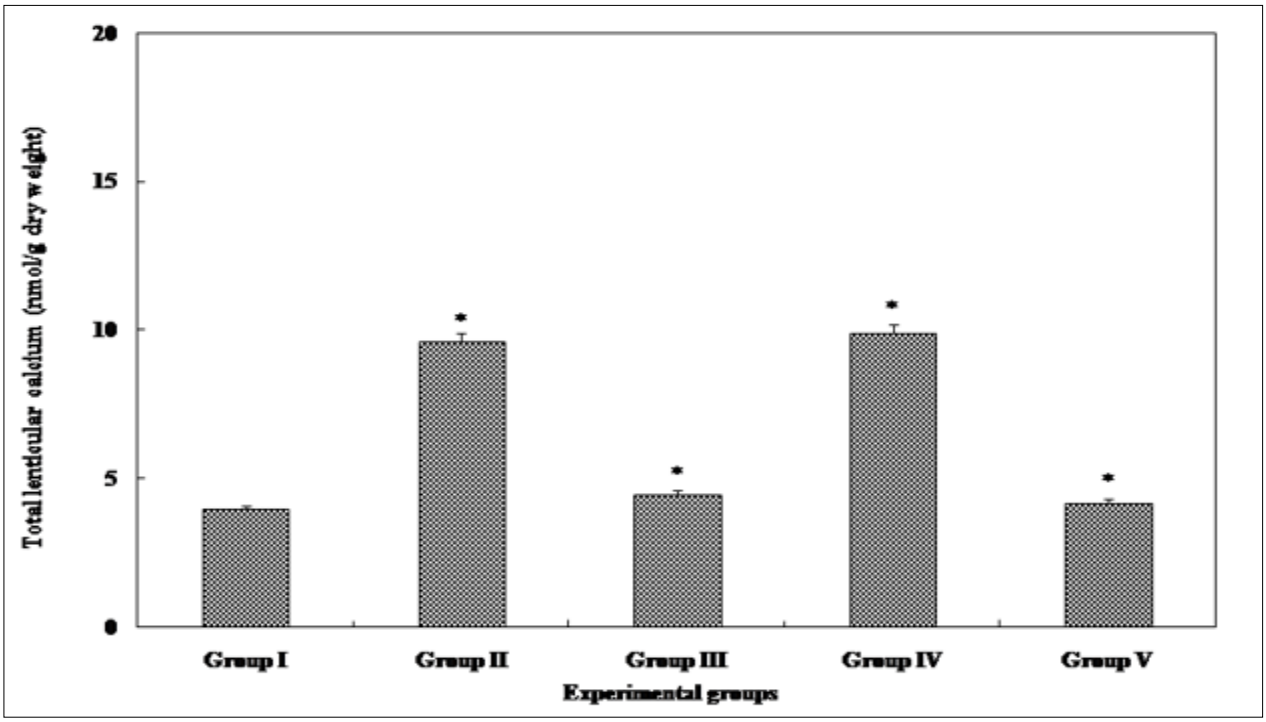

Figure 4. Total calcium levels in the eye lens from different experimental group animals. Each value represents mean ± SD of 4 determinations using samples from different preparations. The difference in total calcium levels observed between groups I & II and groups II & III-V animals were statistically significant at * $p < 0.05$.

