



# Immunochemical detection of glycated $\beta$ - and $\gamma$ -crystallins in lens and their circulating autoantibodies (IgG) in streptozocin induced diabetic rat

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**Purpose:** This study used an immunochemical approach aimed to detect the glycated crystallins ( $\beta$ - and  $\gamma$ -crystallin) in rat lens and their circulating specific autoantibodies in serum during the course of cataractogenesis.

**Methods:** Streptozocin (STZ; 55 mg/kg body mass) induced diabetic male Wistar/NIN rats (2-3 months old) and control nondiabetic rats were used for this study. Plasma glucose, glycated hemoglobin and body weight were evaluated on day zero, and at the interval of every two weeks up to the eighth week of post-injection in both the groups. Other biochemical parameters, such as the levels of nonprotein sulfhydryl (-SH) groups and the activity of  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT) in lens proteins were also estimated. Cataract progress was monitored by measuring the advanced glycation end product (AGE)-like fluorophores in both intact lens as well as in lens homogenate employing digital based image analysis and spectrofluorimetric methods. Similarly, the polyclonal antibodies specific to  $\beta$ -glycated-,  $\gamma$ -glycated-,  $\beta$ -, and  $\gamma$ -crystallins were used to determine the concentration of respective immunogens in lens by noncompetitive ELISA and their respective circulating antibodies by antibody capture assay. The profile of glycated lens protein (soluble and insoluble fractions) during the course of cataractogenesis was assessed by the western blot technique.

**Results:** STZ induced diabetic rats showed typical signs of diabetes (hyperglycemia, increased water and food intake with no increase in body weight). Biochemical analysis of total lens protein showed a significant ( $p < 0.001$ ) decrease in the levels of nonprotein -SH groups. The activity of lenticular  $\gamma$ -GT in diabetic rats was found to be unaltered as compared to the control group. Digital analysis of intact lens illustrated a positive correlation ( $r^2 = 0.888$ ) with the formation of AGE-like fluorophores during the course of cataractogenesis. A similar trend was also observed in the levels of AGE-like fluorophores in the total lens homogenate of diabetic animals during the course of cataractogenesis. The concentration of  $\beta$ - and  $\gamma$ -glycated-crystallins in the rat lens (soluble and insoluble fractions) was analyzed by non-competitive ELISA. The concentration of  $\beta$ - and  $\gamma$ -glycated-crystallins were found to be enhanced by the end of week eight, as compared to the control group. Concomitantly, crystallin-specific ( $\beta$ - and  $\gamma$ -glycated-crystallin) autoantibodies were also detected in the serum of the diabetic rats from week two onwards. Western blot analysis indicated the formation of enhanced glycated lens crystallins ( $\beta$ - and  $\gamma$ -crystallin) in the insoluble fraction.

**Conclusions:** The following was observed during the course of cataractogenesis: (1) there was an enhanced formation of AGEs-like fluorophores in intact lens; (2)  $\beta$ - and  $\gamma$ -glycated-crystallin levels increased in the rat lens (insoluble fraction) by the end of week eight; and (3) release of these glycated lens proteins into peripheral circulation resulted in the production of autoantibodies to  $\beta$ - and  $\gamma$ -glycated-crystallins that could be detected as early as week two, after induction of diabetic status in experimental rats.

Diabetes has been identified as a key risk factor for cataract [1]. There is an uncontrolled diffusion of extracellular glucose into the lens, which is one of the important body parts affected in diabetes mellitus. The proteins in the lens are extremely long lived, and there is virtually no protein turnover, which provides great opportunity for posttranslational modification to occur. Several mechanisms have been implicated in the development of cataract in diabetes [2-4]. The role of Maillard reactions in cataract formation has also been extensively studied in both aged and diabetic lens wherein advanced glycation end products (AGEs) of various derivations and molecular structures have been shown to be markedly elevated [5-8].

Crystallins are the major structural proteins of the eye lens and account for approximately 90% of total proteins. All vertebrate lenses contain  $\alpha$ -,  $\beta$ -, and  $\gamma$ -crystallins. Crystallins undergo nonenzymatic glycation [9] and their degree of glycation have been checked by affinity chromatography [10] as well as immunochemically using polyclonal and monoclonal antibodies to glycated bovine serum albumin (BSA-AGE) in streptozocin (STZ) induced diabetic rats [11,12]. During the course of aging, high-molecular-weight (HMW) and insoluble proteins increase with a concomitant disappearance of  $\gamma$ -crystallins from soluble fraction. This disappearance of  $\gamma$ -crystallins coincides with increased glycation and decrease in sulfhydryl (-SH) groups [13] from soluble fraction. It appears that glycation of lens proteins, disappearance of  $\gamma$ -crystallins, alterations in the -SH groups in the soluble fraction, increase in insoluble fraction, and HMW aggregates are interrelated. Leakage of  $\gamma$ -crystallins into the vitreous humor [14] is one of

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the first chronological events at the time of progression of opacity in diabetic rat that was observed along with the covalent modification of membrane proteins. Immunologically, it has also been observed that apart from the  $\gamma$ -crystallins, there are other subunits of  $\alpha$ - and  $\beta$ -crystallins outside the lens [15]. It has been reported that more than 98% of the cataract patients showed elevated antibody titer against lens crystallins [16]. Few studies have suggested that age-related cataract (ARC) may involve autoimmune phenomena [17-21]. The presence of these circulating autoantibodies are found to be cytotoxic for lens epithelial cells [22]. These observations have explored the therapeutic value of oral administration of lens proteins in ARC [23]. Hence, it is pertinent to note that autoimmune phenomena could play a key role in the initiation and progression of cataract.

