**Assessment of structure, stability and aggregation of soluble lens proteins and
alpha-crystallin upon non-enzymatic glycation: The pathomechanisms underlying
cataract development in diabetic patients.**

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Abstract

Total soluble lens proteins (TSPs) and α -crystallin (α -Cry) were individually subjected to the long-term glycation in the presence of D-glucose. The glycated and non-glycated protein counterparts were incubated under different stress conditions and compared according to their structure, stability and aggregation propensity by various spectroscopic techniques and gel mobility shift analyses. Extensive glycation of the lens proteins was accompanied with structural alteration, reduction in their surface hydrophobicity and increment of their surface tension. Our results suggest that glycation causes lens crystallins to partially resist against structural alteration and aggregation/fibrillation under both thermal and thermochemical systems. The conformational stability of lens crystallins was increased upon glycation, showing the reason behind resistance of glycated proteins against stress-induced structural alteration and aggregation. Due to the resistance of glycated lens crystallins against aggregation, the role of this modification in development of senile cataract can be explained with the associated damaging consequences highlighted in this article.

Keywords: Glycation, Lens crystalins, Aggregation.

1. Introduction

Eye lens is composed of highly stable and long-lived proteins which perform both structural and refractive functions. These proteins (α , β and γ -crystallins) are present at extremely high concentration (>300 mg/mL), accounting for more than ninety percent of the total lens soluble proteins (TSPs) [1]. The short-range spatial order and close packing of these proteins play a vital role in flawless transparency and refractive properties of the mammalian eye lens [2]. These weak forces which usually do not produce any stable complex are thought to be compromised in lens due to accumulation of various types of modification, occurring during normal ageing and cataractogenesis [3]. Due to their remarkable longevity, lens crystallins undergo a number of age-related modifications including non-enzymatic glycation which finally attenuate their ability in the maintenance of lens transparency [4]. Ocular lens does not require insulin for glucose uptake [5], therefore under chronic hyperglycemia, D-glucose and glycolytic intermediates such as glucose 6-phosphate, fructose 6-phosphate and glycer aldehyde 3-phosphate are highly available for irreversible attachment onto amino groups of crystallin proteins, forming stable and structurally divergent adducts which collectively named as advanced glycation end products (AGEs) [6]. Extracellular AGE proteins can potentially bind to the cell surface AGE receptors (RAGE) which up-regulate in the retinas of diabetic patients, activating diverse signal transduction cascades, resulting in generation of the reactive oxygen species (ROS) [7]. Additionally, D-glucose and the above mentioned glycolytic intermediates can enter into other ROS generating pathogenic pathways as indicated in **Scheme 1**.

As a consequence of organelle degradation process during differentiation, the cytoplasmic organelles are absent from the innermost cells of the adult vertebrate lens [8]. Therefore, the damaging effects of hyperglycemia on lens crystallins might be even

intensified in the outer layers of lens with the young fiber cells keeping yet their cellular organelles. During chronic hyperglycemia, AGE formation increases beyond its normal levels and these pro-inflammatory mediators activate inducible nitric oxide synthase (iNOS), leading to overproduction of nitric oxide which can be also achieved during inflammatory condition (**Scheme 1**) [9].

► **Scheme 1 to be inserted here** ◄

Nitric oxide inhibits mitochondrial electron transport chain and subsequently increases the production of superoxide radical which causes strand breaks in nuclear DNA, subsequently activating Poly (ADP-ribose) polymerase 1 (PARP1) [10]. This enzyme which has been indicated to play a role in several nuclear processes can also modify the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which demonstrates a reversible nucleo-cytoplasmic translocation, thus reducing its activity. As a result of GAPDH inhibition, the accumulation of upstream glycolytic metabolites activate multiple pathogenic and ROS-generating pathways including polyol pathway, protein kinase C pathway, hexosamine pathway and AGE pathway (**Scheme 1**) [11]. Additionally, hyperglycemia in diabetic patients pushes more electron donors (NADH and FADH₂) into the electron transport chain which subsequently increases the voltage gradient ($\Delta\mu\text{H}^+$) across the mitochondrial inner membrane beyond a critical threshold [12]. At this point, the electron transfer inside complex III is partially inhibited, resulting in a backup of electrons to coenzyme Q and their donation to molecular oxygen, thereby generating increased amount of superoxide (**Scheme 1**). Overall, the current believes on etiology of cataracts in diabetic patients are related to the progressive glycation of lens crystallins and to the harmful effects of oxidative stress associated with hyperglycemia in eye lens [13]. In this study, TSPs and α -Cry were subjected to long-term glycation and subsequently the structural properties and aggregation/fibrillation

propensity of the modified and non-modified proteins were assessed under various conditions, using different techniques.

2. Materials and Methods

2.1. Materials

Sephacryl S-300 HR, 1-anilino-8-naphthalene sulfonate (ANS), Thioflavin-T (ThT), ortho-phthalaldehyde (OPA), Congo red (CR), fluorescamine and other chemicals were purchased from Sigma. D-glucose and guanidine hydrochloride (GuHCl) were supplied by Merck. All solutions were prepared with double-distilled water and kept at 4 °C before use.