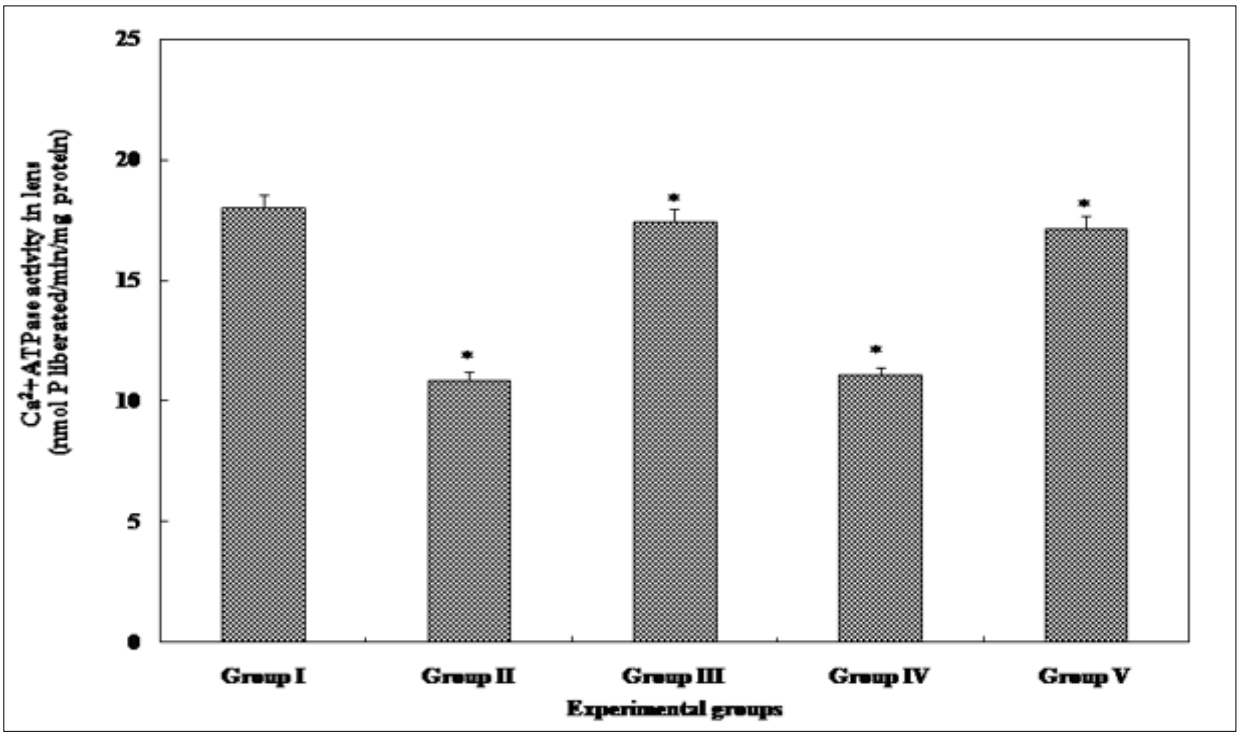

Figure 5. Calcium ATPase activity in the eye lens from from different experimental group animals. Each value represents mean ± SD of 4 determinations using samples from different preparations. The difference in Ca2+ ATPase activity observed between groups I & II and groups II & III-V animals were statistically significant at * $p < 0.05$.

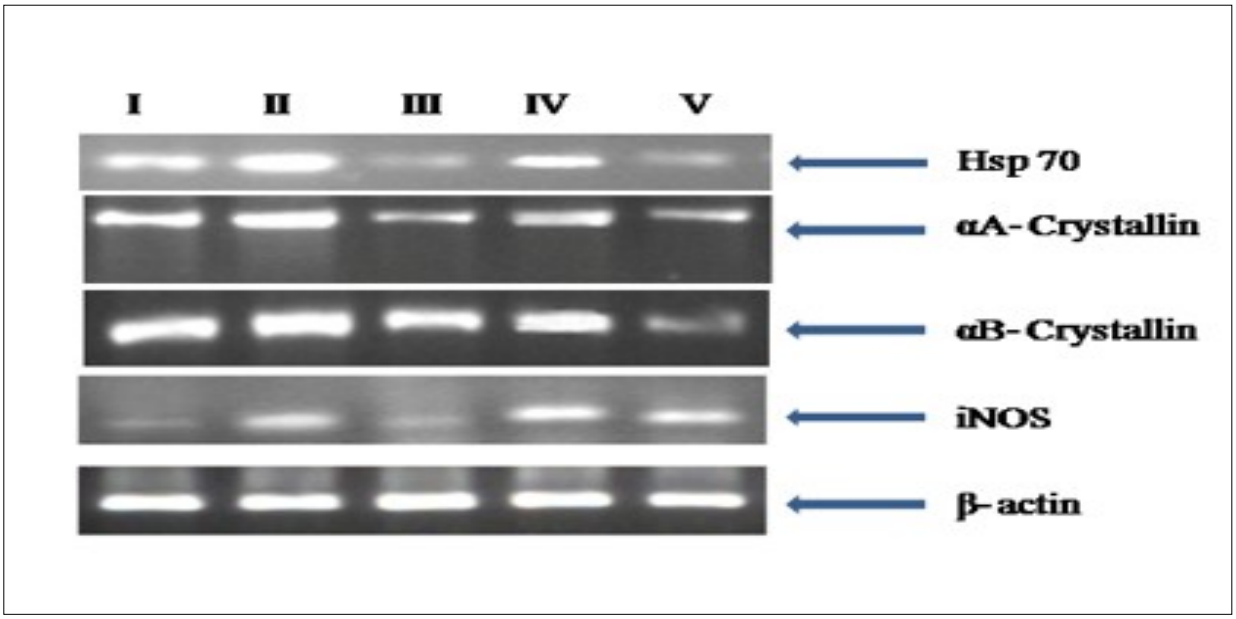

Figure 6. RT PCR analysis for Hsp 70, iNOS, αA and αB crystallin gene expression in the eye lens from different experimental group animals. Reverse transcribed cDNA isolated from- Lane I: control (group I); lane II selenite only (group II); lane III: selenium + resveratrol simultaneously (group III); lane IV: Resveratrol administered 24 h after treatment with selenite (group IV); lane V: Resveratrol administered 24 h before treatment with selenite (group V).

Table 1. Assay for enzymic antioxidant levels in serum and eye lens from the different experimental groups.

Enzymes analyzed (Unit of activity)	Group I	Group II	Group III	Group IV	Group V
Catalase (mmol H_2O_2 consumed/mg protein/min)					
Serum	19.46 ± 1.66	11.97 ± 0.92*	20.26 ± 1.28*	12.00 ± 1.18	19.71 ± 1.83*
Eye lens	6.34 ± 1.02	4.33 ± 0.59*	6.31 ± 1.51*	4.68 ± 1.20	6.89 ± 1.82*
Superoxide Dismutase (50 % inhibition of adrenaline auto oxidation/min)					
Serum	16.42 ± 2.32	11.14 ± 1.60*	16.35 ± 2.22*	11.67 ± 1.33	15.28 ± 1.60*
Eye Lens	3.69 ± 0.51	2.26 ± 0.52*	3.35 ± 1.03*	2.57 ± 0.48	3.47 ± 0.59*
Glutathione peroxidase (mmol glutathione oxidized/ mg protein/min)					
Serum	1.91 ± 0.20	1.04 ± 0.15*	1.85 ± 0.20*	1.02 ± 0.13	1.07 ± 0.30*
Eye lens	1.81 ± 0.16	0.88 ± 0.11*	1.49 ± 0.25*	0.89 ± 0.24	1.40 ± 0.29*
Glutathione-S-transferase (µmol of CDNB conjugated/min/mg/protein)					
Serum	4.0 ± 0.23	2.33 ± 0.48*	3.36 ± 0.57*	2.42 ± 0.56	3.11 ± 0.57*
Eye lens	80.50 ± 2.08	64.44 ± 5.91*	75.59 ± 5.57*	65.89 ± 4.83	73.11 ± 4.80*

Each value represents mean ± SD of 4 determinations using samples from different preparations. The difference in enzymic antioxidant levels observed between groups II and groups I, III and V were statistically significant at *p < 0.05.

