A Review on Research Methodologies of Diabetic Retinopathy

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ABSTRACT
Cataract, opacity of the eye lens is a major complication of chronic diabetes leads to loss of vision and is the leading cause of blindness worldwide. Nearly 19 million people are blind due to cataract in the world. It is a result of increased polyol pathway activity, activation of protein kinase C (PKC), increased oxidative stress, and accumulation of advanced glycation end products (AGEs) due to prolonged hyperglycemia. In vitro and In vivo studies have suggested that the damage to lens is a result of oxidation and generation of free radicals. The antioxidants enzymes might protect the lens against the formation of cataract. Several invasive and non-invasive research methodologies have been in proceeding for the study of diabetic retinopathy. The research methodologies for retinal examinations that improve the cost-effectiveness and noninvasive, allows repeated assessment, and can be performed in a relatively short period of diabetic retinopathy were discussed in this review. Several image processing based methods have been developed in the last decade providing an alternative to first phase examinations by using slitlamp examination and fundoscopy of lens. Estimation of antioxidant enzymes like superoxide dismutase, catalase, aldose reductase, reduced glutathione and glutathione peroxidase and protein products such as malonaldehyde, sorbitol, α-crystalline, protein carbonyl content and estimation of advanced glycation end products and several inflammatory mediators by various approaches were discussed in the present review.

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INTRODUCTION

Diabetes possesses an expanding public health problem across the world. Diabetic retinopathy is a serious complication of diabetes mellitus. This is the commonest cause of blindness in the western world in people between 20 and 74. In the younger onset diabetics [30 years] any retinopathy is present in 29% after 5 years and 80% after 15 years or more. Around 10% of patients with diabetes develop sight threatening retinopathy.

In particular, the increasing prevalence of type II diabetes affects life expectancies and more so obese people. Long-term complications of diabetes mellitus include retinopathy with potential loss of vision, nephropathy leading to renal failure, peripheral neuropathy with risk of foot ulcers, amputation and charcoal joints (1). Cataract, characterized by cloudiness or opacification of the eye lens, is the leading cause of blindness all over the world. In view of the widespread prevalence of diabetes in developing countries like India (2), diabetic cataract may poss a major problem in the management of blindness (3).

Early development of cataract of lens is due to the increased rate of sorbitol formation, caused by hyperglycemia. Glycosylation of retinal proteins and retinal micro vascular abnormalities lead to retinopathy and blindness (4). Glycosylation of lysine residues of lens proteins also causes cataract formation. These reactions modify the structure and function of proteins and may lead to formation of complex cross-links.2 Metal-catalyzed oxidative reactions also give rise to a group of AGEs, classified as glycoxidation products (e.g., *N*(carboxymethyl)lysine [CML] or *N*(carboxyethyl)lysine [CEL]) that also

accumulate on macromolecules with aging and at an increased rate in diabetes.

In the present review we discussed various methods available to screen the retinopathy

1. RETINAL EXAMINATIONS

1.1 Slit lamp examination and cataract grading:

Eyes were examined every week using a slit lamp biomicroscope (Kowa Portable; Kowa, Ltd., Tokyo, Japan) on dilated pupils. Initiation and progression of lens opacity was graded into five categories as described previously. Briefly, lens opacity was graded as clear - clear lenses with no vacuoles; stage 1 - vacuoles cover approximately one-half of the surface of the anterior pole; stage 2 - some vacuoles have disappeared and the cortex haziness; stage 3 - a hazy cortex remains and dense nuclear opacity is present; stage 4 - a mature cataract (5).

% Incidence of cataract was calculated using the following formula:

$$\% \text{ Incidence} = \frac{\text{No of animals in each stage}}{\text{Total no. of animals}} \times 100$$

Opacity index was calculated using the following formula,

$$\text{Opacity index} = \frac{\text{No. of eyes in each stage X stage of the eye}}{\text{Total no. of eyes}}$$

1.2 Retinal examination:

Fluorescein Angiography is the method used to observe retinal blood vessels. Fluoroscein dye was injected into a vein in the leg, as described (6). The dye travels through the blood vessels including those in eyes. The photograph of the retina was taken with Zeiss FF 450 plus IR Camera (Carlzeis Meditec AG, 07740 Jena, Germany). A qualified ophthalmologist required to carry out the pre - and post- treatment fundus examination (7).

