**Preventive role of lens antioxidant defense mechanism against riboflavin-mediated sunlight
damaging of lens crystallins**

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Abstract

The main components of sunlight reaching the eye lens are UVA and visible light exerting their photo-damaging effects indirectly by the aid of endogenous photosensitizer molecules such as riboflavin (RF). In this study, lens proteins solutions were incubated with RF and exposed to the sunlight. Then, gel mobility shift analysis and different spectroscopic assessments were applied to examine the structural damaging effects of solar radiation on these proteins. Exposure of lens proteins to direct sunlight, in the presence of RF, leads to marked structural crosslinking, oligomerization and proteolytic instability. These structural damages were also accompanied with reduction in the emission fluorescence of Trp and Tyr and appearance of a new absorption peak between 300-400 nm which can be related to formation of new chromophores. Also, photo-oxidation of lens crystallins increases their oligomeric size distribution as examined by dynamic light scattering analysis. The above mentioned structural insults, as potential sources of sunlight-induced senile cataract and blindness, were significantly attenuated in the presence of ascorbic acid and glutathione which are two important components of lens antioxidant defense system. Therefore, the powerful antioxidant defense mechanism of eye lens is an important barrier against molecular photo-damaging effects of solar radiations during the life span.

Keywords: Lens crystallins, Sunlight, Riboflavin, Photosensitizer, Oligomerization, Lens antioxidant system.

1. Introduction

Crystallins (α , β and γ) comprise the main component of soluble lens proteins, indicating negligible turnover during the human lifespan [1]. These extremely concentrated proteins constitute a transparent matrix with highly refractive index in the lens fiber cells [2]. A variety of internal and external stresses may influence both native folding and fine short-range order interactions existing among different crystallin subunits, causing formation of the insoluble protein aggregates which subsequently interfere with the proper transmission of light to the retina [3-5]. The eye lenses are continuously subjected to the oxidative stress due to their daily exposure to the sunlight [6, 7]. The damaging effect of ultraviolet (UV) radiation on eye lens has been already well documented [8, 9]. The UV wavelengths of sunlight reaching the eye's surface are UVA (315-400 nm) and UVB (280-315 nm), while the shorter wavelengths (UVC) being completely absorbed by the ozone layer [10]. Some wavelengths in the UVB region are also blocked by the cornea [11, 12]. The photo-oxidation of lens crystallins may occur directly due to the absorption of UVB light by the aromatic residues of these proteins [13]. It can also occurs through sensitization reactions by means of the absorption of either visible light or UV radiation, resulting in their oxidative damages [14]. Riboflavin (RF) is one of the most significant endogenous photo sensitizers existing either in free form or as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in body cells [15, 16]. RF indicates intensive absorption in both UV region and visible light that subsequently cause its excitation and conversion to the triplet state ($^3\text{RF}^*$) (**Scheme 1**) [17]. The photodynamic action of $^3\text{RF}^*$ is generally considered to contribute in the generation of reactive oxygen species (R° , $^\circ\text{OH}$, H_2O_2 , O_2°) via either type-I mechanism (electron transfer) or production of singlet oxygen ($^1\text{O}_2$) with the aid of type-II mechanism (energy transfer) [18-20].

◀Scheme 1 to be inserted here▶

These reactions can mediate the oxidative damages of the cellular components (DNA and protein) especially in tissues like skin and lens which are exposed to sunlight [21-23]. Under these conditions, the eye lens crystallins are appreciably damaged due to oxidation of their sensitive amino acids (Trp, Tyr, Cys, Met and His), leading to protein crosslinking and other structural insults [24-27].

The eye lens has a number of important antioxidant components including glutathione (GSH), ascorbic acid (ASA) and some antioxidant enzymes which form a powerful endogenous defense mechanism against a variety of oxidative damages [28, 29]. The destructive effect of ROS on eye lens crystallins can be prevented by natural antioxidant compounds such as ASA and GSH. ASA is available at high concentration (up to 3 mM) in the normal lens and acts as a hydroxyl and super oxide anion radical scavenger [30]. Also, the high content of GSH in human lens (2-4 mM) can protect sulfhydryl groups of lens proteins from oxidative damages [31]. Under normal condition, the toxic effects of free radicals are well neutralized by the action of antioxidant defense system (**Scheme 1**). However, when levels of the antioxidant components are decreased or as oxidative stress overcomes the antioxidant defense capacity, the oxidative damages to the lens crystallins result in lens opacification and cataract development [32]. In the current study, we investigated, for the first time, the structural damages of eye lens crystallins upon exposure to the direct sunlight in the presence of RF, using different spectroscopic techniques and gel mobility shift assay. Our results indicated that exposure to the sunlight leads to the marked crosslinking, oligomerization and slight proteolytic instability of lens crystallins. Our results also suggest the importance of natural antioxidant defense mechanism against RF-mediated photo-damaging of lens proteins.

2. Materials and methods

2.1. Materials

Riboflavin (RF), 1-anilino-8-naphthalene sulfonate (ANS), Thioflavin T (ThT), β -mercaptoethanol (β -ME), 5, 5'-Dithio bis (2-nitrobenzoic acid) (DTNB), chymotrypsin (EC 232-671-2, 59.3 units.mg⁻¹ protein) and other chemicals were purchased from Sigma Chemical Company.

2.2. Methods

2.2.1. Preparation of bovine soluble lens proteins

The total soluble lens proteins (TSPs) were prepared according to our previous publication [33]. Briefly, the bovine eye 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 15300 g for 30 min at 4°C and the supernatant collected as TSPs. Then, the lens proteins were dialyzed against double distilled water (ddH₂O) and stored at -20°C until use.