Anticrystallin autoantibodies have often been demonstrated in the serum of healthy persons and especially in patients with cataract [15]. In no case, however the specific lens glycosylated crystallins have been identified against their glycosylated crystallin specific antibodies during the course of cataractogenesis. Since, the lens structural proteins have a fairly high degree of organ specificity [24], their elevated concentrations in blood serum might signal impairment of normal lens physiology leading to risk of developing cataract. In order to explore the feasibility of using serum assays of lens proteins to gain information on the physiological status of the lens, we developed a noncompetitive enzyme immunoassay to detect the glycosylated  $\beta$ - and  $\gamma$ -crystallins in lens and their circulating autoantibodies (IgG) in STZ induced diabetic rat, during the course of cataractogenesis.

## METHODS

**Materials:** Bovine serum albumin (BSA-fatty acid free, RIA grade), Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), fish gelatin, hemoglobin, antirabbit immunoglobulin G (IgG) labeled with horseradish peroxidase raised in goat (whole molecule), tetra methyl benzidine (TMB), urea hydrogen peroxide,  $\beta$ -cyclodextrin, antirat IgG labeled with horseradish peroxidase raised in goat, *p*-nitro-blue tetrazolium chloride (NBT), 5-bromo-4-chloro-3-indolyl phosphate toluidine (BCIP), STZ, Immobilon-P transfer membrane and Sepharose 6B were purchased from Sigma (St. Louis, MO). Polystyrene microtiter ELISA plates were from Greiner, Nürtingen, Germany. Other reagents used were of analytical grade.

**Experimental design:** Male, Wistar/NIN rats (2-3 months old) with a mean body mass of 250 g were obtained from the National Center for Laboratory Animal Sciences, National Institute of Nutrition, Hyderabad. All animals were fed on a cereals-plus based semisynthetic stock pellet diet. The control rats (Group I; n=30) received citrate buffer (0.05 M, pH 4.5) as a vehicle, where as the experimental rats (Group II; n=50) received a single intraperitoneal injection of STZ (55 mg/kg body mass) in the same buffer [25].

Animal care protocols were in accordance with and approved by the Institutional Animal Ethics Committee. Animals were housed in individual cages in a temperature and

humidity controlled room having a 12 h light:dark cycle. Food and water were provided ad libitum. Body mass was monitored weekly.

Every two weeks (zero, two, four, six, and eight) four animals each (control and experimental) were sacrificed. Blood was drawn from the retroorbital plexus from each animal for determination of plasma glucose level, total hemoglobin, and glycosylated hemoglobin as indicators of hyperglycemia. Lenses were dissected by posterior approach from each animal and stored at -20 °C until further analysis.

**Digital-based image analysis of rat lens:** This system consisted of a high-resolution charged coupled device (CCD) based camera with absorbance and fluorescence facility. It had an optical zoom lens (12.5x75 mm/f1.8) along with a 49 mm +1 dioptres close-up lens. The sensitivity of the camera was  $10^{-5}$  lux, with negligible signal to noise ratio (<30 db). The CCD camera was housed in a light-tight compact cabinet over a transilluminator and was equipped with UV and IR interference filters. The acquired image is displayed on a built in LCD screen (resolution -8 bit, 256 gray level images). Image acquisition was based on real-time integration in the range of 0.04 to 10 s.

Five lenses from both groups for every time were selected for digital-based image analysis. Digital image acquisition was achieved by placing the intact lens on the flat surface of the transilluminator, housed in the light-tight cabinet. The sample (lens) was excited individually from the base at UV-A ( $\lambda_{\text{ex } 365 \text{ nm}}$ ) region of the electromagnetic spectrum. Lens image formed due to the emission at 450 nm was acquired by real-time integration at 0.04 s. Annotated images were saved in a PC compatible file format (.tif file) to a floppy disk. Later, the digital images of the intact lens were analyzed by the software for determining the net fluorescence density (due to the emitted intensity of the light) measured as peak volume. This was done to measure the AGE-like fluorophores, as indicated by fluorescent intensity (peak volume) at  $\lambda_{\text{ex } 365 \text{ nm}}$  and  $\lambda_{\text{em } 450 \text{ nm}}$  in intact rat lens using high sensitivity CCD-based digital image analyzer (UVItec, Cambridge, UK). Fluorescent intensity (peak volume) was analyzed using UVI image acquisition and analysis software [26].

**Biochemical assessment of diabetic status:** Plasma glucose levels were determined by glucose oxidase-peroxidase assay method [27]. Total hemoglobin and glycosylated hemoglobin were measured by an ion exchange resin method [28]. The concentration of nonprotein -SH groups in the lens proteins was estimated spectrophotometrically by the method of Sedlack and Lindsay [29].  $\gamma$ -GT activity was measured by the method of Tate and Meister [30].

**Preparation of rat lens homogenates:** Lenses (n=2) were homogenized individually in 0.02 M phosphate buffer (pH 7.4; 10% w/v), centrifuged at 10,000x g for 30 min at 4 °C, and separated into supernatant and precipitate. The supernatant was referred to as the "buffer soluble" fraction and precipitate as the "buffer insoluble" fraction. The precipitate was further solubilized in 200 ml of 0.1 N NaOH. Both fractions were used for ELISA and western blot analysis. Total homogenate was also analyzed to detect the level of AGE-like fluorophores

[26]. Protein was estimated by the method of Lowry et al. [31] using BSA as a reference standard.

**Fluorescence measurements:** Fluorescent measurements were performed in total lens homogenate samples, using a spectrofluorimeter (Perkin-Elmer, LS-3B, Norwalk, NJ). AGEs were measured as described in previous research [32,33] at  $\lambda_{ex\ 366\ nm}$  and  $\lambda_{em\ 440\ nm}$ . Results are expressed as fluorescent intensity (FI)/mg of lens protein.