2.2. Methods

2.2.1. Preparation of bovine soluble lens proteins

The total soluble lens proteins (TSPs) were prepared from the bovine eye lens according to our previous publications [14]. In brief, bovine lenses which obtained from a local slaughterhouse were dissected from the eyeballs. A 10% homogenate of the lenses was prepared in 25 mM Tris (pH 8.0), containing 0.1 M NaCl, 0.5 mM EDTA, 0.01% NaN₃, and 10 mM β -mercaptoethanol (β -ME). Then, the homogenate was centrifuged at 14,000 rpm, for 30 min at 4 °C and the supernatant collected as TSPs.

2.2.2. Purification of bovine α -Cry

Purification of lens α -Cry was performed according to our previous publications [15]. In brief, the prepared TSPs were applied on a Sephadex S-300 HR (100×1.5 cm) gel filtration column which pre-equilibrated with the same buffer. The crystallin proteins

were collected in different test tubes at a flow rate of 0.25 mL/min with a fraction size of 2 mL. At the end, the protein concentration was determined by Bradford assay and the purity assessed, using SDS-PAGE (gel 12%) [16]. The pure samples of α -Cry were lyophilized and their powders stored at -20 °C until used.

2.2.3. Glycation of bovine TSPs and α -Cry

The stock of D-glucose was prepared in 100 mM NaPi buffer (pH 7.4). TSPs (10 mg/mL) and α -Cry (5 mg/mL) were incubated with D-glucose (0.5 M) in 0.2 M NaPi buffer (pH 7.4), containing penicillin, streptomycin and 0.01% sodium azide for 4 weeks at 37 °C in dark and under sterile condition. TSPs and α -Cry which incubated under similar conditions, in the absence of D-glucose, served as the control samples. At the end of incubation, the protein preparations were dialyzed against 20 mM NaPi buffer (pH 7.4) to remove any un-reacted sugars [17].

2.2.4. Spectroscopic evaluation of glycation of lens crystallins

Glycation of lens crystallins was evaluated, using OPA and fluorescamine assays. These assays were used to determine the proportion of free/non-reacted α/ϵ -amino groups after glycation of the protein samples [18]. The OPA reagent was prepared immediately before use by mixing 25 mL of 0.1 M sodium borate, 2.5 mL of SDS solution (20%), 100 μ L of β -ME and 40 mg OPA which had previously been dissolved in 1 mL of methanol. The final volume was adjusted to 50 mL with double distilled water. A 50 μ L aliquot of the sample, containing 2 mg/mL protein was added to 1 mL OPA solution in 1mL cuvette. Absorbance of the mixed solution was measured at 340 nm after 2 min incubation at room temperature against a blank containing the OPA reagent, using a T90⁺ UV-Vis spectrophotometer instrument (PG Instrument Ltd., UK). The assay was done in triplicate and the results were averaged. To perform fluorescamine assay, 25 μ L of

protein sample (1 mg/mL) was mixed in a solution containing 500 μ L NaPi buffer (50 mM) and 225 μ L double distilled water. Subsequently, 250 μ L of fluorescamine reagent (1 mM in acetonitrile) were added to the reaction mixture and incubated for 10 min in the dark. The fluorescence measurement was then performed at excitation/emission wavelengths of 390/490 nm with a fluorescence spectrometer instrument (Cary-100, Varian, Australia). The excitation/emission slit widths were set at 10/10 nm.

2.2.5. Non-Trp AGE fluorescence measurement

To further assess the glycation of lens crystallins, non-Trp AGE fluorescence was monitored. In this experiment the protein sample (0.15 mg/mL) in 100 mM NaPi buffer (pH 7.4), was excited at 370 nm, and the emission spectra recorded between 400-500 nm. The excitation/emission slit widths were set at 10/10 nm for TSPs and 20/20 nm for α -Cry [19].

2.2.6. Trp and ANS fluorescence measurement

Both Trp and ANS fluorescence studies were performed to evaluate changes in the protein tertiary structures. The fluorescence measurements of different protein samples were carried out in NaPi buffer (100 mM, pH 7.4). Also the protein samples were diluted to 0.15 mg/mL prior to perform fluorescence assays. In Trp fluorescence assessments, the emission spectra of different TSPs and α -Cry samples were collected between 300-500 nm by using an excitation wavelength of 295 nm [17]. To perform ANS fluorescence study, protein samples were incubated at room temperature with 100 μ M ANS for 30 min in the dark before the measurements. The excitation wavelength was fixed at 365 nm and the emission spectra collected between 400-600 nm. Also, concentration of ANS stock solution in water was determined, using an extinction

coefficient of $4,950 \text{ M}^{-1}\text{cm}^{-1}$ at 350 nm [20]. The slit widths in the excitation were set at 5 and 10 nm for TSPs and α -Cry, respectively and at 10 nm in the emission pathway.