2. LENS OPACITY:

2.1 Evaluation of lens opacity:

The isolated transparent lenses were incubated in artificial aqueous humor (NaCl 140 mM, KCl $_5$mM, MgCl$_2$ 2mM, NaHCO$_3$ 0.5mM, Na$_2$HPO$_4$ 0.5mM, CaCl$_2$ 0.4mM, and glucose 5.5mM) for 72 hours at room temperature. The pH is maintained at 7.8 throughout the incubation period. To prevent microbial contamination, strict aseptic techniques were performed and antibiotic drugs including penicillin 32mg and streptomycin 250mg were added to the culture media. In addition, glucose at concentration of 55mM was added to the media in order to develop the model of diabetic cataract. After 72 hours of incubation, lenses were observed for opacity and photographs were taken by placing the lens on the paper with posterior surface touching the paper, and the number of visible clear squares was observed through the lens during the evaluation of lens opacity (8).

2.2 In vitro analysis of lens opacity:

Rat lenses were dissected from the eyes by posterior approach. Each isolated lens was incubated in 1ml of modified TC-199 medium in the presence of antibiotics *i.e.,* penicillin and amphotericin in a CO$_2$ incubator (95% air and 5% CO$_2$ at 37^{0}C) with 100mM glucose for a period of 10 days according to the method previously described (9) Lens incubated with 5.5mM glucose served as control. Damaged lenses were identified by determining the protein content of an aliquot of the medium after an equilibration period of 2 hrs and were discarded. Test samples were prepared in TC-199 and filtered before adding. Medium was changed every 48 hrs and supplemented with test sample and the standard drug quercetin along with glucose 100mM. All the reagents used in lens culture were filtered through 0.2 μm Millipore disc filters. Lenses were observed for development of generalized haziness or opacity, disruption and other morphological changes. After 10 days of culture the lenses were homogenized in buffer containing 25mMTris, 100mM NaCl, 0.5mM EDTA and 0.01% NaN$_3$, pH 8.0. The soluble fraction of homogenate (10,000×g for 30 min at 4 ∘C) was used for further protein analysis.

3. ENZYME ESTIMATIONS:

Lens collection and processing:

At the end of study period, animals were sacrificed by CO$_2$ asphyxiation, and lenses were dissected by the posterior approach and stored at −70 °C until further analysis. A

10% homogenate was prepared from 3 to 4 pooled lenses in 50 mM phosphate buffer (pH 7.4). Some of the biochemical estimations such as malondialdehyde (MDA), sorbitol cannot be done with two lenses of each rat as the rat lens weighed about 40 mg. Hence we pooled 3–4 lenses for making homogenate not only to estimate MDA and sorbitol but also for other biochemical estimations from the same pool. All the biochemical parameters were analyzed in the soluble fraction of the lens homogenate (15,000× g at 4 °C) except for lens malondialdehyde (MDA) and sorbitol which were determined in the total homogenate (10).

3.1 Measurement of aldose reductase activity:

Rat lenses were removed and homogenized in 12 volumes of a 135 mM Na, K-phosphate buffer (pH 7.0) containing 0.5 mM phenylmethylsulfonyl fluoride and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 100,000 × g for 30 min, and the supernatant fluid was used as the crude rat lens aldose reductase (RLAR) (11-12).The incubation mixture contained 135 mM Na, K-phosphate buffer (pH 7.0), 100 mM lithium sulfate, 0.03 mM NADPH, 1 mM DLglyceraldehyde as a substrate, and 50 μl of enzyme fraction, with or without 25 μl of sample solution, in a total volume of 1.0 ml. The reaction was initiated by the addition of NADPH at 37oC and stopped by the addition of 0.3 ml of 0.5 M HCl. Then, 1 ml of 6M NaOH containing 10 mM imidazole was added, and the solution was heated at 60oC for 10 min to convert NADP to a fluorescent product. Fluorescence was measured using a spectrofluorometric detector (11-12).