2.2.2. Incubation of lens proteins under sunlight in the presence of RF as photosensitizer

Lens proteins solution (2 mg/ml) were treated with different concentrations of RF and then exposed to the sunlight. The control protein sample lacking the photosensitizing agent was also incubated under similar experimental condition. Additionally, a protein sample containing

50 μ M RF was kept in the dark. All protein solutions were left under direct sunlight exposure. During incubations, the UV index, according to standard indicator of UV level by World Meteorological Organization and World Health Organization [34], was about 5-6 and temperature varied between 10-15°C. Also, the perceived power of light (LT) per unit area was about 40000 lux (316 W/m²) [35]. In all experiments, the incubations were done in the glass bottles blocking the majority of UVB and allow the transmission of UVA and visible light to the protein sample [36, 37]. Also, the absorption and transmission spectra of the glass bottles were assessed to assure possessing the above mentioned standard (**Fig. 1**). To assess the inhibitory effect of natural antioxidants, the protein samples containing RF (50 μ M) were individually incubated with ASA and GSH. Also, to evaluate the impact of oxygen on the RF-mediated photo-damaging of lens proteins, different protein samples containing 50 μ M RF were incubated at low oxygen tension. To achieve low oxygen condition, protein samples were bubbled with gaseous nitrogen for 10 min [38]. All solutions were prepared in NaPi buffer (100 mM, pH 7.4). At the end of incubations, the protein samples were extensively dialyzed against this buffer and kept at 4°C.

2.2.3. Fluorescence assessments

Fluorescence analysis of the protein solutions was done in NaPi buffer (100 mM, pH 7.4) using a Cary-Eclipse spectrofluorometer (Varian, Australia). The protein samples were diluted to 0.15 mg/ml prior to perform fluorescence assay. Tyr and Trp fluorescence emission spectra of lens proteins solution were recorded between 300-500 nm with the excitation wavelengths fixed at 280 and 295 nm, respectively. Additionally, ANS fluorescence assessment was performed to study the surface hydrophobicity of different protein samples. The protein samples (0.15 mg/ml)

were incubated with 100 μ M ANS for 30 min in the dark. The changes in emission spectra were collected between 400-600 nm with the excitation wavelength fixed at 365 nm. In order to evaluate the formation of amyloid fibrils, native and modified proteins were treated with ThT (20 μ M) at room temperature for 5 min. In ThT fluorescence analysis, the excitation wavelength was fixed at 440 nm and the emission spectra were obtained between 450-600 nm. The excitation/emission slit widths were set at 10/10 nm for Trp, ANS and ThT fluorescence and 5/10 for Tyr fluorescence measurements [39, 40].

2.2.4. Quantification of free sulfhydryl groups

The protein samples were prepared in NaPi buffer (50 mM, pH 7.4), containing 1 mM EDTA. The protein solutions at a final concentration of 0.4 mg/ml were incubated with or without SDS (2%) for 30 min. DTNB solution was added to samples to yield a protein to DTNB molar ratio of 1:7 and incubated for an additional 90 min in the dark. Quantification of free sulfhydryl groups was done by measuring the absorbance at 412 nm using a Cecil CE 7200 spectrophotometer (Cecil Instruments Ltd., UK). A molar extinction coefficient of $14150 \text{ M}^{-1} \text{ cm}^{-1}$ for the thionitrophenylate anion at 412 nm was used to calculate the amount of free sulfhydryl groups of different protein samples [41].

2.2.5. Proteolysis study

The native and modified lens proteins which incubated in the presence and absence of antioxidants were treated with α -chymotrypsin (59.3 units/mg protein) at a ratio of 50:1 (w/w). All experiments were performed in Tris buffer (20 mM, pH 7.8) containing 100 mM NaCl at

37°C for 3 h. Finally, the proteolytic mixture was boiled at 100°C for 10 min in order to stop the enzymatic activity. The samples (15 µg in each well) were analyzed by SDS-PAGE under reducing conditions on a 12% polyacrylamide gel which followed by a Coomassie Brilliant Blue (CBB) staining protocol [42].

2.2.6. Optical density measurement

In this study, to evaluate the unfolding and aggregation of lens proteins, a Cecil CE 7200 spectrophotometer (Cecil Instruments Ltd., UK) instrument was used. The protein samples were diluted to 0.4 mg/ml in 100 mM NaPi buffer and the change of absorption spectra was recorded between 200-700 nm. In order to distinguish between aggregation and chromophore formation, the modified protein samples were centrifuged at 10000 ×g for 20 min to remove any existed protein aggregates [43]. Then, the absorption spectra were recorded between 200-700 nm.

2.2.7. Gel electrophoresis experiment

Lens crystallins (2 mg/ml) were incubated with a fixed concentration of RF (50 µM) for different times (0.5-4 h) or with different concentrations of this photosensitizing agent (3-50 µM) for 3 h. The experiments were done in NaPi buffer (pH 7.4) under direct sunlight exposure. Then, a 15 µg of each protein sample was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing and reducing conditions on a 12% polyacrylamide separating gel. The protein bands were visualized by a CBB staining method [44].

2.2.8. Dynamic light scattering analysis

Dynamic light scattering (DLS) experiments were used to determine the oligomeric size of different protein samples which carried out with a Nanotracer Wave (Microtrac) dynamic light scattering instrument. The experiments were done at 25°C using a laser wavelength of 780 nm and scattering angle of 90°. DLS data were analyzed by Microtrac FLEX operating software [45].

2.2.10. Protein assay

The concentration of different protein samples were determined according to a standard Bradford protocol using Cecil CE 7200 spectrophotometer instrument [46].