Table 2. Assay for non- enzymic antioxidant levels in serum and eye lens from the different experimental groups.

Enzymes analyzed (Unit of activity)	Group I	Group II	Group III	Group IV	Group V
Reduced glutathione (µmol of NADPH oxidized/min/mg)					
Serum	18.62 ± 3.1	14.01 ± 2.9*	17.27 ± 4.3*	14.37 ±1.89	17.24 ± 4.7*
Eye lens	2.20 ± 0.31	1.12 ± 0.14*	2.22 ± 0.27*	1.64 ± 0.53	2.24 ± 0.62*

Each value represents mean ± SD of 4 determinations using samples from different preparations. The difference in non-enzymic antioxidant levels observed between groups II and groups I, III and V were statistically significant at *p < 0.05.

Table 3. Assay for cytochrome c reduction in the eye lens from the different experimental groups.

Test condition	**Cytochrome *C* reduction (OD 550 nm/ 15 min)**
Control (group I)	0.312 ± 0.026
Selenite only (group II)	0.560 ± 0.054*
Selenite + SOD (65 units)	0.458 ± 0.045
Selenite + resveratrol administered simultaneously (group III)	0.318 ± 0.041*
Resveratrol gavaged 24 h prior to Selenite treatment (group IV)	0.584 ± 0.079
Resveratrol 24 h post selenite treatment (group V)	0.477 ± 0.072*

Each value represents mean ± SD of 4 determinations using samples from different preparations. The difference in cytochrome C reduction observed between groups II and groups I, III and V were statistically significant at *p < 0.05.

lens samples from groups III and V displayed an improvement in the levels of such cellular anti-oxidants that was also statistically significant when compared to group I.

Gene and Protein Expression Levels of Cellular Stress Mediators are Modulated by Resveratrol

Cell stress mediators including the heat shock protein Hsp 70, lens - αA-, αB-crystallins, iNOS and p65/ NF-κB were analysed both for their relative gene and protein expression levels among all the different experimental groups. The expression was compared with the untreated group and normalized to the house keeping gene, β- actin.

RT-PCR results revealed an up-regulation in Hsp 70, lens αA- and αB-crystallins and iNOS levels in group II (Fig. 6) that was reduced with resveratrol administration in groups III and V. The protein level expression for these molecular chaperones also mimicked those obtained by us earlier with gene expression study. Upon Western analysis, it was revealed that inducible nitric oxide synthase (iNOS), p65/NF-κB and αA- and αB-crystallins (Fig. 7) were significantly elevated in group II rats, while such expression was found to be decreased upon resveratrol pre- or co-treatments (groups III and V).

Resveratrol Reduces Selenite Mediated Superoxide Anion Generation and Formazan Deposition in Lens Tissue

The generation of potent free radical mediators was assayed via the NBT method wherein the production of superoxide anion was positively correlated with group II lens homogenates (Fig. 8). This also reflected in increased deposition of the deep blue formazan deposits in the whole lens from the same group (group II; Fig. 8 B). In contrast, both lens and lenticular homogenates from groups III and V (Fig. 8 C and E) demonstrated a significant reduction in superoxide anion generation as well as formazan deposition indicative of cytoprotective potential of resveratrol. The results of the NBT assay were also observed by the cytochrome C reduction method (Table 3) with higher reduction observed with group II rats, whereas reduction was found to be lower in group III and V rats. This indicates higher levels of free radical generation in group II rats when compared to resveratrol administered rats.

Resveratrol Diminishes iNOS and αA-, αB-crystallin Protein Levels

The expression of iNOS and αA- and αB-crystallins in the lens sections from the different experimental groups was studied via immunohistochemical analysis. Group II lens exhibited markedly a higher degree of staining for iNOS and crystallin proteins (Figs. 9 and 10). Further, immunohistochemical analysis of lenses groups III and V showed that the staining was minimal and the sections were comparable to the lens from control group.