2.2.7. The Far UV-CD measurement

Far UV-CD spectra (190-260) were recorded at 25 °C with a circular dichroism spectrophotometer instrument (Aviv, Model-215 and USA) in NaPi buffer (100 mM, pH 7.4). The results are expressed as mean residue ellipticity at wavelength λ , $[\theta]_{\text{mrw}\lambda}$ ($\text{deg cm}^2 \text{ dmol}^{-1}$) which is given by the following equation:

$$[\theta]_{\text{mrw},\lambda} = \text{MRW} * \theta\lambda / 10 * d * c \quad (1)$$

where MRW stands for the mean residue weight of the peptide bond, $\theta\lambda$ is the observed ellipticity (milli degrees) at wavelength λ , d is the path length (cm), and c is the protein concentration (mg/mL) [21]. Also, $\text{MRW} = M / (N^{-1})$, where M is the molecular mass of the polypeptide chain (Da) and N is the number of amino acids of the chain. The spectrum of the buffer without protein was subtracted from each recorded spectrum. The secondary structure content was calculated with the CDNN CD spectra deconvolution software [22].

2.2.8. The ThT fluorescence study

The ThT dye demonstrates enhanced fluorescence emission upon binding to the amyloid fibrils; therefore this dye is commonly used to diagnose the protein amyloid-like entity [23]. The protein samples (0.15 mg/mL) were prepared in 100 mM NaPi buffer, pH 7.4 and the final concentration of ThT was 20 μM . The emission spectra were collected between 450-600 nm, while the excitation wavelength was fixed at 440 nm. An increase in the fluorescence emission intensity at 485 nm was taken as the indicative of amyloid fibril formation. The slit widths in the excitation and emission were 5 and 10, respectively.

2.2.9. Surface tension analyses

The surface tension analyses was done in 100 mM NaPi buffer pH 7.4 with a Krüss K100 tensiometer using Du Noüy ring method at 25 °C [24]. Prior to perform the measurements, the protein samples were diluted to a final concentration of 1 mg/mL. Also, a digital tensiometer (KRÜSS, K100) was used and careful cleaning at each solution change was done, after the O-ring had been immersed into a sample solution.

2.2.10. The electrophoresis and western blot analyses

To detect formation of high molecular weight (HMW) protein aggregates, both native poly acrylamide gel electrophoresis (PAGE) and SDS-PAGE under non-reducing and reducing conditions were performed (12% gel) [25]. In addition, the results of both non-reducing and reducing SDS-PAGE (12% gel) were compared to distinguish the involvement of disulfide bridges in formation of HMW crystallin aggregates. Ten micrograms of protein were loaded into each well of polyacrylamide gel and the protein bands were stained using Coomassie Brilliant Blue (CBB) staining method. To confirm the presence of α -Cry, the protein bands were electrophoretically transferred onto nitrocellulose membrane by wet blotting. For protein transfer, the Trans-Blot electrophoretic transfer cell (Padideh Nojen Pars, Iran) was used [26]. The membrane was incubated overnight at 4 °C in a blocking solution (5 % skimmed milk, in PBS) and then washed three times with washing buffer (0.05 % Tween 20 in PBS). The polyclonal primary antibody raised in rabbit against bovine α -Cry (Generously provided by Dr Samuel Zigler, the Johns Hopkins School of Medicine) was diluted 1:500 in PBS, containing 1% skimmed milk and the membrane incubated for 1 h with the antibody buffer. The membrane was washed three times with the washing solution to remove unbound antibody and followed by incubation for 1 h in a conjugate solution (5%

skimmed milk in PBS containing 1:2000 diluted secondary antibody). The horse radish peroxidase conjugated goat anti-rabbit IgG was used as the secondary antibody. The membrane was washed three times with the washing solution and incubated with 3, 3'-diaminobenzidine color developer until brown bands appeared. At the end, the color development was stopped by immersing the membrane in distilled water.

2.2.11. Assessment of the conformational stability

In order to measure the conformational stability of lens proteins, a fixed concentration (0.1 mg/mL) of each protein sample was incubated with various concentrations of GuHCl (0-5 M) overnight, at room temperature. Then, their fluorescence emission intensities were recorded between 310 and 400 nm, using an excitation wavelength of 295 nm. To determine denaturation process, the fluorescence intensity ratio of I_{365}/I_{335} was obtained and drawn versus concentration of GuHCl. In order to quantify the stability parameters, all denaturation profiles were analyzed with the aid of a global three-state fitting procedure, according to the following equation:

$$F = \frac{F_N + F_I \exp(-\Delta G_1^0 + m_1 [\text{GuHCl}])/RT) + F_U \exp(-\Delta G_2^0 + m_2 [\text{GuHCl}])/RT}{1 + \exp(-\Delta G_1^0 + m_1 [\text{GuHCl}])/RT) + \exp(-\Delta G_2^0 + m_2 [\text{GuHCl}])/RT)} \quad (2)$$

where F_N , F_I and F_U indicate the fluorescence ratios (I_{365}/I_{335}) for folded, intermediate and unfolded states of protein, respectively. ΔG_1^0 indicates the standard free energy changes between native and intermediate states, while ΔG_2^0 shows the standard free energy changes between intermediate and unfolded states. ΔG^0 (the sum of ΔG_1^0 and

ΔG°_2) shows the free energy changes between folded and unfolded states (free energy changes of unfolding) in the absence of GuHCl [27, 28].