**Production and characterization of polyclonal antibodies to crystallins ( $\beta$ - and  $\gamma$ -crystallin) and glycated crystallins ( $\beta$ -gly- and  $\gamma$ -gly-crystallin):** Lens  $\beta$ - and  $\gamma$ -crystallins were isolated from one-month-old male Wistar/NIN rats using Sepharose-6B column chromatography, by following a technique previously described by Bhat [34]. Glycated  $\beta$ - and  $\gamma$ -crystallins were synthesized in vitro as described elsewhere in the literature [35]. Briefly, proteins ( $\beta$ - and  $\gamma$ -crystallins; 0.1 g) were dissolved along with glucose (2.9 g) and ribose (0.1 g) in 5 ml of 0.4 M sterile sodium phosphate buffer (pH 7.4). Samples were processed under sterile condition using the laminar-flow cabinet. Vials were sealed and placed in an air-circulating incubator at 37 °C for 12 weeks. After incubation, the mixture was dialyzed extensively against 0.02 M sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl. The degree of glycation was assessed by the trinitrobenzene sulfonic acid method [36].

$$\% \text{ conjugation} = \frac{(\text{Concentration of } \epsilon\text{-amino group in BSA}) - (\text{Concentration of } \epsilon\text{-amino group in BSA after glycation})}{\text{Concentration of } \epsilon\text{-amino group in BSA}}$$

Polyclonal antibodies for crystallins ( $\beta$ - and  $\gamma$ -crystallin) and glycated crystallins ( $\beta$ -gly- and  $\gamma$ -gly-crystallin) were raised in rabbits following guidelines in the literature [32]. A primer dose of 250-300  $\mu\text{g/kg}$  of body mass of the purified crystallins ( $\beta$ - and  $\gamma$ -crystallin) and synthesized glycated crystallins ( $\beta$ -gly- and  $\gamma$ -gly-crystallin) were injected at multiple sites (intradermal injection). It was dissolved in sterile saline and emulsified with Freund's complete adjuvant in a 1:1 ratio. Later, three booster doses were given (100-150  $\mu\text{g/kg}$ ) intramuscularly in Freund's incomplete adjuvant. Blood was collected from the animals after 30 days of primer dose and 10-12 days after the booster doses. At the end of the immunization schedule the animals were sacrificed and blood was collected by cardiac puncture. Serum was separated, lyophilized, and stored at -20 °C until further use. Immune response to these immu-

nogens was checked by the Ouchterlony double diffusion technique [37]. Antisera titer for preimmune, primer, I, II, and III boosters were determined by checkerboard analysis using antibody capture assay. The cross-reactivity of these antisera was checked against glycated-hemoglobin.

**Noncompetitive ELISA:** The polyclonal antibodies specific to  $\beta$ -,  $\gamma$ -,  $\beta$ -gly- and  $\gamma$ -gly-crystallins were used to determine the levels of respective immunogens in lens by noncompetitive ELISA as described in detail previously with minor modifications [38]. Briefly, a 96 well microtiter plate was coated with a concentration of antigens ( $\beta$ -,  $\gamma$ - crystallins, 500 ng/50  $\mu\text{l}$ /well and  $\beta$ -gly-,  $\gamma$ -gly-crystallins, 100 ng/50  $\mu\text{l}$ /well) dissolved in 0.1 M carbonate buffer, pH 9.6, overnight at 37 °C and washed thrice with washing buffer (phosphate buffer saline [PBS-T] 0.01 M, pH 7.2 with 0.05% tween-20 and 0.01% sodium azide). The wells were blocked for nonspecific binding with 100  $\mu\text{l}$ /well of 0.1% fish gelatin in PBS (0.01 M, pH 7.2) and washed three times as earlier. To each well was added 50  $\mu\text{l}$  of diluted respective polyclonal antibodies ( $\beta$ -, 1:7,000;  $\gamma$ -,  $\beta$ -gly-, and  $\gamma$ -gly-crystallin, 1:10,000), which had been preincubated with test (lens sample) and standard at different concentrations (1-1000 ng) of their respective antigens for 1 h at room temperature prior to addition to the plate. After 1 h incubation, wells were washed thrice and later incubated at 37 °C for 1 h with 50  $\mu\text{l}$ /well of 1:6,000 diluted (0.5% fish gelatin in PBS) horseradish peroxidase labeled antirabbit IgG raised in goat. The plates were washed thrice and 150  $\mu\text{l}$ /well of substrate (450  $\mu\text{l}$  of TMB [10 mg/ml of dimethyl sulfoxide] in 15 ml of 0.1 M acetate buffer pH 5.0 containing 0.25% (w/v)  $\beta$ -cyclodextrin and 0.015% (w/v) of urea hydrogen peroxide) was added. The reaction was terminated after 10 min by adding 50  $\mu\text{l}$ /well of 1.25 M  $\text{H}_2\text{SO}_4$ . The absorbance was recorded at 450 nm using a SLT Spectra ELISA reader (SLT Lab Instruments, Salzburg, Austria). Percent binding of antibodies versus concentration of analyte (1-1000 ng) was used to generate inhibition plot using linear regression analysis. The concentration of respective antigens ( $\beta$ -,  $\gamma$ -,  $\beta$ -gly-, and  $\gamma$ -gly-crystallins) in lens was expressed as  $\mu\text{g/mg}$  of lens protein. Concentration was defined as the amount of proteins ( $\beta$ -,  $\gamma$ -,  $\beta$ -gly-, and  $\gamma$ -gly-crystallins) required to inhibit 50% ( $\text{IC}_{50}$ ) of their respective antibody binding.

Crossreactivity of the respective antibodies ( $\beta$ -,  $\gamma$ -,  $\beta$ -gly-, and  $\gamma$ -gly-crystallins) to  $\beta$ -,  $\gamma$ -,  $\beta$ -gly-, and  $\gamma$ -gly-crystallins

TABLE 1.