2.2.11. Data analysis

Data were analyzed by Sigma Plot 12.0 software. The differences between multiple groups were compared using parametric one-way ANOVA, followed by Tukey's *post-hoc* test [41]. The differences were considered statistically significant when $p < 0.05$

3. Results

3.1. The photo-induced oligomerization of lens crystallins after direct exposure to sun light

In this study the lens proteins were incubated, under direct sun light, in the glass bottles which are capable to block the majority of UVB and allow the transmission of UVA and visible light [36, 37]. Also, the absorption and transmission spectra of the glass bottles were assessed (Fig. 1).

◀Fig. 1 to be inserted here▶

As shown, the glass bottles indicate an important transmission ability in the regions of UVA and visible light. The gel mobility shift assay under reducing and non-reducing conditions was applied to examine formation of HMW protein species upon direct exposure of lens proteins solution to sun light. As shown in Fig. 2A₁, incubation of lens proteins with 50 μ M RF leads to protein crosslinking and oligomerization in a time-dependent manner.

◀Fig. 2 to be inserted here▶

The formation of HMW protein oligomers were also accompanied with disappearance of the bands corresponding to the native proteins. In addition, as the incubation proceed for 2 hours, due to the size limits, a major quantity of HMW protein oligomers was retained either in the well of electrophoresis gel, or in the border of staking and resolving gels. The solutions of lens proteins which incubated in the absence of RF and under direct exposure to the sun light or even those protein samples treated with RF and kept in the dark did not show significant changes in the electrophoresis pattern. The effect of RF concentration on the photo-induced oligomerization of lens proteins was also examined (Fig. 2A₂). Lens proteins which treated with various concentrations of RF (3-50 μ M) were incubated under sun light for three hours. As indicated in Fig. 2A₂, a significant amount of photo-induced protein crosslinking was seen even at the lowest

concentration of RF which is slightly higher than its physiological level in human eye lens (4.5 μM) [17]. When the RF levels reach 20 μM and higher, the photo-oxidation of lens proteins leads to formation of larger oligomers, retaining in the wells of electrophoresis gel. The reducing SDS-PAGE was also applied (**Fig. 2A₃**) with the aim to assess the possible participation of disulfide crosslinking in photo-induced oligomerization of lens proteins. The decrease in intensity of the protein bands retained in the wells of reducing SDS-PAGE was accompanied with restoring the intensity of the native proteins. These results suggest that photosensitized reaction of RF induces oligomerization of lens proteins mainly by non-disulfide covalent crosslinking and to some extent by disulfide bonds. The RF-mediated photo-oxidation of proteins (Type-II reaction) also occurs in an oxygen dependent reaction (**Scheme 1**). To evaluate the effect of oxygen, protein samples containing 50 μM RF were irradiated under normal (atmospheric) and low oxygen conditions. As shown in **Fig. 2B₁**, RF-mediated protein oligomerization occurs more intensively under normal oxygen than at low oxygen level. The eye lens has a well-designed antioxidant defense system against oxidative stress [47]. In order to evaluate the preventive capacity of lens antioxidant defense system against photo-damaging effects of sun light, the protein samples treated with 50 μM RF were incubated in the presence of various concentrations of ASA and GSH for 3 h (**Figs. 2B₂-2B₃**). As shown, the intensity of HMW protein bands formed by the photo-damaging of lens proteins was diminished gradually by increasing the concentration of ASA (**Fig. 2B₂**) and GSH (**Fig. 2B₃**). In this study ASA and GSH indicate significant protective capacity against photo-induced protein oligomerization. Our results indicate that ASA and GSH with a concentration of 10 mM display the most promising inhibitory effect against photo-damaging of lens proteins as they were exposed to direct sun

light. Moreover, treatment of lens crystallins with a combination mixture of ASA and GSH did not show significant improvement compared to the presence of ASA or GSH alone (**Fig. 3A**).

3.2. The exposure of lens crystallins to sunlight leads to significant reduction of their free sulfhydryl contents

Disulfide crosslinking of lens proteins, leading to protein aggregation and insolubilization, has been indicated in the pathogenesis of cataract diseases [48]. Photo-oxidation by UV light has been already reported to induce disulfide-mediated protein oligomerization [49]. To estimate the free sulfhydryl groups in lens proteins, DTNB assay was applied [41]. The estimation was done in the absence and presence of SDS (2%) with the aim to measure the sulfhydryl contents in folded and unfolded states of lens proteins, respectively.

◀Fig. 3 to be inserted here▶

As shown in **Fig. 3B**, no significant changes were observed in sulfhydryl content of the native protein and samples which incubated in dark with RF or under sun light without RF. Incubation of lens proteins with RF (50 μ M) under sunlight for 3 hours results in the marked reduction of their sulfhydryl contents. Also, in the presence of ASA or GSH, a significant increase in the content of the protein sulfhydryl groups was observed. Additionally, ASA (10 mM) protects free sulfhydryl groups from oxidation more effectively than either GSH or a combined mixture of both antioxidants. The content of sulfhydryl groups in the protein solutions incubated with a combined mixture of both antioxidants was not altered compared to the samples incubated with GSH. Our results suggest that ASA and GSH, two important antioxidant components of eye lens, significantly protect sulfhydryl groups of lens proteins against photo-

oxidation under direct exposure to sun light. In addition, upon treatment with SDS, the level of sulfhydryl groups in most protein samples was significantly increased. This observation suggests exposure of buried sulfhydryls upon SDS mediated unfolding of lens proteins, indicating their increased accessibility for the reaction with DTNB. In the absence of antioxidant, when lens proteins were treated with 50 μ M RF under sunlight exposure for 3 hours, no significant changes were observed between sulfhydryl contents of lens proteins in their folded and unfolded states. These findings suggest that exposure to direct sun light can oxidize both buried and exposed sulfhydryl groups in these proteins.