Resveratrol Down-regulates p65/NF-κB Expression

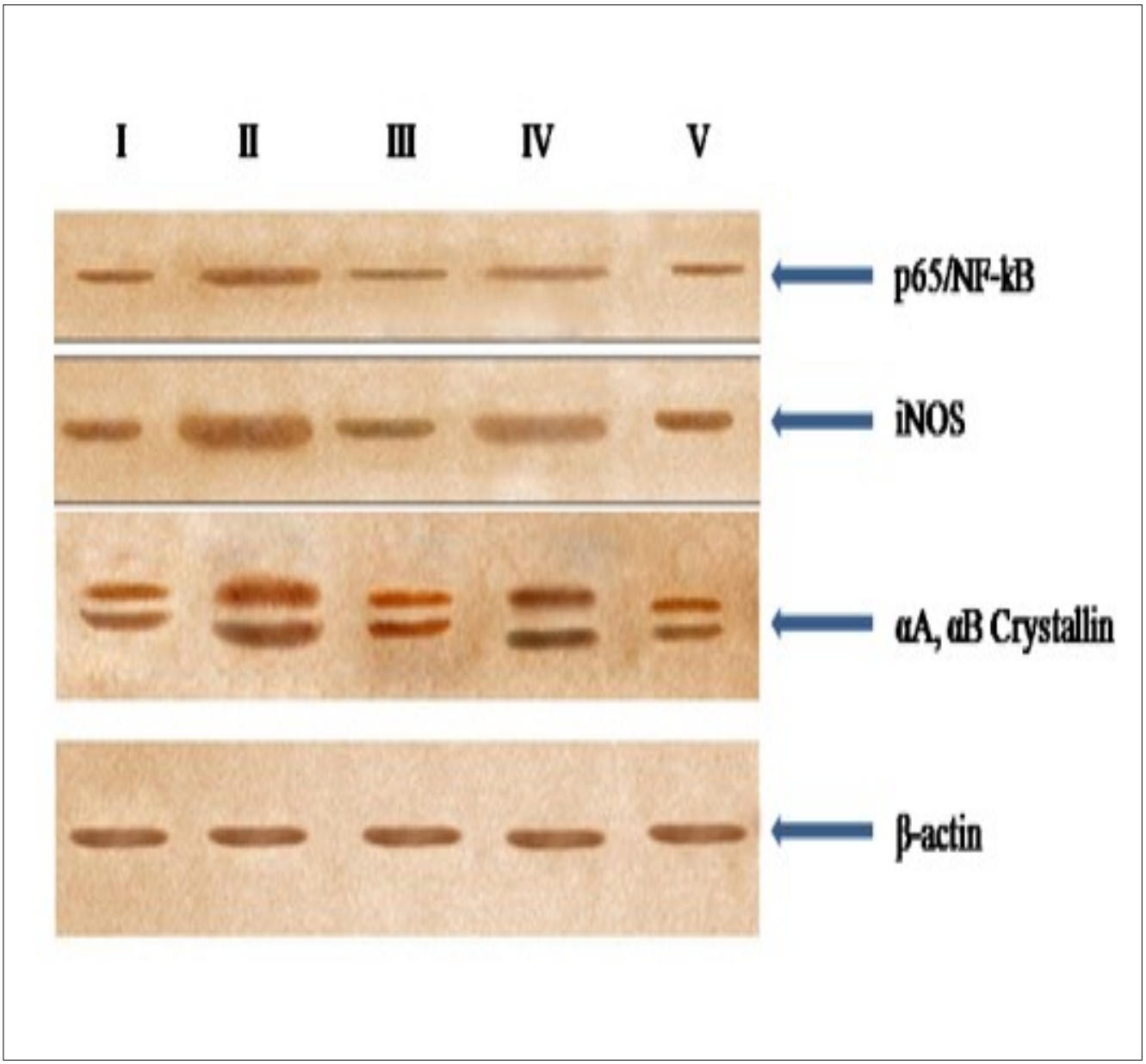

Figure 7. Western blot analyis for p65/NF-kB, iNOS, αA and αB crystallin protein expression in the eye lens from different experimental group animals. Lens homogenates from from- Lane I: control (group I); lane II selenite only (group II); lane III: selenium + resveratrol simultaneously (group III); lane IV: Resveratrol administered 24 h after treatment with selenite (group IV); lane V: Resveratrol administered 24 h before treatment with selenite (group V).). Separated lens protein was pre-incubated with anti-iNOS, p56/NF- kB and αA, αB crystallin polyclonal rabbit antibody (1: 500 dilution) and subsequently with goat anti-rabbit IgG-HRP (1: 2500 dilution). The immunoreactivity was developed with 0.01% DAB and H2O2.