The transition midpoint ($C_{1/2}$) values (M) and ΔG values (kcal/mol) for glycated and non-glycated TSPs/ α -Cry were estimated.

2.2.12. Quantification of protein concentration

The protein samples were prepared in 100 mM NaPi buffer, pH 7.4 and their concentration was measured on a T90⁺ UV-Vis spectrophotometer instrument. TSPs concentration was measured, using a standard Bradford protocol [29]. The concentration of α -Cry was determined, using an extinction coefficient of 0.85 for 1 mg/mL at 280 nm [30].

3. Results and Discussion

3.1. Analyses of non-enzymatic glycation of lens crystallins

During ageing and in diabetic patients, lens crystallins are subjected to non-enzymatic glycation and several other modifications [31]. Due to their negligible turnover, these long-lived proteins accumulate various modifications in their structures throughout the lifespan which eventually disrupt lens architecture and change the optical properties, leading to senile cataract formation [32]. In this study, both TSPs and purified sample of α -Cry were maintained in the high glucose environment (0.5 M) for two months. Purification of α -Cry was performed, using gel filtration column. The results of both purification and western both analyses are indicated in **Fig.1A**.

► **Fig.1 to be inserted here** ◀

As shown in **Fig.1B**, the color change of TSPs sample after two months exposure to D-glucose is consequence of the later steps (protein cross-linking) of protein glycation pathway. Similarly, the fluorescent yellow and brown colors seen in human cataracts could derive from the similar steps in the pathway of non-enzymatic browning of glycated crystallins. Also, native PAGE analyses and SDS-PAGE electrophoresis under non-reducing and reducing conditions for α -Cry (**Fig.1C**) and TSPs (**Fig.1D**) are shown. Due to the ability of glycation to alter charge and charge distribution in the modified proteins, native PAGE in which the electrophoretic mobility depends on both charge and hydrodynamic size of protein was performed [33]. As shown in **Figs.1C & 1D**, fresh samples of α -Cry and TSPs reveal different pattern of electrophoretic mobility shift than their respective samples that incubated for longer time, either in presence or in absence of D-glucose. Additionally, no significant variation was observed between glycated and control samples of non-glycated α -Cry/TSPs which can be explained with their intrinsic ability for oligomeric assemblies. As shown in **Fig.1C**, due to non-enzymatic glycation, the band corresponding to the α -Cry monomer has disappeared, and at the same time new bands that stand for HMW protein entities appeared in the border of stacking/separating gel and in the wells of each SDS-PAGE gel. We also investigated the nature of interactions involved in aggregation of proteins through gel electrophoresis under reducing condition. As shown, reducing condition does not change significantly the intensity of the bands, corresponding to HMW protein species, indicating formation of AGE covalent cross-linking products. As shown in this figure, α -Cry was glycated to a larger extent than TSPs. The poor glycation extent of TSPs may suggest that many of basic residues in the protein mixture do not react with D-glucose and this event could arise from the buried state of some cationic residues in the protein mixture. As shown, most of these HMW proteins can not enter the non-reducing SDS gel. Overall, glycation

of lens crystallins in the presence of D-glucose may lead to formation of both inter- and intra-subunit cross-linkings which subsequently produce HMW protein species. To further characterize the non-enzymatic modification of lens crystallins, both OPA and fluorescamine assays were performed (**Fig.1B**). OPA and fluorescamine react with free amine groups of proteins, leading to the enhancement of OPA absorption and fluorescence emission of the fluorescent dye. When D-glucose is attached to the amino group of protein, it prevents OPA and fluorescamine combination to protein and causes a reduction in the corresponding dyes signal intensity [34]. As indicated in **Fig.2**, a significant reduction in OPA/fluorescamine intensities of glycated crystallins suggests the extensive attachment of D-glucose to these proteins during non-enzymatic glycation.

► **Fig.2 to be inserted here** ◀

Reduction in OPA/fluorescamine signal intensities of the control samples compared to the fresh protein counterparts can also be explained with folding variation and subsequently inaccessibility of the target residues during long-term incubation of these proteins at 37 °C. Overall, the results of OPA and fluorescamine assays indicate a significant level of protein glycation both in TSPs and α -Cry. In this study, after *in vitro* glycation of lens crystallins, non-Trp fluorescence was performed to further clarify the modification of these proteins by D-glucose. Previous studies have shown that AGE levels are positively correlated with yellow/brown pigmentation, non-Trp fluorescence, and cross-linked aggregates in both aged and cataractous lenses [35]. As indicated in **Fig.2**, the significant increment in non-Trp fluorescence of glycated crystallins suggests the formation of new fluorophore(s) during modification of these proteins.

3.2. Structural assessment of lens crystallins upon glycation

Due to their limited turn over, long-lived proteins such as crystallins are prone to age-related accumulation of modifications which result in significant structural alteration and formation of light scattering aggregates that are observed during cataractogenesis [36]. In this study, Trp fluorescence which is an important and sensitive reporter of protein structural changes was used for the structural evaluation of lens crystallins after glycation (**Fig.3A**).