3.3. Analysis of the oligomeric size distribution of lens crystallins by DLS

In the current study, DLS analysis was used to assess the changes in the oligomeric size distribution of lens proteins upon exposure to direct sunlight in the presence of lens antioxidant components [50]. Lens crystallins populate a range of inter-converting oligomeric states which may influence their structural and functional features. The results of DLS measurement are indicated in **Fig. 4**.

◀Fig. 4 to be inserted here▶

The relative intensity versus size (inset figure) suggests at least two populations for the lens proteins. Also, based on their volumes, the native proteins demonstrate one major population with the average size diameter of 18.03 ± 0.84 nm. In addition, lens proteins that incubated without RF under sun light or with RF in the dark indicate the oligomeric forms with the average sizes of 20.09 ± 0.58 and 20.90 ± 0.29 , respectively. In the presence of RF, the average diameter of this population was increased to 42.63 ± 0.60 nm upon exposure to direct

sun light. However, the size was significantly reduced in the presence of lens antioxidant compounds. The average diameters of this population was reduced to 22.42 ± 1.03 nm, 18.58 ± 0.50 nm and 19.31 ± 0.14 nm in the presence of ASA, GSH and ASH/GSH mixture, respectively. Overall, our results suggest that exposure to direct sun light leads to the significant increment in average size of lens crystallins. The photo-induced size enhancement of lens crystallins was effectively prevented by ASA and GSH. These results are in well agreement with the results of gel mobility shift analysis as indicated in **Fig. 2**.

3.4. UV-Vis spectroscopic assessment of photo-damaging of crystallins in the presence of lens antioxidants

Crosslinking and aggregation of eye lens crystallins upon exposure to the UV irradiation has been already indicated [51]. In order to monitor the structural changes, unfolding and aggregation of lens proteins, UV-Vis spectroscopic analysis was applied. The exposure of lens proteins solution to direct sunlight resulted in formation of new peak, insensitive to the high speed centrifugation, at the wavelength region of 300-400 nm. Therefore, this peak can be related to the formation of chromophores whose absorptions were gradually enhanced as a function of time of exposure to direct sun light (**Fig. 5A₁**). Also, the inset figures demonstrate the changes in the optical density at 322 nm which is related to a Trp derived chromophore (N-formyl kynurenine). Similar results were also observed as these proteins were incubated with increasing concentration of RF (**Fig. 5A₂**).

◀**Fig. 5 to be inserted here**▶

Moreover, the absorption intensity of these chromospheres was gradually reduced in the presence of increasing concentrations of ASA (**Fig. 5B₁**) and GSH (**Fig. 5B₂**). The protein samples which irradiated in the presence of 50 μ M RF were also subjected to a high speed centrifugation. According to our results, similar absorption spectra of the protein samples before and after centrifugation may suggest that new peak in the visible region is appeared as a result of chromophore formation (**Fig. 5C**). Overall our results suggest that these antioxidants compounds are capable to significantly prevent the photo-induced structural damages of lens crystallins.

3.5. Fluorescence analysis of photo-damaging of lens crystallins in the presence of lens antioxidants

In this study, different fluorescence assessments were used to investigate the structural damages of lens proteins upon direct exposure to the sun light (**Fig. 6**). As solution of lens proteins was exposed to sun light in the presence of a fixed concentration of RF (50 μ M), a time-dependent reduction in both Trp and Tyr fluorescence intensities was observed (**Figs. 6A₁-6A₂**). Also these experiments were repeated with various concentration of RF for 3 hours (**Figs. 6B₁-6B₂**). As shown, the fluorescence intensities of these aromatic residues were reduced as a function of RF concentration.

◀Fig. 6 to be inserted here▶

Our result indicates the exposure of aromatic residues to the aqueous solvent, suggesting a significant protein structural alteration upon RF-sensitized photo-oxidation. Also an important reduction in ANS fluorescence intensity was observed for the lens proteins which incubated either for different times (**Fig. 6A₃**) or with various concentrations of RF (**Fig. 6B₃**), under sun

light. The ANS fluorescence reduction can be explained with decrease in the solvent-exposed hydrophobic patches of the photo-damaged lens proteins. The ThT dye binds to cross-beta-sheet structure, which is a defining feature of amyloid fibril, but also existed elsewhere [40]. The ThT fluorescence emission was significantly reduced upon direct exposure of lens proteins to sun light, as a function of time (**Fig. 6A₄**) or upon increasing the concentration of RF (**Fig. 6B₄**). These results can be also explained with the structural alteration of lens proteins which induced by sun light. Also, a reduction in ANS and ThT fluorescence intensities of the protein samples which irradiated without RF or those incubated with this photosensitizing agent in the dark was observed (**Fig. 6**). These changes might be explained with direct damaging effect of UVA on the lens crystallins [52, 53]. Also, RF can bind to proteins via hydrophobic interaction [54, 55]. Therefore, under dark condition, the hydrophobic interaction of RF with protein may occupy the ANS-binding sites and leads to reduction of the ANS fluorescence intensity. Similarly, this interaction may also influence the binding of ThT to the protein, leading to reduction of ThT fluorescence intensity. In the current study, the lens proteins which treated with different concentrations of either ASA or GSH (0-10 mM) were exposed to direct sun light for 3 hours. At the end, different fluorescence assessments were performed (**Fig. 7**). The inset figures show the changes in maximum emission fluorescence of each protein sample versus concentration of antioxidant compounds.