Weeks	Glucose (mg/dl)		Glycated hemoglobin (% GHb)	
	Control	STZ	Control	STZ
0	108± 8.7	117± 21.2	8.0±1.01	6.8±0.36
2	104±10.6	355±125	8.3±0.91	15.4±2.00
4	92±16.4	388± 85.2	7.2±1.63	17.3±4.09
6	108±20.1	381± 93.4	5.7±1.26	16.0±2.48
8	112±17.5	349±117.4	6.5±1.75	16.7±4.31

Plasma profile of glucose and glycated hemoglobin in control and streptozocin-induced diabetic rats.

TABLE 2.

Week	(μmoles mg <sup>-1</sup> of lens protein)			
	Control (-SH) group	STZ (-SH) group	Control $\gamma$ -GT	STZ $\gamma$ -GT
0	0.22±0.036	0.22 ±0.018	0.3 ±0.01	0.23±0.035
2	0.22±0.029	0.066±0.009*	0.29±0.081	0.25±0.057
4	0.22±0.018	0.039±0.007	0.26±0.035	0.29±0.028
6	0.2 ±0.001	0.06 ±0.014	0.24±0.028	0.31±0.075
8	0.23±0.015	0.08 ±0.017	0.31±0.012	0.28±0.014

Levels of lens nonprotein sulfhydryl groups and membrane  $\gamma$ -GT level in control and experimental (STZ) rats. Values are mean of five animals (mean±SD) at every given time. The asterisk indicates a  $p<0.001$  when compared with the 0 week value.

were evaluated by noncompetitive ELISA wherein these antigens ( $\beta$ -,  $\gamma$ -,  $\beta$ -gly-, and  $\gamma$ -gly-crystallins) were used as analytes to displace the antibody. Cross-reactivity studies were carried out at 50% inhibition ( $IC_{50}$ ) level of the respective parent antigens.

**Antibody capture assay:** The concentration of circulating antibodies to  $\beta$ -,  $\gamma$ -,  $\beta$ -gly-, and  $\gamma$ -gly-crystallins in serum from experimental rats were analyzed by antibody capture assay as described previously [38] using their respective antigens ( $\beta$ -,  $\gamma$ -,  $\beta$ -gly-, and  $\gamma$ -gly-crystallins) from the same species. Microtiter plates were coated with their respective concentration of antigens ( $\beta$ - and  $\gamma$ - crystallins separately, 500 ng/50  $\mu$ l/well and  $\beta$ -gly- and  $\gamma$ -gly-crystallins separately, 100 ng/50  $\mu$ l/well) dissolved in 0.1 M carbonate buffer, pH 9.6, incubated overnight at 37 °C and washed thrice with washing buffer. The wells were blocked for nonspecific binding with 100  $\mu$ l/well of 0.1% fish gelatin in PBS (0.01 M, pH 7.2). Plates were washed thrice, and different dilution of antisera (experimental and control) from rats were added and incubated for 2 h at 37 °C. After washing, plates were incubated at 37 °C for 1 h with 50  $\mu$ l/well of 1:6,000 diluted (0.5% fish gelatin in PBS) horseradish peroxidase labeled anti-rat IgG raised in goat. The plates were washed thrice and 150  $\mu$ l/well of substrate (450  $\mu$ l of TMB [10 mg/ml of dimethyl sulfoxide] in 15 ml of 0.1 M acetate buffer pH 5.0 containing 0.25% (w/v)  $\beta$ -cyclodextrin and 0.015% (w/v) of urea hydrogen peroxide was added. The reaction was terminated after 10 min by adding 50  $\mu$ l/well of 1.25 M  $H_2SO_4$ . The absorbance was recorded at 450 nm using SLT Spectra ELISA reader (SLT Lab Instruments, Salzburg, Austria). Rat IgG was used to generate the calibration plot for crystallin specific antibodies ( $\beta$ -,  $\gamma$ -,  $\beta$ -gly-, and  $\gamma$ -gly-crystallins). Results were expressed as  $\mu$ g/ml of serum.

**Western blot analysis:** Western blot analysis was carried out according to the Towbin method [39] with a modification that involved using 20  $\mu$ g of lens protein (soluble and insoluble

fractions) of week eight from both the groups, to assess the degree of glycation. Briefly, proteins separated by 12.5% SDS-PAGE were electrophoretically transferred to Immobilon-P transfer membrane (polyvinylidene fluoride membrane) using a Consort electroblotter system (Consort N.V., Turnhout, Belgium). The membrane was soaked in 100% methanol for 30 s and washed with water for 2 min. It was then soaked in Towbin's transfer buffer (0.025 M Tris, 0.192 M glycine and 20% methanol) for 30 min. Filter papers for sandwiching the membrane and gel were cut to the dimensions of gel and were also soaked in Towbin's transfer buffer for 30 min. The transfer was carried out at 1.1 mA/cm<sup>2</sup> of gel, 50 V, 150 W for 2 h at 21 °C. The membrane was washed thrice with washing buffer (0.01 M PBS with 0.05% Tween-20) and incubated in blocking buffer (3% fish gelatin in 0.01 M PBS) for 30 min and washed with washing buffer. It was incubated overnight with blocking buffer containing 1:1,000 diluted polyclonal antibodies to  $\beta$ - and  $\gamma$ -glycated-crystallins with gentle shaking at 21 °C. The membrane was washed thrice with washing buffer to remove unbound antibodies and incubated for 1 h in alkaline phosphatase assay buffer (0.1 M NaCl, 0.005 M  $MgCl_2$ , 0.1 M Tris, pH 9.5) containing 1:5,000 goat antirabbit IgG coupled with alkaline phosphatase. The membrane was washed thrice with washing buffer and incubated with BCIP/NBT developing solution (115 mmoles of BCIP and 61 mmoles of NBT) until purple bands appeared. Color development was arrested by immersing the membrane in water. The membrane was air dried for 20 min and stored. It was documented using a digital based image analyzer (UVItec, Cambridge, England).

**Statistical analysis:** The data was statistically analyzed by using Sigma-plot software version 5.0. The test of significance was based on Student's T-test.