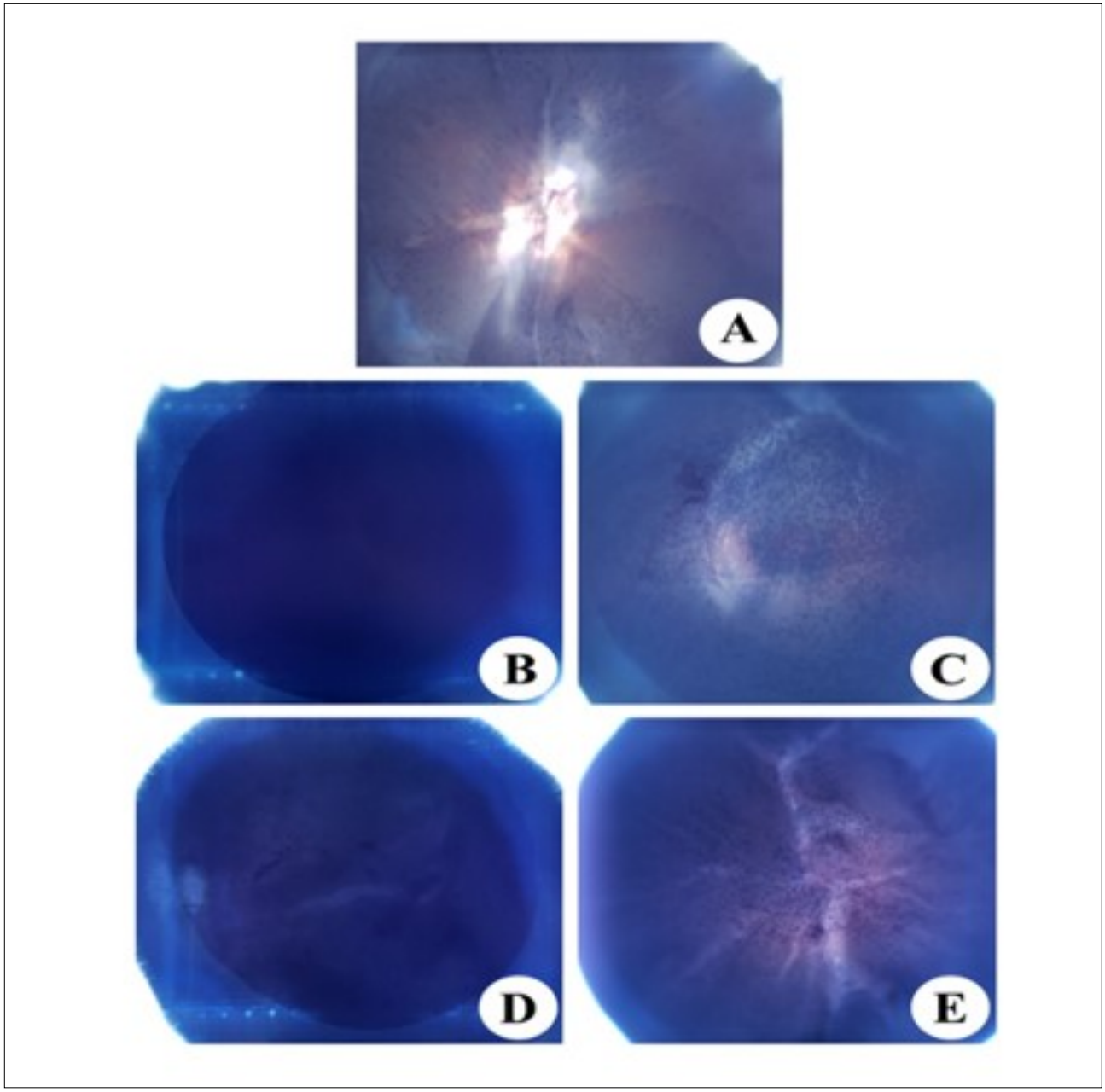

Figure 8. NBT reduction in the eye lens of from different experimental group animals to assay for superoxide anion generation. Blue colour formation upon incubation with 0.3% NBT for observed after 45 min with intense blue colour formation indicative of superoxide anion generation. Inset (A): Physiological saline treated (group I); (B) Selenite treated (group II); (C) Selenium and resveratrol administered simultaneously (group III); (D) Resveratrol administered 24 h after treatment with selenite (group IV); (E) Resveratrol administered 24 h before treatment with selenite (group V).