◀Fig. 7 to be inserted here▶

As shown in this figure, all types of fluorescence emission of the photo-damaged proteins are effectively restored in the presence of the lens antioxidant components. Overall, our results suggested that photo-oxidation of lens proteins in the presence of RF leads to protein structural damages which can be efficiently prevented by both ASA and GSH.

3.6. The proteolytic stability analysis of photo-damaged lens proteins

Proteolytic instability of lens proteins is associated with opacification of eye lens and cataract development [56]. In this study, proteolysis of the photo-damaged lens proteins was examined after incubation of the protein samples (1mg/ml) with α -chymotrypsin, at a ratio of 50:1 (w/w). The experiment was done in Tris buffer pH 7.8 for 3 h, at 37°C. Then, the digestibility of these proteins was examined on a reducing electrophoresis gel (**Fig. 8**).

◀**Fig. 8 to be inserted here**▶

As indicated in this figure, the photo-damaged lens proteins display significantly increased susceptibility for chymotrypsinolysis compared to the native protein counterparts. In addition, ASA and GSH partially restore the native form of these proteins against photo-damaging effects of solar radiation; subsequently increasing their stability to some extent against enzymatic digestion.

4. Discussion

The solar UV irradiation is one of the most important environmental stresses that causes a number of human diseases such as skin cancer and eye diseases [57, 58]. The total flux of UVA at the earth's surface vastly exceeds than UVB, with all the UVC being completely absorbed by the stratospheric ozone (**Scheme 2**). While a partial blockage of UVB occurs by the ozone layer, normal glasses block all of UVB and allow UVA and visible light to come through [36, 37, 59].

Depending on the time of the day and the season of the year, the terrestrial spectrum of solar UV radiation consists of 95-99% of UVA and 1-5% of UVB [60]. In addition, the major components of eye lens including crystallins, enzymes and antioxidant agents have a maximum absorption in the UVB and UVC region but not in the UVA and visible region [13]. Thus, UVB can directly damage the eye lens crystallins but the main mechanism behind UVA and visible light-induced cataract disorders is indirect through the endogenous photosensitizer molecules such as RF (Schemes 1& 2) [61].

◀ Scheme 2 to be inserted here ▶

RF is a normal component of the eye lens with the concentration of 4.5 μM in the human lenticular tissues. This photosensitizer molecule absorbs UVA (315-400 nm) and visible light (400-500 nm) [62] and subsequently converts into its triplet state ($^3\text{RF}^*$) indicating important damaging effects on the sensitive amino acid residues of protein (Scheme 2). As shown in Scheme 2, the triplet state of RF converts Trp, Tyr and Cys into kynurenine, dityrosine and cystine. While, kynurenine is a photosensitizing agent; dityrosine and cystine play important role in protein-protein covalent crosslinkings. In this study, the incubation of eye lens crystallins in the presence of RF was done in glass bottles that block UVB light and transmit UVA and visible light (Fig. 1) [37]. Therefore, in our experimental set up, the RF-mediated photo-damaging of lens crystallins most likely comes from their exposure to UVA and visible lights. Additionally, the samples irradiated without RF were used to study the damaging effect of only UVA and visible light compared to RF-mediated photo-damaging of these proteins. As shown in Fig. 2, the samples that irradiated without RF or proteins which incubated with RF in the dark display similar electrophoresis patterns. This result shows that UVA and visible light cannot directly bring important damages to the lens proteins. Gel mobility shift assay of the modified samples

indicated that protein crosslinking occurs in the time and concentration dependent manners. Our results clearly indicated that both irradiation time and concentration of RF have important effect on the lens protein crosslinking (**Figs. 2A₁-2A₃**). As shown in **Fig. 5**, a new absorption peak between 300-400 nm can be related to formation of new chromophores such as dityrosine and N-formyl kynurenine [25, 63]. The reduction in the emission fluorescence of Trp and Tyr residues may be due to formation of the chromophores indicated in **Scheme 2**. On the other hand, RF-induced photo-oxidation of lens crystallins leads to increase in their oligomeric size distributions (**Fig. 4**) that is also in consistent with the formation of HMW oligomers indicated by gel electrophoresis analysis (**Fig. 2**).

Incubation of lens crystallins with ASA and GSH significantly prevented the RF-mediated photo-damaging (crosslinking and oligomerization) of these proteins. Our results suggest a more effective role for GSH than ASA against the photo-damages which induced by solar radiation in lens proteins (**Fig. 3B₂-3B₃**). The weak protection of ASA can be explained with its photodecomposition which can takes place in the presence of RF [64]. Overall our results suggest that UVA and visible light induce important levels of RF-mediated photo-oxidation, crosslinking, oligomerization and proteolytic instability in lens crystallins. These structural insults of lens crystallins are generally potential sources of senile cataract and blindness. Our results also suggest that lens antioxidant components (ASA and GSH) are effectively capable to reduce the structural damages of lens crystallins happening upon their direct exposure to the sunlight. Therefore, the powerful antioxidant defense mechanism of eye lens is an important barrier against molecular photo-damages occurring by the solar radiations during the life span. This important defense mechanism is attenuated during pathological conditions such diabetes mellitus and also in aging [65]. Therefore, the protective capacity of

lens antioxidant defense system on damaging effects of solar radiations can be varied between individuals, depending on their age and health status.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

References

1. H. Bloemendal, W. de Jong, R. Jaenicke, N.H. Lubsen, C. Slingsby, A. Tardieu, *Prog. Biophys. Mol. Biol.* 86 (2004) 407.
2. R. Michael, A.J. Bron, *J. Boil.Sci.* 366 (2011) 1278.
3. S. Meehan, Y. Berry, B. Luisi, C.M. Dobson, J.A. Carver, C.E. MacPhee, *J. Boil. Chem.* 279 (2004) 3413.
4. S.M. Salvi, S. Akhtar, Z. Currie, *Postgrad. Med.* 82 (2006) 581.
5. U.P. Andley, *Int. J. Biochem. Cell Biol.* 40 (2008) 317.
6. N. Azzam, A. Dovrat, *Exp. Eye Res.* 79 (2004) 903.