► **Fig.3 to be inserted here** ◄

As shown, Trp fluorescence of modified crystallins was decreased in the presence of D-glucose, suggesting that upon protein modification, these residues move to more polar environment. These observations can be explained with the structural variation of lens crystallins which occurs during glycation. Additionally, in the case of TSPs, the fluorescence changes may suggest variation in the pattern of fine interactions which normally exist among different classes of lens crystallins [37]. As reported earlier, these short range interactions, among lens crystallin subunits, play important role in the proper function of lens to focus the light rays onto the back of the eye (retina) [38]. For further investigation of the effect of glycation on structure of lens crystallins, ANS fluorescence assessment was done to characterize the solvent exposed hydrophobic patches. As shown in **Fig.3A**, a significant reduction in ANS fluorescence emission intensity of lens proteins upon glycation indicates a substantial decrease in their solvent exposed hydrophobic patches. These results can be explained with either shielding of hydrophobic regions from the aqueous solvent and/or structural alteration of these proteins during glycation. Since, surface hydrophobicity plays a significant role in the protein aggregation propensity, its reduction may substantially reduce propensity of these

proteins for self-aggregation. The interaction between hydrophobic patches on chaperone's surface and exposed hydrophobic sites of partially unfolded target protein is of highly importance for the suppression of target protein aggregation. Significant reduction in the surface hydrophobicity of glycated α -Cry, as indicated in this study, may explain the reduction of its chaperoning activity which has been indicated in the previous study to occur as a result of glycation [39]. However, according to literature a quantifiable relationship between hydrophobicity and chaperone activity of α -Cry is not always the case.

Surface tension which is an important feature of protein hydrophobicity at the air/water interface has been already used to determine the protein structural stability and conformation [40]. In this study, the surface tension of lens crystallins before and after glycation was measured and compared. As shown in this study, the increment in the surface tension of the protein samples (**Fig.3B**) was accompanied with the reduction in solvent exposed hydrophobic patches of these proteins (**Fig.3A**) [41]. Our results are in agreement with previous studies suggesting an inverse correlation between the surface tension and surface hydrophobicity of protein [19]. Also in this study, we collected the CD spectra of lens crystallins in the far UV region which is indicative of protein's secondary structures (**Fig.3C**).

The peptide chains of lens crystallins are mainly made up of the antiparallel β -pleated sheets, reflected by the presence of a negative CD band centered at 218 nm [42]. As shown in **Fig.3C**, while glycation of lens α -Cry results in significant increment in the ellipticity value of this band, only a little variation was observed between CD signal intensities of glycated and non-glycated TSPs. In fact, circular dichroism spectroscopy revealed that glycation increases β -sheet content of α -Cry to a significant level which is in agreement with the result of previous report [43]. Due to non-enzymatic glycation,

while beta-sheet content of α -Cry increases from 34.45 to 40.36; the alpha-helix content of this protein indicates an important reduction from 9.05 to 5.92. The small changes in of CD signal of TSPs after non-enzymatic glycation can also be explained with different impacts of this modification on structure of individual protein in the mixture of various crystallins.

3.3. Aggregation propensity of the glycated and non-glycated lens crystallins under stress conditions

As previously indicated, during aging and cataractogenesis, the structural proteins of human lens indicate an increased amount of aggregation [44]. Over the years, as a result of non-enzymatic modifications, the partially denatured crystallins form HMW aggregates with ability to scatter light. In this study, the aggregation propensity of glycated and non-glycated lens crystallins was assessed in both thermal and thermochemical systems (**Fig.4A**). While in the thermal system, the protein samples were incubated at 60 °C, in the thermochemical system they were incubated with either 1 M GuHCl or TFE (10%) at 60 °C. The incubation time was 2h and the protein concentration adjusted at 1 mg/mL. Before collecting the UV-Vis absorption spectra, the protein sample was diluted to 0.15 mg/mL and the absorption spectra were collected in the range of 200-600 nm. The increment of absorbance at 360 nm was also used as an indication of the protein aggregation. As shown in **Fig.4A**, neither glycated nor fresh/non-glycated TSPs indicate signal of aggregation when they were incubated under only thermal stress.

► Fig.4 to be inserted here ◀

α -Cry which accounts for approximately 60% of the soluble lens proteins has been already indicated to possess temperature-dependent chaperone activity, acting as stronger chaperone at the elevated temperatures [45]. Thus, absence of protein aggregation under