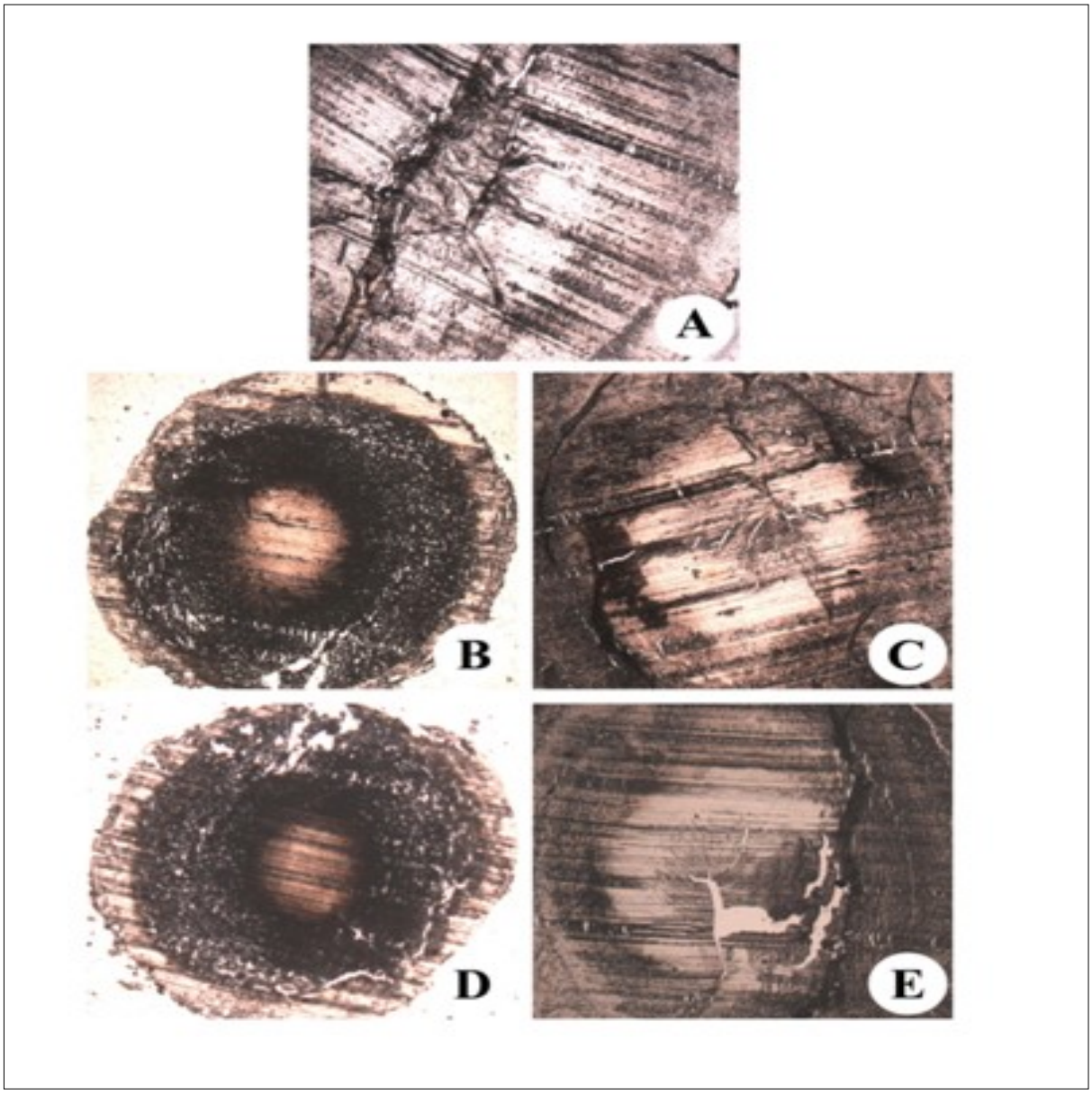

Figure 9. Immunohistochemical analysis of iNOS in the eye lens from different experimental group animals. Lens sections were preincubated with anti-iNOS polyclonal rabbit antibody (1: 500 dilution) and subsequently with goat anti-rabbit IgG-HRP (1: 3000 dilution). The immunoreactivity was developed with 0.01% DAB and H2O2. Note brown color formation indicative of peroxidase reaction in the nucleus. Inset (A): Physiological saline treated (group I); (B) Selenite treated (group II); (C) Selenium and resveratrol administered simultaneously (group III); (D) Resveratrol administered 24 h after treatment with selenite (group IV); (E) Resveratrol administered 24 h before treatment with selenite (group V).

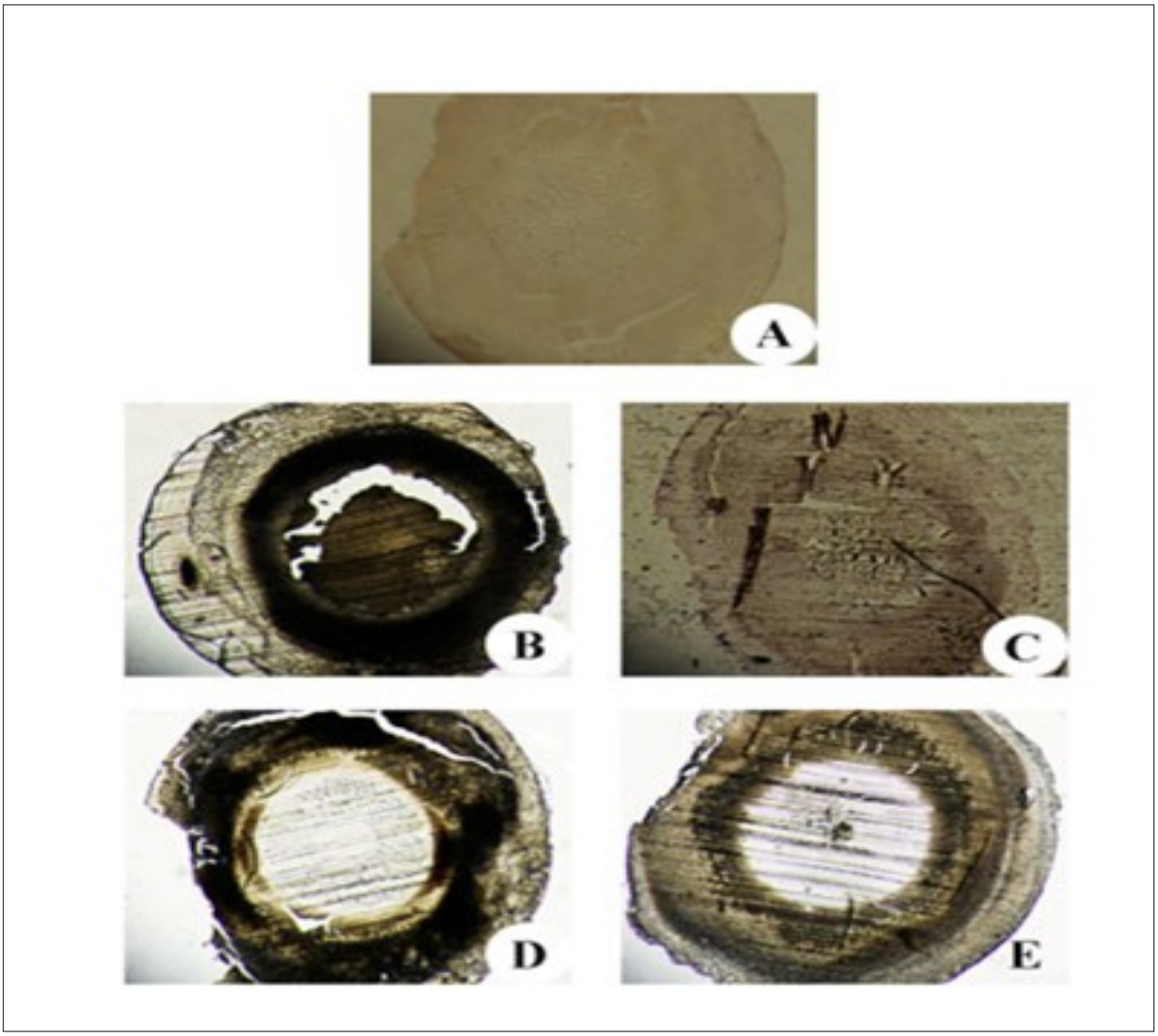

Figure 10. Immunohistochemistry of αA and αB crystallin in the eye lens from different experimental group animals. Lens sections were preincubated with anti- αA, αB crystallin polyclonal rabbit antibody (1: 500 dilution) and subsequently with goat anti-rabbit IgG-HRP (1: 3000 dilution). The immunoreactivity was developed with 0.01% DAB and H2O2. Note the brown colour formation that is indicative of peroxidase reaction in the nucleus. Inset (A): Physiological saline treated (group I); (B) Selenite treated (group II); (C) Selenium and resveratrol administered simultaneously (group III); (D) Resveratrol administered 24 h after treatment with selenite (group IV); (E) Resveratrol administered 24 h before treatment with selenite (group V).