3.2 Superoxide dismutase (SOD):

To 0.5 ml of the lens sample added 0.5ml of distilled water. To this chilled ethanol 0.25ml and chloroform 0.15ml was added. The mixture was shaken for 1 min and centrifuged at 2000 × g for 10 min. The PMS 0.5ml was added with 1.5ml PBS buffer n (PH 7.2). The reaction initiated by the addition of 0.4ml epinephrine and change in optical density OD (min 1) was measured at 470 nm (13). SOD activity was expressed as U/mg of protein. Change in OD (min-1) at 50% inhibition to adrenochrome transition by the enzyme was taken as one enzyme unit.

3.3 Catalase (CAT):

To 0.1 ml of tissue homogenate add 1.0 ml of 0.01 M phosphate buffer (PH 7.0) and 0.4 ml of 2 M H_2O_2. The reaction was stopped by the addition of 2.0 ml dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).Then the absorbance was measured at 530 nm; CAT activitiy was expressed as μM of H_2O_2 consumed/min/mg protein (14).

3.4 Glutathione peroxidase (GPx):

To 0.2 ml of tissue homogenate in 0.4M phosphate buffer P^H 7.0 and 0.1ml 10 mM sodium azide, 0.2 ml reduced glutathione , 0.1 ml 0.2 mM hydrogen peroxide. The contents were incubated for 10 min at 37o c, 0.4 ml 10% TCA was added to stop the reaction and centrifuged at 3200 × g for 20 min. The supernatant was assayed for glutathione content using Ellman's reagent (19.8 mg 5, 5'-dithiobisnitrobenzoic acid (DTNB) in 100 ml 0.1% sodium nitrate). activities were expressed as μg of GSH consumed/ min/mg protein (15).

3.5 Glutathione (GSH):

The homogenate was centrifuged at 5,000 rpm for 15 min at 4°C. To the supernatant, 0.5 ml of 10% trichloroacetic acid was added and recentrifuged. The protein-free supernatant thus obtained was reacted with 4 ml of 0.3 M of Na_2HPO_4 (pH 8.0) and 0.5 ml of 0.04% (wt/vol) 5, 5'-dithiobis-2-nitrobenzoic acid. The absorbance of the resulting yellow color was measured in the spectrophotometer at 412 nm. A parallel standard was also maintained (16).

3.6 Malondialdehyde (MDA):

The tissue homogenate was mixed with 0.15 M KCl and centrifuged at10, 000 rpm for 10 min. To the supernatant, 0.2 ml was reacted with 0.2 ml of 8.1% of sodium dodecyl sulfate, 1.5 ml of 20% acetic acid (pH 3.5), and 1.5 ml of thiobarbituric acid. All the samples were heated in a boiling-water bath for 60 min. After cooling, 5 ml of n-butanol/ pyridine mixture was added to each sample. The solution was shaken vigorously in a vortex and centrifuged at 5,000 rpm for 10 min. Organic layer was separated, and absorbance was observed in the spectrophotometer at 515 nm. Simultaneously various amounts of 1, 1′, 3,

3′- tetra methoxypropane was used as a standard to obtain a standard curve for the calculation f unknown MDA in the samples (17).

3.7 Sorbitol:

Sorbitol was extracted by homogenizing RBC in nine volumes of 0.8 M perchloric acid. The homogenate was centrifuged at 5,000 g at 4 °C for 10 min, and the pH of the supernatant was adjusted to 3.5 with 0.5 M potassium carbonate. The sorbitol content of the supernatant was measured by the fluorometric method as described previously (18) using a fluorometer (Jasco-FP-6500, Tokyo, Japan). One ml reaction mixture, consisted of 50 μmol glycine buffer, pH 9.4, 2 μmol magnesium chloride, 0.2 μmol nicotinamide adenine dinucleotide (NAD) and protein-free supernatant, was incubated for 5 min at 37 °C and reaction was initiated by the addition of 0.6 U of sorbitol dehydrogenase. The relative fluorescence due to NADH formation was measured in a fluorometer with an excitation wavelength at 360 nm and an emission wavelength of 452 nm. Sorbitol standards, ranging from 0.2 to 9.0μg/ml, were analyzed by the same way to generate a standard curve. For extraction of sorbitol, the lens was homogenized in 9 volumes of 0.8M perchloric acid. The homogenate was centrifuged at 5,000x g at 4 °C for 10 min and the pH of the supernatant was adjusted to 3.5 with 0.5 M potassium carbonate. The sorbitol content of the supernatant was measured by an enzymatic method as described previously (19).