7. M.N. Ayala, R. Michael, P.G. Söderberg, *Invest. Ophthalmol. Vis. Sci.* 41 (2000) 3539.
8. A. Midelfart, *Acta.Ophthalmol. Scand.* 83 (2005) 642.
9. C. Delcourt, I. Carriere, A. Ponton-Sanchez, A. Lacroux, M.J. Covacho, L. Papoz, *Arch. Ophthalmol.* 118 (2000) 385.
10. J.P.G. Bergmanson, P.G. Söderberg, *Ophthalmic. Physiol.Opt.* 15 (1995) 83.
11. D.H. Sliney, *Invest. Ophthalmol. Vis. Sci.* 27 (1986) 781.
12. U.P. Andley, R.M. Lewis, J.R. Reddan, I.E. Kochevar, *Invest. Ophthalmol. Vis. Sci.* 35 (1994) 367.
13. D. Balasubramanian, X. Du, J.S. Zigler, *Photochem. Photobiol.* 52 (1990) 761.
14. D. Balasubramanian, *J. Ocul. Pharmacol. Ther.* 16 (2000) 285.
15. L. Vergani, M. Barile, C. Angelini, A.B. Burlina, L. Nijtmans, M.P. Freda, C. Brizio, E. Zerbetto, F. Dabbeni-Sala, *Brain* 122 (1999) 2401.
16. I.Q.B.A.L. Ahmad, F.H. Vaid, *Flavins Photochem. Photobiol.* 13 (2006).
17. A. Rochette, E. Silva, I. Birlouez Aragon, M. Mancini, A.M. Edwards, P. Morlière, *Photochem. Photobiol.* 72 (2000) 815.
18. A.M. Edwards, E. Silva, *J. Photochem. Photobiol. B.* 63 (2001) 126.
19. E.Silva, M. Jopia, A.M. Edwards, E. Lemp, E. Lissi, *Photochem. Photobiol.* 75 (2002) 585.
20. A. Besaratinia, S.I. Kim, S.E. Bates, G.P. Pfeifer, *Proc. Natl. Acad. Sci. U S A.* 104 (2007) 5953.

21. L. Rittié, G.J. Fisher, Ageing Res. Rev. 1(2002) 705.
22. U.P. Andley, B.A. Clark, Exp. Eye Res. 47 (1988) 1.
23. G. Viteri, A.M. Edwards, J. De la Fuente, E. Silva, Photochem. Photobiol. 77 (2003) 535.
24. U.P. Andley, Exp. Eye Res. 46 (1988) 531.
25. J.Bhatia, L.D. Stegink, E.E. Ziegler, J. Parenter. Enteral. Nutr. 7 (1983) 277.
26. L. Shen, H.F. Ji, J. Photochem. Photobiol B. 92 (2008) 10.
27. S.D. Varma, S. Kovtun, K.R. Hegde, Eye Contact Lens 37 (2011) 233.
28. Y. Chen, G. Mehta, V. Vasiliou, Ocul. Surf. 7 (2009) 176.
29. B. Kisic, D. Miric, L. Zoric, A. Ilic, I. Dragojevic, Oxid. Med. Cell. Longev. 2012(2012).
30. M.F. Lou, Prog. Retin. Eye Res. 22 (2003) 657.
31. M.Y. Jung, S.K. Kim, S.Y. Kim, Food Chem. 53 (1995) 397.
32. A. Taylor, T. Nowell, Adv. Pharmacol. 38 (1996) 515.
33. R. Yousefi, S. Jalili, Colloids Surf. B. 88 (2011) 497.
34. V.E. Fioletov, J.B. Kerr, A. Fergusson, Can. J. Public. Health. 101 (2010) 5.
35. M.S. Islam, M.R. Hasan, Int. J. Environ. Sustain. Dev. 15 (2016) 146.
36. C. Tuchinda, S. Srivannaboon, H.W. Lim, J. Am. Acad. Dermatol. 5 (2006) 845.
37. F. Almutawa, R. Vandal, S.Q. Wang, H.W. Lim, Photodermatol. Photoimmunol. Photomed. 29(2013) 65.
38. C. Bukh, M. Lund, M.J. Bjerrum, J. Inorg. Biochem. 100 (2006)1547.

39. A. Hawe, M. Sutter, W. Jiskoot, *Pharm. Res.* 25 (2008) 1487.
40. L.S. Wolfe, M.F. Calabrese, A. Nath, D.V. Blaho, A.D. Miranker, Y. Xiong, *Proc. Natl. Acad.* 107 (2010) 16863.
41. K. Khoshaman, R. Yousefi, A.M. Tamaddon, L. Saso, A.A. Moosavi-Movahedi, *Free Radic. Biol. Med.* 89 (2015) 819.
42. M. Ghahramani, R. Yousefi, K. Khoshaman, M.M. Alavianmehr, *Colloids Surf. B.* 125 (2015) 170.
43. M. Ghahramani, R. Yousefi, K. Khoshaman, S.S. Moghadam, B.I. Kurganov, *Int. J. Biol. Macromol.* 87 (2016) 208.
44. H. Schagger, G. von Jagow, *Anal. Biochem.* 166 (1987) 368.
45. M.B. Datiles, R.R. Ansari, K.I. Suh, S. Vitale, G.F. Reed, J.S. Zigler, F.L. Ferris, *Arch. Ophthalmol.* 126 (2008) 1687.
46. B. Raman, T. Ramakrishna, C.M. Rao, *J. Biol. Chem.* 270 (1995) 19888.
47. S.D. Varma, S. Kovtun, K.R. Hegde, *Eye Contact Lens.* 37 (2011) 233.
48. A.J. Bron, G.F.J.M. Vrensen, J. Koretz, G. Maraini, J.J. Harding, *Ophthalmol.* 214 (2000) 86.
49. M.J. Davies, R.J. Truscott, *J. Photochem. Photobiol. B.* 63 (2001) 114.
50. S. Amin, G.V. Barnett, J.A. Pathak, C.J. Roberts, P.S. Sarangapani, *Curr. Opin. Colloid Interface Sci.* 19 (2014) 438.