such stress condition can be explained with the improved chaperoning action of α -Cry, preventing strongly the aggregation of substrate proteins. However, in the thermochemical system with either GuHCl or TFE, while non-glycated TSPs get simply aggregated, glycated protein counterparts demonstrate significant resistance against aggregation, indicating that a combination of thermal and chemical stresses would need to overcome the thermodynamic barrier preserving the structure/folding state of glycated crystallins. This figure also shows aggregation propensity of the glycated and non-glycated α -Cry under similar stress systems. As shown, both glycated and non-glycated α -Cry resist against aggregation under thermal- and thermochemical system, as the later contains chemical denaturant agent, GuHCl. Aggregation of α -Cry was observed only in the thermochemical system containing TFE and in this system glycated protein counterpart markedly resists against stress-induced aggregation. The inability of the stress system containing GuHCl to induce aggregation of α -Cry can be due to the specific mode of denaturing effect of this chemical agent [46]. According to data of sequence modeling and physical analyses, the secondary structure of small heat shock/ α -Cry proteins is predominately beta-pleated sheet. While, GuHCl is a strong chaotrope, disrupting ordered structure of proteins, TFE induces helix formation [47]. Therefore, the results can be explained with the specific mode of action of these two denaturant agents on the ordered structure of α -Cry. Also, the different patterns of aggregation which observed for TSPs and α -Cry under the above mentioned stress systems can be explained with the dissimilar chaperoning action of α -Cry in the crude and pure protein samples. Additionally, the significant resistance of glycated TSPs/ α -Cry against the above mentioned stresses can be explained with the increment of their stability upon glycation. This observation is in agreement with the results of previous study suggesting that glycation in general improves the conformational stability of protein [43].

3.4. ThT fluorescence assessment of the glycated and non-glycated lens crystallins under stress conditions

Cataract is often characterized by the gradual progressive destabilization of lens crystallins and their self-assembly to aggregates or to form amyloid-like fibrils. In this study the formation of amyloid-like entities was studied using benzothiazole dye ThT as a sensitive probe in detecting the amyloid fibrils [48]. The values of maximum emission intensity ($F_{\max}$) were used to compare the ThT fluorescence emission properties of different protein samples. As shown in **Fig.4B**, the ThT fluorescence emissions of TSPs were increased as a result of incubation under thermal and thermochemical incubation systems. The results may suggest that these stresses are capable to induce formation of amyloid fibril entities in the lens proteins. As shown, under different stress conditions, the glycated TSPs demonstrated lower ThT fluorescence emissions than other protein counterpart samples; suggesting that this modification results in partial resistance of the lens proteins against stress-induced amyloidogenesis (**Fig.4B**). Also, the ThT fluorescence emission intensities of different samples of α -Cry are displayed in **Fig.4B**. As shown, no significant variation can be seen between ThT emission intensity of fresh, control and glycated α -Cry before and after incubation under thermal- and thermochemical system. However when these protein samples were treated with a combination of thermal stress and TFE, the values of ThT fluorescence emission intensity were enhanced to the significant levels (**Fig.4B**). The ThT fluorescence emission intensity was increased for the α -Cry samples which incubated for 2 h under this thermochemical system. As shown in this figure, under different stress conditions, the glycated form of α -Cry demonstrates significantly lower ThT fluorescence emission, possibly reflecting the fact that glycation causes α -Cry to resist against stress-induced formation of amyloid-like entities.

3.5. ANS and far UV-CD analyses of the glycated and non-glycated lens crystallins under stress conditions

Since solvent exposed hydrophobic surface plays an important role in aggregation/fibrillation propensity of proteins, in this study the ANS fluorescence measurements were applied for the estimation of hydrophobic surfaces of different crystallin samples which incubated under the thermochemical stresses, containing either GuHCl or TFE as the chemical denaturant agents. As shown in **Fig.5**, owing to incubation under stress condition (60 °C plus 1 M GuHCl), different samples of either TSPs or α -Cry demonstrated significant increment in their ANS fluorescence intensities. These results reflect the fact that thermochemical stress is capable to enhance the protein's surface hydrophobicity which is in well agreement with their aggregation propensity as discussed above.

► **Fig.5 to be inserted here** ◄

Additionally, under the stress condition, glycated forms of TSPs and α -Cry expose the minimum amount of hydrophobic surface to the aqueous solvent compared to their corresponding non-glycated protein counterparts. As the experiments were repeated under other thermochemical system (60 °C plus TFE), similar results were observed for different samples of TSPs. However under this condition, the α -Cry samples indicated approximately similar values of ANS fluorescence intensity before and after treating with this type of stress. In conclusion, non-enzymatic glycation partially prevents stress-induced exposure of the hydrophobic surfaces in TSPs and α -Cry. Also, far UV-CD spectra were collected for different samples of lens crystallins which were incubated under these thermochemical systems (**Fig.6A**). As shown, the stress systems containing GuHCl and TFE are respectively capable to perturb the ordered structures of TSPs and α -Cry protein samples.

► **Fig.6 to be inserted here** ◄

As shown, a combination of thermal stress and GuHCl was capable to significantly reduce the CD signals around 218 nm in both glycosylated and non-glycosylated TSPs, suggesting the reduction of β -sheet contents in these proteins. However, as shown in this figure, the reduction of β -sheet contents is less significant in the case of glycosylated TSPs compared to non-glycosylated protein counterparts. As indicated in **Fig.6A**, a combination of thermal stress and TFE was capable to induce significant structural alteration both in glycosylated and non-glycosylated α -Cry. The extent of alteration was significantly higher in the case of non-glycosylated proteins compared to glycosylated α -Cry counterparts. Overall, the results of this study notify that glycosylation partially prevents the perturbation of ordered structures of lens crystallins under the stress conditions.