4. PROTEIN ESTIMATION:

4.1 Lens Protein carbonyl content estimation:

Aliquot (0.5 ml) of protein samples was treated with an equal volume of 0.1% (wt/vol) 2, 4- dinitrophenylhydrazine in 2 M HCl and incubated for 1 h at room temperature. This mixture was treated with 0.5 ml of 20% trichloroacetic acid (w/vol, final concentration), and after centrifugation, the precipitate was extracted three times with ethanolyethyl acetate (1:1, vol/vol). The protein sample was then dissolved with 2 ml of 8 M guanidine hydrochloride, 13 mM Ethylene diamine tetra acetic acid (EDTA), and 133 mM Tris solution (pH 7.4). The UV absorbance was measured at 365 nm. **(20).**

4.2 Retinal Advanced Glycation End Products (AGEs):

The lens tissue was homogenized in Tween buffer pH-8, followed by centrifugation at 8000g for 15 min at 4⁰C. Estimate the protein levels in soluble protein in homogenate. Dilute the protein up to 1mg/ml and the amount of AGE was measured in a spectroflurometer at an excitation/ emission wavelength of 370/440 nm against buffer blank. BSA (bovine serum albumin) preparation (1 mg/ml in distilled water) was used as a reference, and its fluorescent intensity was defined as 1 arbitrary unit (AU) (21). The fluorescence intensities of the samples were measured and expressed as arbitrary units (AU)/mg protein.

4.3 Primary retinal microglia culture:

The retinas were collected into 0.01 M PBS and digested with 0.125% trypsin for 3–5 min before mixing with Dulbecco's Modified Eagle Medium (DMEM)/ F12 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Retina pieces were then filtered through a mesh (100 μm), collected by centrifugation, re suspended in culture medium and plated onto T150 cell culture flasks (Corning, NY) at a density of 2×105 cells/cm. After 2 weeks, microglial cells were harvested by shaking the flasks at 100 rpm for 1 h. Immunocytochemical studies showed that more than 95% cultured cells stained positively for Iba1. Almost none of these cells showed positive staining for Glial fibrillary acidic protein (GFAP), indicating that majority of the isolated cells were microglia and were not contaminated with astrocytes or Muller cells (data not shown). For microglia activation, glycated albumin was added to each well in Cellgro Complete media for indicated time. Non-glycated albumin was used as a control. Cell viability was determined by counting the number of trypan blue–excluding cells under an inverted microscope, using a hemocytometer. Cells were homogenized for western blot analysis. Culture media were used for tumor necrotic factor-α (TNF-α) release determination by ELISA (22).

4.3.1: ELISA for TNF-α in vitreous or culture media:
TNF-α level in the samples were estimated with ELISA (R&D) per the manufacturer's instructions. Briefly,

standards and samples were bound by the immobilized antibody, and an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells followed by a substrate solution yielding a colored product. The intensity of the color was measured at 450 nm. The sample levels were calculated from a standard curve and were corrected for protein concentration.

4.4 Quantitative Real Time-PCR:

Total RNA was isolated from rat retina using Promega SV Total RNA Isolation System. Subsequently, cDNAs were generated from 1 µg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The resulting cDNA was subjected to a 40 cycle PCR amplification using manufacturer's Taq Man Universal PCR Master Mix protocol. Quantification of *Iba-1*, *TNF-α*, and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were performed by relative quantitative real-time RT–PCR with TaqMan Probe-based Assays and Applied Biosystems 7300 Sequence Detection system. The ready-made primer and probe sets were ordered from Applied Biosystems (Catalog #: Iba1:Rn01525935m1; TNF-α: Rn99999017m1; GAPDH: Rn01775763g1). Three replicates were run for each gene for each sample in a 96-well plate. The relative quantitation method was used, with the ratio of target mRNA, normalized respect to GAPDH mRNA and relative to a calibrator sample. PBS-normal, nondiabetic, retinas were used as calibrators.

4.5 Western blot analysis:

Retinal homogenates was subjected to western blot analysis according to procedure El-Remessy AB et al., 2008. Antibodies for β-actin, glycated albumin, phospho-tyrosine, phospho-ERK and ERK, phospho-P38 and P38 were detected with a horseradish peroxidase-conjugated antibody and enhanced chemiluminescence detection system (Amersham BioSciences). Intensity of immunoreactivity was measured by densitometry (22).