## RESULTS

### Physical and biochemical assessment of diabetic model:

There was no change in body mass at the end of week eight

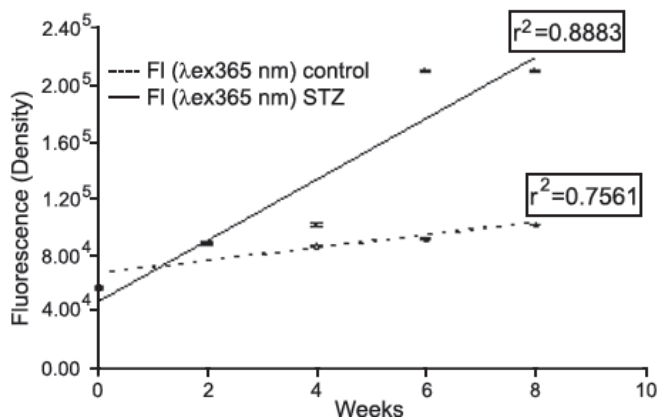


Figure 1. Correlation between advanced glycation end product-like fluorophores (fluorescence intensity at  $\lambda_{ex\ 365\ nm}$  and  $\lambda_{em\ 450\ nm}$ ) and cataract progression in intact diabetic rat lens, using digital based image analysis ( $r^2=0.888$ , confidence interval 95%). On the y-axis, values are in exponential. Values are mean $\pm$ SD (n=5).

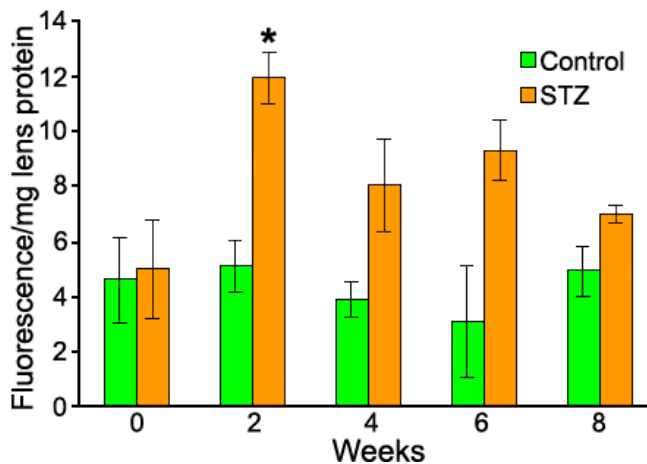


Figure 2. advanced glycation end product-like fluorophores in the total lens homogenate as a function of cataract progression in diabetic rats ( $\lambda_{ex\ 366\ nm}$ ;  $\lambda_{em\ 440\ nm}$ ). Values are mean $\pm$ SD (n=5). The asterisk indicates a p<0.001 when compared with the zero week value.

between control (299±42 g) and STZ rats (274±52 g). Table 1 depicts the plasma glucose and glycated hemoglobin levels in control and experimental animals. The plasma glucose level in the diabetic group was found to be enhanced from week two onward to a hyperglycemic state (in the range of 350-450 mg/dl), with a concomitant increase in the concentration of glycated hemoglobin (15%). In addition, a three-fold decrease in lens protein-free -SH groups was also observed on week two as compared to week zero sample, in the diabetic animals. Further, there was no alteration in the activity of lens  $\gamma$ -GT during cataract progression (Table 2).

**Fluorometric determination of advanced glycation end product-like fluorophores ( $\lambda_{ex\ 366\ nm}$ ,  $\lambda_{em\ 440\ nm}$ ) in lens:** AGE-like fluorophores were analyzed in both intact lens (Figure 1) as well as in lens homogenate (Figure 2). A positive correlation ( $r^2=0.888$ ) was found between the progression of cataract and fluorescent intensity in the diabetic lens, although this was not observed in the control group (Figure 1). Similarly, AGE-like fluorophores showed a statistically significant

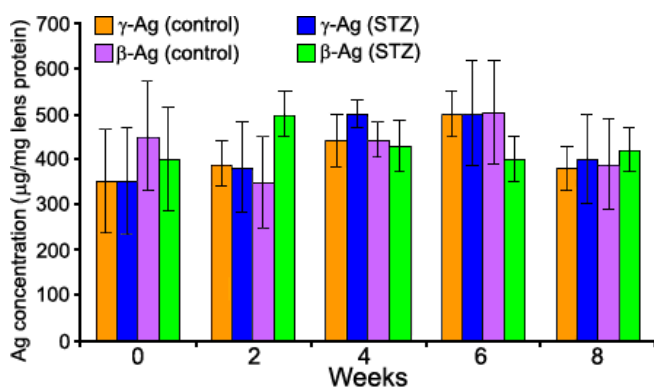


Figure 3. Profile of rat lens crystallins ( $\gamma$ - and  $\beta$ -crystallin) during the experimental period as determined by noncompetitive ELISA using their respective polyclonal antibodies. Values are mean±SD at every given time (n=5).

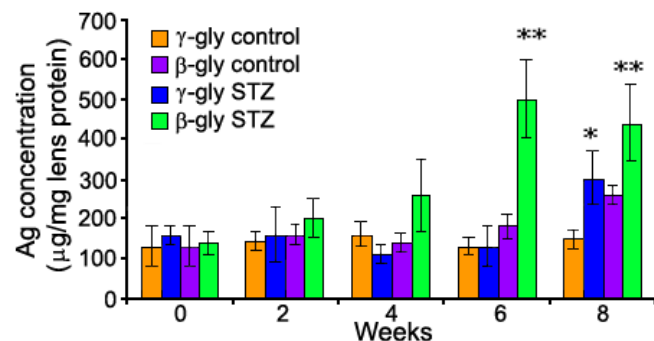


Figure 5. Profile of glycated rat lens crystallins ( $\gamma$ - and  $\beta$ -crystallin) in insoluble fractions during the experimental period as determined by noncompetitive ELISA using their respective polyclonal antibodies. Values are mean±SD at every given time (n=5). The asterisk indicates a  $p<0.005$  at week eight for  $\gamma$ -glycated-crystallin, and the double asterisk represents a  $p<0.001$  for  $\beta$ -glycated-crystallin at weeks six and eight.