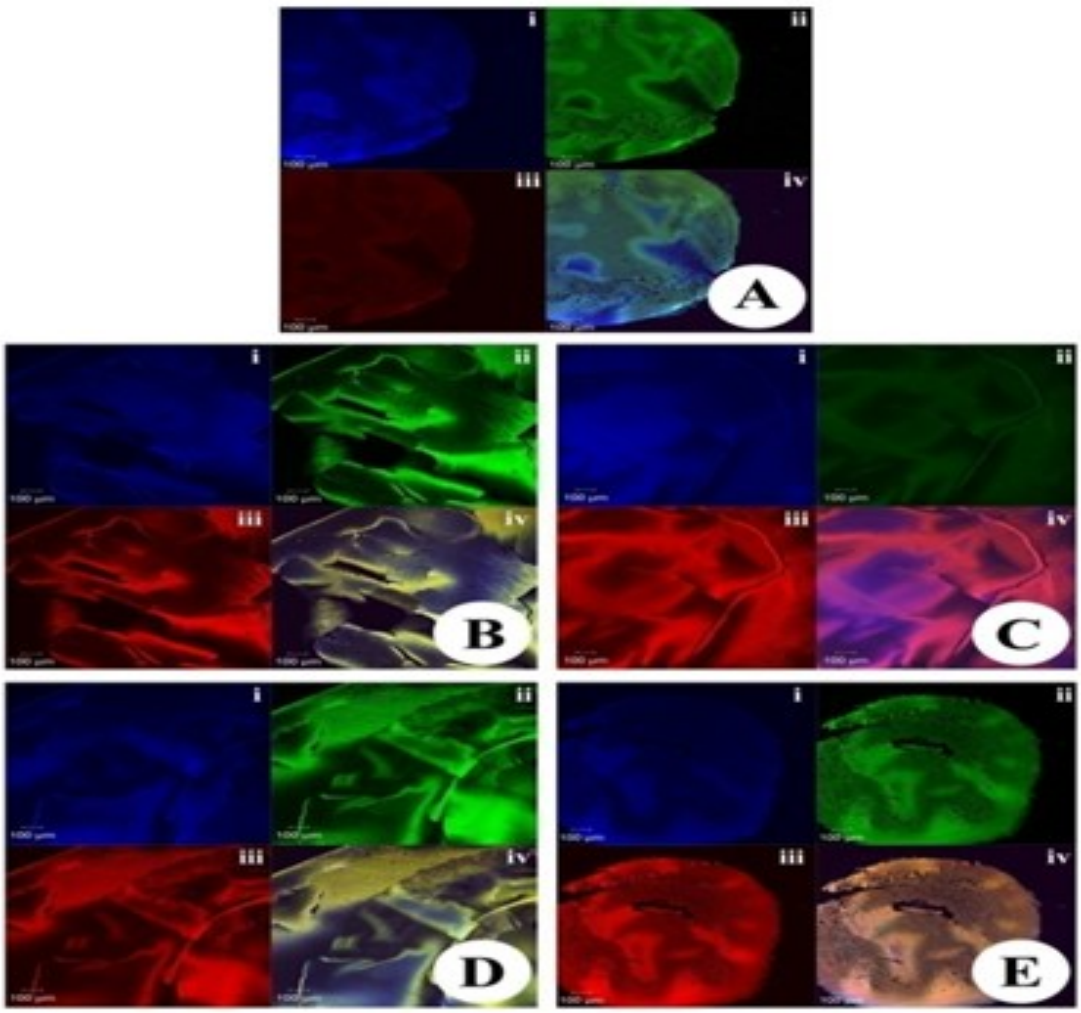

Figure 11. Immunofluorescence analysis for the expression of NF-kB in the eye lens from different experimental group animals. Lens section were pre-incubated with anti-p65/ NF-κB (at a dilution of 1: 500) and then with secondary antibody conjugated to FITC (at 1: 40 dilution) in the dark at room temperature for about 2 h. The sections were then counter-stained with either PI or DAPI. Please note the sub-insets i, ii, iii and iv in each of the insets indicate staining with DAPI (in blue), NF-kB (in green), PI (Red) and merged images respectively. The expression levels of NF-kB and PI were observed to be higher in group II in comparison to groups I, III and V. Inset (A): Physiological saline treated (group I); (B) Selenite treated (group II); (C) Selenium and resveratrol administered simultaneously (group III); (D) Resveratrol administered 24 h after treatment with selenite (group IV); (E) Resveratrol administered 24 h before treatment with selenite (group V).

An attempt was also made to analyse the expression of p65/NF-κB and relate it to late stage apoptotic cells (propidium iodide staining) by immunofluorescence in isolated rat lenses from different treatment groups. As can be seen from the results (Fig. 11), the co-expression level of both p65/NF-κB and apoptosis marker propidium iodide was found to be higher in lenses from group II rats, when compared to controls. However, such expression was minimal in the case of resveratrol administered rat lenses (groups III and V), indicating the ability of resveratrol in preventing NF-κB induced inflammatory changes.

Discussion

The anterior lens epithelium and it underlying fibre cell mass are of utmost importance in the regulation of calcium level and its transport which has been highlighted in several different studies. For instance, Rhodes and Sanderson [71] reported that lens epithelium and fibre cells concentration of calcium to be in minuscule amounts ranging from 100 nM and 10 μM respectively under normal conditions. Failure to maintain this extremely low level (as seen with cataract lenses reaching 900 μM concentrations) exposes the lens to calcium mediated damage. It is in this light we investigated both the intracellular calcium levels in lens tissue besides the functioning of the Ca^{2+} ATPase. As expected, treatment with sodium selenite (group II) resulted in both an increase in intracellular calcium levels and a reduction in the Ca^{2+} ATPase activity (Figs. 4 and 5) that was mitigated by treatment with resveratrol in groups III and V. A direct effect to elevated calcium load in lens tissue leads to calpain activation and consequently the lens crystallin degradation, a characteristic feature in cataract formation.