3.6. Comparing stability of the glycosylated and non-glycosylated forms of TSPs and α -Cry

Glycosylation has been already indicated to increase conformational stability against chemically induced denaturation [43]. In order to determine the impact of glycosylation on structural stability of TSPs and α -Cry, the conformational stability of different crystallin samples was investigated, using chemical unfolding method. The unfolding was estimated by monitoring the intrinsic Trp fluorescence intensity of these protein samples at various GuHCl concentrations. The ratio of fluorescence emission intensities (I_{365}/I_{335}) was plotted against GuHCl concentration (**Fig.6B**). The intensity values of I_{365} and I_{335} represent the λ_{max} intensities at complete unfolded and complete folded states of proteins, respectively. The denaturation profiles were extracted from the sigmoidal unfolding curve and data were indicated as **Table 1**.

► **Table 1 to be inserted here** ◀

Assuming a three-state protein folding performance, the midpoint of denaturation is defined as denaturant concentration at which both folded and unfolded states of protein

are equally populated at equilibrium [49]. According to the parameters calculated from the chemical denaturation curves (**Table 1**), the transition midpoint ($C_{1/2}$) values of glycosylated crystallins need a higher GuHCl concentration than the unmodified protein counterparts to denature. The transition midpoints for glycosylated and non-glycosylated TSPs were 2.72 and 2.03 M, respectively. Accordingly, these values for glycosylated and non-glycosylated α -Cry were 2.21 and 1.63 M. The higher level of GuHCl which is needed for the chemical unfolding of glycosylated crystallins can suggest that this type of modification would improve the ability of the lens proteins to resist against stress-induced unfolding. Also in this study, as an index of protein conformational stability, the value of free energy (ΔG) at 25 °C in the absence of GuHCl (denaturant), ΔG_{H_2O} , was obtained. As shown in **Table 1**, the values of ΔG_{H_2O} for GuHCl induced denaturation of glycosylated and non-glycosylated TSPs were 6.26 and 4.07 Kcal mol⁻¹, respectively. Accordingly, the values of ΔG_{H_2O} for glycosylated and non-glycosylated α -Cry were 5.30 and 3.82 Kcal mol⁻¹. These data suggest that glycosylation can increase the conformational stability of lens crystallins against chemical denaturation. Glycosylation can, in principle, stabilize proteins by affecting their underlying energy landscapes [50]. According to the results of previous studies the stabilization by non-enzymatic glycosylation is due to the formation of new interactions between the sugars and the amino acids in the native state. The increased conformational stability of lens crystallins upon glycosylation can explain the reason behind resistance of these proteins against stress-induced structural alteration and aggregation.

Conclusion

In the context of diabetes, the most important causative players are formation of advanced glycation end-products and generation of reactive oxygen species which are intensified during chronic hyperglycemia. In this study, as α -Cry and TSPs were subjected to the extensive non-enzymatic glycosylation, they demonstrated significant

structural alteration which was accompanied with the reduction in their solvent exposed hydrophobic patches and indicated an important increment in the surface tension. Additionally, glycated proteins displayed larger conformational stability than non-glycated protein counterparts. Also, as incubated under different physicochemical stresses, glycated proteins demonstrated significant resistance against stress-induced structural alteration/aggregation. Therefore, the impact of glycation of lens crystallins on development of senile cataracts can be explained with the effect of this modification on structure and short-range interactions among lens crystallins and attenuation of α -Cry chaperone activity, leading to reduction of protection mechanism of lens against aggregation of other proteins. Additionally, the over-production of ROS during chronic hyperglycemia can directly damages lenticular proteins and attenuates the antioxidant defense system which is necessary in proper function of the eye lens.

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Conflict of interest

The authors certify that there is no conflict of interest relevant to this article to declare.

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Table 1: Free energy and transition midpoint of GuHCl denaturation of glycated (G) and non-glycated (N) α -Cry/TSPs.

Protein	ΔG_D (H ₂ O)	[GuHcl] _{1/2}
	Kcal mol ⁻¹	M
TSPs (G)	6.26	2.72
TSPs (N)	4.07	2.03
α -Cry (G)	5.30	2.21
α -Cry (N)	3.82	1.63

The denaturation profiles were analyzed with the aid of a global three-state fitting procedure.

Figure legends

Scheme 1. Activation of multiple pathogenic ROS-generating pathways under chronic hyperglycemia.

Under chronic hyperglycemia and inflammation the mitochondrial overproduction of superoxide radical causes the nuclear DNA strand break which subsequently results in activation of PARP-1 enzyme. This enzyme modifies the glycolytic enzyme GAPDH which possesses a reversible nucleo-cytoplasmic translocation. Accordingly, accumulation of the upstream glycolytic metabolites results in activation of multiple pathogenic and ROS-generating pathways including polyol pathway, protein kinase C pathway, hexosamine pathway and AGE pathway.

Fig.1. Electrophoresis and western-blot analyses of lens crystallins.