( $p<0.001$ ) increase in diabetic lens homogenate from week two onwards, as compared to the control group (Figure 2).

**Trinitrobenzene sulfonate assay:** The degree of glycation of crystallins ( $\beta$ - and  $\gamma$ -crystallin) was monitored by trinitrobenzene sulfonate (TNBS) assay and was observed to be 86% at the end of 12 weeks of incubation.

**Noncompetitive ELISA:** ELISA analysis was carried out to quantitate the changing levels of glycated crystallins ( $\beta$ - and  $\gamma$ -crystallin) in lens during cataractogenesis. There was no noticeable change in the levels of  $\beta$ - and  $\gamma$ -crystallins during the experimental duration in both control and experimental groups (Figure 3). Figure 4 and Figure 5 indicated a significantly elevated level of  $\gamma$ -glycated-crystallins (1.6 fold and 2.1 fold) in both the fractions (soluble [ $p<0.005$ ] and insoluble [ $p<0.005$ ]), respectively, at the end of week eight in comparison to week zero sample of the experimental group. These changes were not observed in the control group. Between the control and experimental groups, a significant ( $p<0.001$ ) alteration in levels of  $\gamma$ -glycated-crystallins, at the end of week eight (in both soluble and insoluble fractions; Figure 4 and Figure 5) was observed. Significant changes in the levels of  $\beta$ -glycated-crystallins were noticed between week zero and week six onwards in the insoluble fraction ( $p<0.001$ ) and week zero and week eight in the soluble fraction ( $p<0.001$ ). The

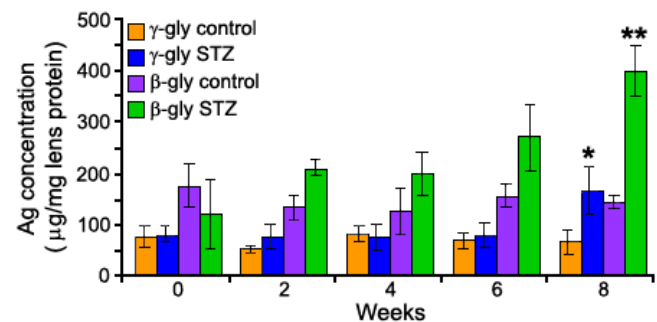


Figure 4. Profile of glycated rat lens crystallins ( $\gamma$ - and  $\beta$ -crystallins) in soluble fractions during the experimental period, as determined by noncompetitive ELISA using their respective polyclonal antibodies. Values are mean±SD at every given time (n=5). The asterisk indicates a  $p<0.005$  at week eight for  $\gamma$ -glycated-crystallin, and the double asterisk represents a  $p<0.001$  for  $\beta$ -glycated-crystallin.

TABLE 3.

Crystallins	Crystallin antibodies			
	$\beta$ -	$\gamma$ -	$\beta$ -Gly	$\gamma$ -Gly
$\beta$ -	100%	ND	8%	ND
$\gamma$ -	ND	100%	ND	ND
$\beta$ -Gly	ND	3%	100%	6%
$\gamma$ -Gly	ND	26%	2.6%	100%

Percent cross-reactivity of crystallins ( $\beta$ - and  $\gamma$ -crystallin) and glycated crystallins ( $\beta$ -gly- and  $\gamma$ -gly-crystallin) with their respective antibodies.

levels of  $\beta$ -glycated-crystallins in the insoluble fraction of lens showed a gradual increase from week four onwards, although this trend was not true for soluble fraction of lens (Figure 5).

The inhibitory concentration at 50% antibody binding ( $IC_{50}$ ) for  $\beta$ -gly- and  $\gamma$ -gly-crystallins was found to be 7 ng and 6.1 ng, respectively (regression analysis,  $\beta$ -gly-crystallins,  $r^2=0.944$ ;  $\gamma$ -gly-crystallins,  $r^2=0.952$ ). The crossreactivity of these crystallins ( $\beta$ - and  $\gamma$ -crystallin) and their glycated crystallins ( $\beta$ -gly- and  $\gamma$ -gly-crystallin) are shown in Table 3. The results suggest that the immunogen  $\gamma$ -gly-crystallins showed 26% cross-reactivity with antibodies raised against  $\gamma$ -crystallins.

**Antibody capture assay:** This assay was able to detect the crystallin-specific ( $\beta$ -gly-,  $\gamma$ -gly-,  $\beta$ -, and  $\gamma$ -crystallin) antibodies in serum of the diabetic group (Figure 6, Figure 7)

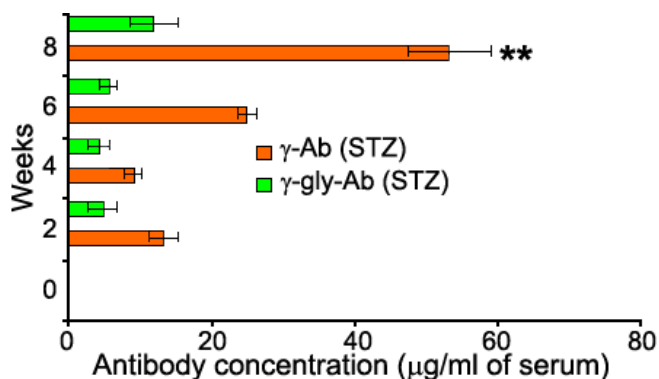


Figure 6. Profile of circulating autoantibodies in rat serum to  $\gamma$ - and  $\gamma$ -glycated-crystallins as determined by antibody capture assay during the experimental period. Autoantibodies were not detected in control groups throughout the experimental duration. Values are mean $\pm$ SD at every given time (n=5). The double asterisk indicates a  $p<0.001$  for  $\gamma$ -crystallin autoantibodies between weeks two and eight.

from week two onwards. Antibody concentration against  $\gamma$ -crystallin was three-fold higher at the end of week eight in comparison to the concentration of antibody against  $\beta$ -crystallin. The concentration of antibody against  $\gamma$ -glycated-crystallin was also found to be higher (1.2 fold) than that of  $\beta$ -glycated-crystallin antibody at the same time.