51. K.O. Muranov, O.I. Maloletkina, N.B. Poliansky, K.A. Markossian, S.Y. Kleymenov, S.P. Rozhkov, A.S. Goryunov, M.A. Ostrovsky, B.I. Kurganov, *Exp. Eye Res.* 92 (2011) 76.
52. S., Zigman, T. Paxhia, T. McDaniel, M.F. Lou, N.T. Yu, *Invest. Ophthalmol. Vis. Sci.* 32 (1991) 1723.
53. A. Dovrat, O. Weinreb, *Invest. Ophthalmol. Vis. Sci.* 36 (1995) 2417.
54. F. Diarrassouba, L. Liang, G. Remondetto, M. Subirade, *Food. Chem.* 119 (2010) 1550.
55. J. Steczko, W. Ostrowski, *Biochim. Biophys. Acta* 393 (1975) 253.
56. Y. Nakamura, C. Fukiage, M. Shih, H. Ma, L.L. David, M. Azuma, T.R. Shearer, *Invest. Ophthalmol. Vis. Sci.* 41 (2000) 1460.
57. B.K. Armstrong, A. Kricker, J. Photochem. Photobiol. B. 63 (2001) 8.
58. F.J. Van Kuijk, *Environ. Health Perspect.* 96 (1991) 177.
59. E.F. Bernstein, M. Schwartz, R. Viehmeyer, M.S. Arocena, C.P. Sambuco, S.M. Ksenzenko, *laser. Surg. Med.* 38 (2006) 337.
60. W.A. Bruls, H. Slaper, J.C. van Der Leun, L. Berrens, *Photochem. Photobiol.* 40 (1984) 485.
61. S. Zigman, *J. Ocul. Pharmacol. Ther.* 16 (2000) 161.
62. D. Balasubramanian, X. Du, J.S. Zigler, *Photochem. Photobiol.* 52 (1990) 761.
63. M. Mancini, A.M. Edwards, M.I. Becker, A. De Ioannes, E. Silva, *J. Photochem. Photobiol. B.* 55 (2000) 9.

64. R. Kannan, A. Stolz, Q. Ji, P.D. Prasad, V. Ganapathy, Exp. Eye Res. 73 (2001) 159.
65. A.C. Maritim, A. Sanders, J. Watkins, J. Biochem. Mol. Toxicol. 17 (2003) 24.

Figure legends

Figure 1. The absorption and transmission profiles of the glass bottles used in this study for the incubation of lens proteins under direct sunlight.

These profiles confirm the significant transmission capability of the glass bottles for UVA and visible light. This experiment was done by using a Cecil CE 7200 spectrophotometer (Cecil Instruments Ltd., UK).

Figure 2. The effects of sunlight on RF-mediated crosslinking and oligomerization of lens crystallins

(A₁) Reducing SDS-PAGE profile (12% gel) of lens crystallins (2 mg/ml) after incubation with RF (50 µM) under sunlight for different times. (A₂) and (A₃) respectively indicate non-reducing and reducing SDS-PAGE profiles of lens crystallins (2 mg/ml) that incubated with different concentrations of RF (3-50 µM) for 3 h. (B₁) Reducing SDS-PAGE profile of lens crystallin incubated with RF (50 µM) upon exposure to the sun light for 3 h under low (lanes 2-4) and normal (lanes 5-7) oxygen levels. The arrows indicate the formation of new HMW oligomers compared to the samples which incubated with RF in the dark (lanes 3 & 6) or without RF under

exposure to sun light (lanes 2 & 5). Under normal oxygen level a very intense band formed in the well (lane 7) compared to the sample which incubated at low oxygen condition (lane 4). (**B₂/B₃**) The protein samples (2 mg/ml) were incubated with RF (50 μ M) in the presence of ASA (1-10 mM) (**B₂**) and GSH (1-10 mM) (**B₃**) for 3 h. All experiments were done in NaPi buffer (100 mM, pH 7.4). A 15 μ g of each protein sample was loaded into wells of electrophoresis gel and the bands were visualized by CBB staining.

Figure 3. The protective effect of lens antioxidants against sunlight-induced protein crosslinking and sulfhydryl oxidation

Different protein samples (2 mg/ml) were incubated upon exposure to the sunlight for 3 h with RF (50 μ M), in the presence of the lens antioxidant compounds. (**A**) The protective effects of ASA and GSH on the RF-mediated protein crosslinking and oligomerization were studied. (**B**) The analysis of free sulfhydryl groups were done for native and unfolded states of these proteins according to protocol which described in the method section. The data were analyzed from three independent experiments and expressed as mean $\pm$ SD. The free sulfhydryl groups of native and unfolded states of lens proteins samples lacking antioxidant and incubated with RF under sunlight were compared with their corresponding samples. Also protein samples which treated with ASA and GSH or ASA/GSH were compared with each other's. The statistical significance was expressed as asterisk, indicating $P < 0.001$.