A. Electrophoresis and western-blot analyses of bovine lens crystallins. Lanes **1** and **2**, respectively stand for TSPs and α -Cry. **B.** Browning of lens crystallins (TSPs) as result of incubation for two months in the presence of 0.5 M D-glucose. **C&D** respectively indicate the gel electrophoretic analyses of α -Cry and TSPs as a result of glycation. The experiments were performed under native condition, and with non-reducing and reducing SDS-PAGEs. While "m" stands for the molecular mass markers, **1**, **2** and **3**, respectively demonstrate fresh protein, non-glycated protein sample and glycated protein counterpart. The electrophoresis experiments were done with a 12% polyacrylamide gel and a 10 μ g protein sample was applied into each well of the electrophoresis gels. At the end, the vitalization of protein bands was done using CBB staining protocol.

Fig.2. Spectroscopic assessment of the bovine lens crystallins glycation

A. Fluorescamine and OPA analyses were done to measure the free amine groups in lens crystallins upon glycation. To perform fluorescamine test, the protein sample (0.15 mg/mL) was mixed in an appropriate concentration with fluorescamine reagent (1 mM in acetonitrile). The excitation/emission wavelengths were 390/490 nm and the slit widths fixed at 10/10 nm in the both channels. The OPA assessments (inset figures) were done with measuring the absorbance of OPA/protein solution at 340 nm. **B.** The non-Trp fluorescence assessments of different crystallin samples were done. Prior to the measurements, the protein sample was diluted to 0.15 mg/mL and the excitation performed at 370 nm. The excitation/emission slit widths were also set at 10 nm. All experiments were done in 100 mM NaPi buffer (pH 7.4). Moreover F, N and G respectively stand for fresh protein sample, non-glycated and glycated crystallin samples.

Fig.3. Fluorescence, surface tension and far UV-CD analyses of lens crystallins glycation.

A. Trp and ANS fluorescence analyses were done as the protein samples were individually diluted to 0.15 mg/mL. To perform Trp and ANS fluorescence analyses, the excitation wavelengths were set at 295 and 365 nm, and the emission spectra were collected in the indicated ranges. **B.** The surface tension assessment of different lens crystallin samples was done. Prior to the measurements, the protein samples were individually diluted to a final concentration of 1 mg/mL. Also, the data represent the average of decuple measurements. **C.** The far UV-CD spectra were collected for glycated and non-glycated samples as the protein concentration was fixed at 0.2 mg/mL. All these measurements were done in 100 mM NaPi buffer, pH 7.4. Also **F**, **N** and **G** respectively stand for fresh protein, non-glycated and glycated crystallins.

Fig.4. Assessment of aggregation of lens crystallins in the thermal and thermochemical systems

The individual protein sample (1 mg/mL) was incubated under different stress conditions, including thermal stress at 60 °C, thermal stress plus GuHCl (1M) and thermal stress plus TFE (10% v/v). The incubations were done for two hour in 100 mM NaPi buffer, pH 7.4. **A.** The protein samples were diluted to 0.15 mg/mL and their absorption spectra were collected. The absorption intensity at 360 nm was used as indication of the protein aggregation. **B.** The protein samples were diluted to 0.15 mg/mL with same buffer in the presence of 20 µM ThT fluorescence dye. While the protein samples were excited at 440 nm, the emission spectra were collected. The slit widths in the excitation and emission were also set at 5 and 10 nm, respectively. **F, N** and **G** respectively stand for the fresh protein sample, non-glycated and glycated protein samples.

Fig.5. ANS fluorescence study of glycated and non-glycated crystallins after incubation under thermal/chemical stresses

The protein samples (1mg/ml) were incubated with either GuHCl (1M) or TFE (10%v/v) at 60 °C for 2h. At the end, the protein samples were diluted with the same buffer (100 mM NaPi buffer, pH 7.4) to 0.15 mg/mL. The excitation wavelengths of protein/ANS complex occurred at 365 nm and the emission spectra were collected at the indicated ranges. While, **A** and **B** show before and after incubation; **F, N** and **G** respectively stand for fresh protein, non-glycated and glycated crystallin samples.

Fig.6. Far UV-CD and stability analyses of lens crystallins treated with the thermal/thermochemical stresses.

A. The protein samples (1 mg/mL) were incubated in 100 mM NaPi buffer, pH 7.4 under different stress conditions, including thermal stress plus GuHCl (1M) and thermal stress plus TFE (10% v/v). At the end, the protein samples were diluted to 0.2 mg/mL in the same buffer and their spectra were collected in the far region. B. Each protein sample (0.1 mg/mL) was incubated with different concentrations of GuHCl (0-5 M) for 24 h in 100 mM NaPi buffer, pH 7.4. At the end, the protein samples were diluted to 0.15 mg/mL in the same buffer and then Trp fluorescence emission were collected in a similar way as described earlier. The results were presented as the ratio of fluorescence intensity of 365/335 nm versus GuHCl concentration. The denaturation parameters were obtained according to a three-state fitting procedure and the obtained data were presented as **Table 1**.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Graphical abstract

The assessment of structure, stability and aggregation of soluble lens proteins and alpha-crystallin upon non-enzymatic glycosylation: The pathomechanisms underlying cataract development in diabetic patients.

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