**Western blot analysis:** Since immunologically, week eight diabetic lens sample showed higher concentration of glycated crystallins in both the fractions, these samples were used for western blot analysis. Figure 8 shows the comparison of glycation between soluble and insoluble fractions at the end of week eight with respect to the diabetic (STZ) and control group rats. The insoluble fraction of  $\beta$ -glycated-crystallins showed enhanced degree of glycation in comparison to the same fraction of  $\gamma$ -glycated-crystallin with respect to their re-

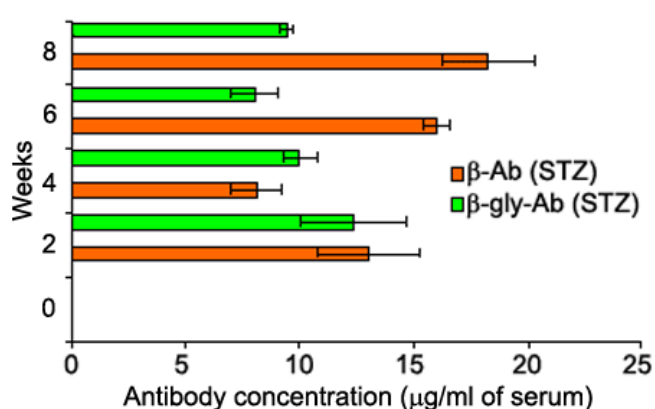


Figure 7. Profile of circulating autoantibodies in rat serum to  $\beta$ - and  $\beta$ -glycated-crystallins as determined by antibody capture assay during the experimental period. Autoantibodies were not detected in control groups throughout the experimental duration. Values are mean $\pm$ SD, at every given time (n=5).

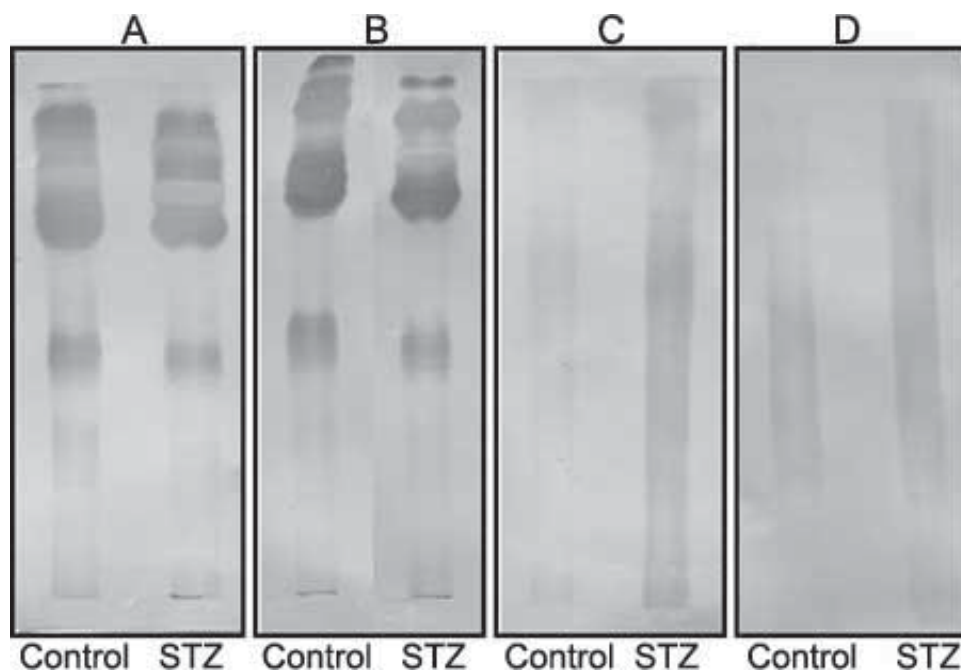


Figure 8. Western blot analysis of rat lens crystallins indicating the degree of glycation in control and experimental animals at the end of week eight. **A:** Soluble fraction of  $\gamma$ -glycated-crystallin. **B:** Soluble fraction of  $\beta$ -glycated-crystallin. **C:** Insoluble fraction of  $\gamma$ -glycated-crystallin. **D:** Insoluble fraction of  $\beta$ -glycated-crystallin.

spective controls (Figure 8). However soluble fractions of  $\beta$ -glycated- and  $\gamma$ -glycated-crystallin did not show any obvious change in the degree glycation with their respective controls at the same time (Figure 8).

## DISCUSSION

The biochemical data presented here strongly suggests the induction of diabetes in STZ-diabetic rat model (Table 1). Similar levels of blood glucose and glycated hemoglobin for cataract development at different time intervals were reported by Abraham et al. [40]. One of the earliest changes observed in the lens of diabetic rat was the fall in nonprotein -SH groups (78%) by week two of post-injection (Table 2). This observation was similar to the earlier reports [5,41]. The fall in the levels of -SH groups brought about by the diabetic status of the animal, decreased the ability to maintain protein -SH groups in the reduced state. This could lead to the formation of intra- and interchain disulfide bond, which is reported to be associated with high molecular weight (HMW) protein aggregate and cataract formation [42]. It has been suggested that rather than osmotic stress, oxidative stress and lens membrane damage are the major factors responsible for lens opacification [40]. The present experimental results indicated no alteration in the activity of lenticular  $\gamma$ -GT in both the groups. Serum  $\gamma$ -GT might be useful in understanding these oxidative stress-related issues rather than cytosolic  $\gamma$ -GT as reported earlier [43].