Figure 4. DLS analysis after exposure of lens crystallins to the solar radiation in the presence of lens antioxidants.

The particle size characterization was done by DLS analysis. The size distribution of different protein samples was reported as their relative volumes. Also, the insets stand for their relative scattering intensity. The data were analyzed from three independent experiments and expressed as mean $\pm$ SD. The experiments were done in 100 mM NaPi buffer and the concentration of lens proteins was fixed at 4 mg/ml.

Figure 5. The UV-Vis spectroscopic analysis of RF-mediated photo-oxidation of lens proteins

The absorption spectra of lens proteins incubated with different chemical compounds (RF, ASA and GSH) in NaPi buffer (100 mM, pH 7.4) were collected by UV-Vis spectroscopic analysis. The protein samples were diluted to 0.4 mg/ml and UV-Vis absorption spectra were collected between 200-700 nm. (**A₁/A₂**) **A₁** and **A₂** indicate the absorption spectra of lens protein samples which incubated for different times and in the presence of different concentrations of RF, respectively. (**B₁/B₂**) **B₁** and **B₂** stand for absorption spectra of the protein samples which incubated with RF (50 μ M) in the presence of different concentrations (0-10 mM) of ASA and GSH, respectively. The insets indicate the value of optical density (OD) at 322 nm which is related to formation of N-formyl kynurenine chromophore. (**C**) UV-Vis spectra of protein sample which incubated with 50 μ M RF under sunlight before and after centrifugation.

Figure 6. Fluorescence assessments of lens proteins after exposure to sun light

The fluorescence assessments are indicated as following: (**A₁, B₁**) Trp fluorescence, (**A₂, B₂**) Tyr fluorescence, (**A₃, B₃**) ANS fluorescence and (**A₄, B₄**) ThT fluorescence. The concentrations of lens proteins, ANS and ThT were fixed at 0.15 mg/ml, 100 μ M and 20 μ M, respectively. The insets indicate maximum emission wavelength (λ_{max}) of fluorescence spectra corresponding to different protein samples. The fluorescence intensity was decreased as a function of incubation time and with increasing RF concentration. All solutions were prepared in NaPi buffer (100 mM, pH 7.4). The excitation/emission wavelengths were set as 295/300-500 nm for Trp, 280/300-500 nm for Tyr, 365/400-600 nm for ANS-protein complex and 440/450-650 nm for mixture of ThT-protein samples.

Figure 7. Fluorescence assessments of lens proteins in the presence of lens antioxidants

(**A₁, B₁**) Trp fluorescence, (**A₂, B₂**) Tyr fluorescence, (**A₃, B₃**) ANS fluorescence and (**A₄, B₄**) ThT fluorescence. All experiments were measured at excitation/emission wavelengths as described in **Fig.6**. The insets indicate maximum emission wavelength (λ_{max}) of fluorescence spectra corresponding to different protein samples. The fluorescence intensity was enhanced with increasing concentration of ASA and GSH.

Figure 8. The SDS-PAGE profile of chymotryptic digestion of photo-oxidized lens proteins

(**A**) The lens proteins were treated with RF under sunlight in the presence and absence of the antioxidant compounds. The protein-chymotrypsin mixtures were prepared at a ratio of 50:1

(w/w) and incubated for 3 h at 37°C. The incubations were done in Tris buffer (20 mM, pH 7.8) containing 100 mM NaCl. The protein bands on reducing SDS-PAGE were visualized by CBB staining.

Scheme 1. The possible pathway of riboflavin-mediated lens proteins damaging upon exposure to UV and visible light.

Following UV and visible light absorption, riboflavin (RF) reaches its singlet excited state ($^1\text{RF}^*$) and then converts into the triplet state ($^3\text{RF}^*$) by intersystem crossing (ISC). The later excited molecule ($^3\text{RF}^*$) can react with hydrogen atoms of protein through the electron transfer (Type-I reaction), forming protein radical ($\text{P}^{\cdot}$) and riboflavin radical ($\text{RFH}^{\cdot}$) that subsequently react with molecular oxygen to generate ROS. Also, formation of protein radical leads to protein crosslinking. On the other hand, $^3\text{RF}^*$ can react directly with molecular oxygen through energy transfer (Type-II reaction) to generate singlet oxygen ($^1\text{O}_2$). Finally, these molecular events lead to protein crosslinking and protein oxidation which are potential sources of cataract development. Antioxidant defense mechanism (ADM) is likely to protect lens proteins against ROS-induced structural and functional insults.

Scheme 2. Effect of UVA and visible light on riboflavin photosensitization and subsequent damage of sensitive amino acids

(A) This panel demonstrates the sunlight spectra reaching the eye lens which exert their damaging effects through riboflavin (RF) photosensitization. While UVC is completely absorbed by the atmospheric gases, only partial blockage of UVB occurs by the ozone layer. Normal

glasses block all UVB radiations but allow UVA and visible light to come through. UVA and visible light exert their photo-damaging effects indirectly through photosensitization of riboflavin. **(B)** The RF-mediated photo-oxidation products of different amino acids are indicated. The triplet state of riboflavin ($^3\text{RF}^*$) converts Trp, Tyr and Cys into kynurenine, dityrosine and cystine, respectively. ISC stands for the intersystem crossing.

Figure 1

Fig. 1

Figure 2

Figure 3

Fig. 3

Figure 4

Figure 5

Fig. 5

Figure 6

Fig. 6

Figure 7

Fig. 7

Figure 8

Fig. 8

Scheme 1

Scheme 2

B

Graphical abstract

Preventive role of lens antioxidant defense mechanism against riboflavin-mediated sunlight damaging of lens crystallins

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