4.6 Immunodetection of AGE-antigens:

Lens soluble proteins were resolved under reducing conditions on 12% SDS–PAGE, proteins were transferred onto a nitrocellulose membrane (NC) and NC was blocked with 5% skimmed milk powder. NC membrane was incubated with partially purified antiserum of AGE-BSA, MGO-BSA and CML-BSA (1:100 dilutions) later with HRP-conjugated goat anti-rabbit antibody (1:500). Subsequently, detection was performed with diaminobenzidine in the presence of hydrogen peroxide.

4.7 Immunoprecipitation of CML and MGO-AGE antigens:

Immunoprecipitation (IP) was performed using 1 mg of reconstituted insoluble fraction of lens in 500 µl of IP buffer (50 mM sodium phosphate buffer pH 7.4). Partially purified polyclonal antiserum of CML-BSA or MGO-BSA was added at 1:100 dilutions in IP buffer and incubated at 4°C overnight. To this, 50 µl of slurry of Protein-A was added and incubated for 3 h at 4°C. Immunocomplex was collected by centrifugation at 10,000× g for 1 min at 4°C and complex was washed 3 times with 1 ml of IP buffer, spinning at 10,000× g each time for 1 min. Finally, pellet was washed with 50 mM Tris-HCl pH 7.9 and re-suspended in sample buffer. Immunoblotting and detection was performed as described above using anti-CML-BSA or anti-MGO-BSA antiserum (1:100 dilutions).

4.8 Boronate affinity chromatography:

The percentage of glycated protein was estimated in the soluble fraction of lens as described previously (23-24). Briefly, 5 mg of glycated lens protein was passed through a phenyl boronate affinity column equilibrated with 0.25 M ammonium acetate buffer (pH 8.5) containing 0.05 M $MgCl_2$. The unbound fraction containing non-glycated protein was washed with the above buffer, while bound glycated protein was eluted using 0.1 M 2-amino-2-hydroxymethyl-propane-1, 3-diol-HCl (pH 7.5) containing 0.2 M sorbitol.

4.9 SDS-PAGE and Size Exclusion Chromatography:

The cross-linking of soluble proteins was analyzed on 10% polyacrylamide gels in the presence of SDS under reducing conditions. Crystalline distribution in the soluble protein fraction was performed by size-exclusion chromatography on a 600 ×7.5 mm column (TSK-G4000 SW; Tosoh Co., Tokyo, Japan) in an HPLC system (Class-VP; Shimadzu, Kyoto, Japan). The column was equilibrated with 0.1 M sodium phosphate

buffer (pH 6.7) containing 0.1 M sodium chloride at a flow rate of 1 mL/min. The soluble protein samples (20 µL of 1 mg/mL solution) were loaded onto the column, and protein peaks were detected at 280 nm.

4.10 Electrophoresis:
An aliquot of total lens homogenate equivalent to 0.5 mg of protein was precipitated with equal volume of 10 % TCA and the washed pellets were treated with 5 % SDS under either reducing conditions in the presence of 5 % 2-mercaptoethanol or non-reducing conditions. Electrophoresis was performed according to Laemmli, 1970 (25) with stacking and separating gels containing 4 % and 10 % acrylamide, respectively. The gels were stained in 0.1 % Coomassie blue, and destained in 10 % acetic acid in 25 % methanol. The dried gels were scanned and the relative abundance of protein bands was evaluated by UN-SCAN-IT software.

4.11 Sulfhydryl determination:
Aliquots of total lens homogenate of approximately 3 mg of proteins were precipitated with equal volume of 4 % sulfosalicylic acid (SSA); the pellets obtained after centrifugation were washed with 1 ml of 2 % SSA to remove free thiols. The washed pellets were taken up to 0.2 ml of 6 mol/l guanidine (pH 7.4) and read spectrophotometrically at 412 nm and 530 nm on Labsystems Multiscan RC Spectrophotometer, before and after 30 min incubation in the dark with 50 µl of 10 mmol/l 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). Content of protein sulfhydryls was calculated using a calibration curve prepared with reduced glutathione (26).