Previous studies have clearly shown protein glycation plays an important role in the development of cataract in the diabetic rat model [13,42,44,45]. Abraham et al. [40] used Scheimpflug imaging and densitometry to quantify the opacity of lens in terms of blood glucose level and glycation of proteins during the progression of cataract in situ in lens. This is the first report of AGE-like fluorophores being analyzed in intact rat lens by digital based image analysis during the progression of cataract. Digital data (Figure 1) clearly shows an increase of AGE-like fluorophores in diabetic rats as compared to the control group. This observation was similar to the one made in aging human lens [26]. It is notable that digital analysis of intact lens and lens homogenate indicated a similar increasing trend of fluorescent intensity due to AGE-like fluorophores in diabetic rats as compared to that of control rats from week two onwards (Figure 1, Figure 2). Both intact lens and lens homogenate data suggests a significant increase in the levels of AGE-like fluorophores in diabetic rats. This argues well with the earlier reports [42,44,46], where protein glycation played an important role in the development of cataracts in this diabetic rat model. The spectrofluorometric studies suffer from the spurious contribution to total fluorescence of non-AGE adducts, such as lipid- or sugar-derived oxidative products that have similar fluorescence spectra [47,48]. It is therefore difficult to characterize lens crystalline-specific AGE products present in lens by their fluorescence properties. This limitation has been overcome by using immunological techniques, wherein specific antibodies were used to detect lens AGE products both in animals [8,12,38] and in human [32,33,35] that increase with aging of the tissue.

In mature cataracts longstanding leakage of crystallins through the damaged capsule is likely to occur. The lens crystallins being sequestered antigens are exposed to the immune surveillance and the resultant autoantibody response. However, those crystallins that normally occur in small amounts outside the lens would be recognized as self, and therefore normally do not evoke an autoimmune response. Only the truly lens-specific crystallins would be recognized as non self antigen, thus triggering autoantibodies formation. In this respect, for the first time the lens-specific antibodies to glycated crystallins ( $\beta$ -gly- and  $\gamma$ -gly-crystallin) were used to probe their levels during the course of cataractogenesis in the diabetic model. The immunoanalytical results of our study clearly demonstrate the specificity of polyclonal antibodies raised against  $\beta$ - and  $\gamma$ -crystallins and their respective glycated proteins (Table 3). The higher percentage of crossreactivity (26%) between  $\gamma$ - and  $\gamma$ -glycated-crystallins indicates that both share few common antigenic determinants. Due to this crossreactivity, the autoantibodies to  $\gamma$ - and  $\gamma$ -glycated-crystallins show preponderance over the serum level of autoantibodies to  $\beta$ - and  $\beta$ -glycated-crystallins in STZ-rats (Figure 6, Figure 7). The higher antibody titer (1:10,000) of  $\gamma$ -,  $\gamma$ -glycated and  $\beta$ -glycated-crystallins, indicate that these are more immunogenic proteins than  $\beta$ -crystallins (1:7,000). Both  $\gamma$ - and  $\beta$ -glycated-crystallins elicit an enhanced immunogenic response than their nonglycated ones which may be due to their modified forms. This augurs well with an earlier report wherein AGE-modified proteins have been found to be more immunogenic [49].

It has been well established that during the glycation of lens crystallins, the disappearance of reactive sulfhydryl groups and formation of HMW aggregates, are all interrelated and are responsible for cataractogenesis [42]. Consistent with these possibilities, our ELISA and western blot analysis indicated increasing concentrations of both  $\beta$ - and  $\gamma$ -glycated-crystallins in the insoluble fraction at the end of week eight in the diabetic lens. This indirectly reflects the role of these glycated crystallins during the course of cataractogenesis (Figure 4, Figure 5, Figure 6, Figure 7, Figure 8), which may ultimately enhance the opacity as supported by an earlier report [42]. Interestingly, the presence of antibodies to these glycated as well as nonglycated  $\beta$ - and  $\gamma$ -crystallins in serum of diabetic animals at week two, indirectly reflects the leakage of these proteins at an early stage of cataract development. Our experimental results support the hypothesis that other than osmotic stress, oxidative stress and lens membrane damage results in the leakage of crystallins, which ultimately leads to lens opacity. Possibly, the earlier observation by Nayak et al. [38] on osmotic stress due to galactose-fed rat (galactose-induced cataract animal model) could not lead to the membrane damage, hence no autoantibodies to  $\beta$ - and  $\gamma$ -crystallins were detected in the rat serum. Immunologically,  $\gamma$ -crystallins tend to be more immunogenic than the  $\beta$ -crystallins [15,38]. Intriguingly, in the present investigation, the experimental evidence suggests that the glycated form of  $\gamma$ -crystallin was more immunogenic than the glycated form of  $\beta$ -crystallin that is similar to the observation recorded for their native forms

[15,38]. It is appealing to note that the immunogenic property of crystallins does not change due to glycation and both glycated ( $\beta$ - and  $\gamma$ -) crystallins can elicit immune response at an early stage (week two) of cataractogenesis. The leakage of the glycated crystallins at an early stage and subsequent specific autoantibody response may play an imperative role during the course of cataractogenesis.

Our experimental investigation is the first to highlight the presence of autoantibodies to glycated crystallins ( $\beta$ - and  $\gamma$ -) during the course of cataractogenesis in an animal model. Earlier, autoantibodies to AGE-modified IgG were also documented in patients of rheumatic arthritis [50]. This observation needs to be further substantiated in the human subjects during the course of cataractogenesis.

Our experimental findings may have practical relevance in detecting the glycated crystallin ( $\beta$ - and  $\gamma$ -crystallin) specific autoantibodies as a biochemical marker for early detection of cataract in human subjects.

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