The lens crystallin proteins are the major structural proteins that maintain lens transparency, which is a principal function. The α-crystallin is known to prevent protein aggregation and functions as a molecular chaperone that is especially important during conditions of cellular stress. This ability is progressively compromised in aging lens tissue and is the prime target of calpain mediated truncation (due to elevated intracellular calcium levels) that affects its structure and solubility (increased cross-linking of subunits results in lower chaperone activity [72]. This overwhelms the ability of lens to overcome protein aggregation including the β-, γ-crystallins and other cytoskeletal proteins that lead to light refraction as well as eventually clouding of the lens tissue. We therefore sought to shed light in the fate of crystallins both at the gene expression and protein regulation across the different experimental groups. The gene expression levels of cell stress mediators including heat shock protein Hsp 70, lens - αA -, αB-crystallins and iNOS were markedly upregulated by treatment with sodium selenite (group II) and mitigated with resveratrol administration (again in groups III and V that was comparable with group I; Fig. 6). A similar trend in the upregulation of iNOS and αA-, αB- crystallin proteins were observed in group II treated lenses that were down-regulated with resveratol treatment (Fig. 7). We would also like to bring attention to the major transcription factor that regulates cell inflammatory signaling, NF-κB, and probing for the p65 subunit revealed an increase in the level of this protein in group II lenses that was down-regulated in those administered with resveratrol (groups III and V that match untreated lenses from group I).

The ocular lens tissue is subjected to a milieu of oxidants including photo-oxidative stress inducers. Metabolic stress to mitochondria as a result of any imbalance in the cell's redox status can pave the way for induction of apoptosis via different mechanisms. Páramoa et al. [73] for instance reported glucose deprivation leads to oxidative stress and in turn activation of calpain via elevated calcium levels that can trigger apoptosis. An elevated and sustained generation of free radicals has been known to damage cellular components that include those that regulate intracellular calcium levels [74]. It was therefore important in our study we assessed the levels of major antioxidants and also prominent free radicals in addition to malondialdehyde (MDA), a well known marker for oxidative stress, to get a complete picture of the lens redox status from different experimental groups. Nearly a two-fold increase in both the levels of MDA, hydroxyl radical and at least a three-fold elevation in nitrite levels were recorded in group II lenses that were significantly reduced in groups III and V (Fig. 1). Similarly, the assays for cellular antioxidant enzymes revealed the loss of activity including catalase, SOD, glutathione

peroxidase and glutathione S-transferase in the sodium selenite treated group II pups both in serum and lenticular tissue. This was significantly abrogated upon treatment with resveratrol wherein a comparable improvement in the levels of all assayed antioxidant enzymes undertaken here was observed (Table 1). We also report a similar pattern that was exhibited in the assay for reduced glutathione level across the different experimental groups (Table 2).

In our continued investigation into the cellular oxidants, we also undertook the NBT cytochemical detection test to identify the localization of superoxide anion (O_2^-), an important cellular oxidant that can form the highly reactive peroxynitrite anion ($ONOO^-$) in the presence of NO. The cell damage caused here can be extensive including the impairment in mitochondrial function, generation of other free radical intermediates and cellular protein nitration [75]. As observable in Fig. 8, the intense NBT staining in the lens central core in group II was typical of sodium selenite induced oxidative stress that was ameliorated in lenses from groups III and V.

Dietary polyphenols have been known to possess numerous beneficial effects including their potential in regulating inflammation. The nicotinamide adenine dinucleotide (NAD^+) dependent family of enzymes, the sirtuins, are a known target of the polyphenol, resveratrol [76]. Sirtuins carry out a variety of cellular functions including maintenance of cellular homeostasis, regulating the cell cycle, apoptosis, DNA repair and also in cellular stress signaling mechanisms. The major transcription factor, NF-κB, is widely known to promote pro-inflammatory responses including those such as iNOS, TNF-α, IL-8, MCP-1 and IL1β and resveratrol is known to regulate NF-κB expression [77]. Only very few reports exist that investigate the anti-cataract effects of resveratrol. The sodium selenite model of cataract formation in rat pups has been well established and is believed to closely resemble the age related cataract development in human that is typified with heightened oxidative stress, aberrations in intracellular calcium and protein aggregation. One such investigation by Doganay et al. [78] demonstrated the potential of resveratol to mitigate cataract through restoring antioxidant defense systems and also reduce the level of malondialdehyde. The focus will need to be devising novel therapies using phyto-compounds such as resveratrol that could both be effective and also avoid the unwanted side-effects of currently available drugs such as the NSAIDs (non-steroidal anti inflammatory drugs).

Conclusion

The result concluded that the cytosolic level of calcium is tightly regulated under normal conditions. The pro-oxidant effects of sodium selenite have been established and elevated levels of free radicals can lead to cell damage including cellular mechanisms that regulate intracellular calcium levels. This ultimately leads to mitochondrial dysfunction, activation of pro-inflammatory signalling and activation of the cell death cascade. We postulate, using the sodium selenite induced Wistar rat pup model of cataractogenesis, that administration of resveratrol restores the redox balance disrupted by selenite in addition to modulating the levels of lens crystallin proteins and p65/NF-κB to alleviate cataract formation.

Conflict of Interest

The author reports no conflict of interest.

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