4.12 MALDI-TOF-TOF Mass Chromatographic Analysis and Database Search:
Completely freeze-dried lens samples dissolved in 0.1% trifluoroacetic acid (TFA; Sigma, USA) were analyzed using an MS-T100LP/LC mass spectrometer. Mass spectra were identified using a mixed spotting method. Samples (0.4 μL) were spotted onto 384-well stainless steel plates, and 0.4 μL of 10mg/mL α- cyanocinnamic acid substrate solution was added. Matrix-assisted laser desorption/ionization (MALDI) with time of flight (TOF) analysis

(MALDI TOF/TOF) was used to reveal the amino acid sequence of peptides by using post source decay or high-energy collision-induced dissociation. Samples underwent mass-spectrometry (MS) after being allowed to naturally air dry. The MS system was comprised of a nitrogen gas laser (337 nm) using reflection technology with positive ion detection. Scans were taken at 800–4000 Da using a standard peptide mixture as an external standard for calibration. The MS signal for each sample was the result of 600–800 individual laser shots. The 5 peaks with the strongest signal to noise ratio (>100) were selected from each sample as precursor ions for second-order MS analysis (27). The MS signal for each selected precursor ion was the result of 900–1200 individual laser shots. The resulting first-order and second-order MS were analyzed using GPS Explore V3.6 software. The first- and second-order MSs data for each sample were integrated into a single file, and proteins were identified using the MASCOT (v2.1) database.

4.13 AR Gene Expression by RT-PCR:
4.13.1 Extraction, Isolation, and Detection of Total Lens RNA.
Rat lens specimens were placed on a mortar with ice and ground to form a homogenate. Homogenates were lysed in an ice bath containing Trizol (Invitrogen, USA), and total RNA was extracted. RNA purity was assayed using ultraviolet absorption assay, and RNA integrity was assayed using 1.2% formaldehyde degeneration gel electrophoresis.
25.2. Primer and cDNA Design and Synthesis. Primer sequences of AR and β-actin were designed and synthesized by Shanghai Boya Biotechnological (China). The PCR upstream primer (P1) for AR was 5'-AGC GGT TTA GGT ACC ATG GGT TTT-3', and the downstream primer (P2) was 5'-AGG GTA AGC TTC GAA TTC TCA GGC GCG GAT TTG TTG TGA-3'. The fragment length was 262 bp. P1 for the β-actin primer was 5'-GAGACCTTCAACACCCAGCC-3', and P2 was 5'-GCGGGGCATCGGAACCGTCA-3'. The fragment length was 420 bp.
4.13.2 PCR Analyses. Fluorescent quantitation PCR was conducted using the TaK aRa ExTaq HS enzyme (TaK, USA) and SYBR Green I fluorochrome on an ABI PRISM

7000 quantitative real-time PCR (qRT-PCR) amplifier. The PCR solution (25 µL) contained Taq enzyme (12.5 µL), P1 (0.5 µL), P2 (0.5 µL), fluorescent probe (0.5 µL), the cDNA template (2.0 µL), and Diethyl pyrocarbonate (DEPC) water solution (9.0 µL). The amplification procedure included predegeneration at 95^0C for 10 s, followed by cycle degeneration at 90^0C for 5 s and at 60^0C for 31 s, repeated for 40 cycles. The cycle threshold C (t) for the 40 cycles was recorded (27). The relative content of object mRNA copies was calculated using α-actin as an internal standard. Changes in gene expression level were evaluated by comparing the results from each group. The expression of the AR gene was normalized using the housekeeper gene (β-actin) that exhibits even expression in rat lens.

DISCUSSION

In vivo and *in vitro* studies have suggested that the damage to lens is a result of oxidation due to the generation of free radicals, and that antioxidants might protect the lens against the formation of cataract (28). Superoxide dismutase, catalase and reduced glutathione are present in almost all the mammalian cells and protect the cell from oxidative damage by superoxide radical, H_2O_2 and hydroxyl radical. The specific activity of aldose reductase is a keyenzyme of the polyol pathway. Lipid peroxidation is an autocatalytic process, which is a common cause of cell death in retina (29). The by-products of lipid peroxidation are the toxic compounds MDA involvement in cataractogenesis, mainly due to its cross linking ability. Free carbonyls and SH were used as markers of oxidative modifications and cross linking of lens proteins. Decomposition of lipid peroxide initiates the chain reactions that produce an increase in protein carbonyl content and advanced glycation end products, and a decrease in protein SH content in the lens (30).

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