Background: With age, the human lens accumulates variety of substances that absorbs and fluorescence, which explains the color of yellow, brunescent and nigrescent cataract in terms of aging. The aim of this study was to assess lens fluorophores with properties comparable to those of advanced glycated end products (AGEs) in relation to age in human lenses. These fluorescent compounds are believed to be involved in the development of cataract.

Methods: Spectroscopic (UV-Vis-NIR) and fluorescence photography (CCD-Digital based image analysis) studies were carried out in randomly selected intact human lenses (2–85 years). AGE-like fluorophores were also measured in water soluble and insoluble (alkali soluble) fractions of human lenses (20–80 years).

Results: Our experimental findings suggest that there was a progressive shift in the absorbance characteristic of intact lens in the range of $\lambda_{210\text{ nm}}$-$\lambda_{470\text{ nm}}$. A relative increase in the absorptivity at $\lambda_{(511-520\text{ nm})}$ with age, was also observed. In addition, the ratio of absorptivity at $\lambda_{(511-520\text{ nm})}$ versus the maximum absorbance recorded at blue-end cut-off (210–470 nm) was also found to increase, with age. The fluorescent intensity in the intact lens at both UV-B ($\lambda_{\text{Ex312 nm}}$) and UV-A ($\lambda_{\text{Ex365 nm}}$) were found to be positively correlated ($r^2 = 0.91 & 0.94$, respectively; Confidence interval 95%) upto 50 years of age. In addition, a concomitant changes in AGE- like fluorophores were also observed in the processed lens samples (soluble and insoluble fractions) along the age. A significant increase in the concentration of AGE- like fluorophores, both in intact and processed lens was observed during the period of 40 – 50 years.

Conclusion: Based on the present investigation, it was concluded that significant changes do occur in the AGE-like fluorophores of human lenses during the period of 40–50 years.
yellow and fluorescent [3-5]. The age-related increase in lens coloration and fluorescence is associated with the major proteins of the lens, the crystallins, which are particularly prominent in the lens nucleus [4,5]. The lens proteins are as old as the individual since there is little or no turn over of these proteins [6]. Numerous chemical and photochemical processes may account for these changes, which include the (i) photochemical modification of tryptophan [7] (ii) lipid peroxidation [8] and (iii) chemical linking of sugar or ascorbic acid through the Maillard reaction [9,10].

The Maillard reaction, a non-enzymatic reaction between ketones or aldehydes and primary amino groups of macromolecules, contributes to the aging of proteins and to complications associated with diabetes [11-13]. Advanced glyated end products (AGEs) were originally characterized by a yellow-brown fluorescent color and their ability to form cross-links with and between amino groups [14]. Fluorescence is one of the qualitative properties classically used to estimate AGE formation in addition to their brown coloration. There is considerable evidence that AGE of lens proteins are involved in browning and fluorophore formation in the lens [15,16]. Several different AGEs (fluorescent cross-links and non-fluorescent cross links) have been detected in the human lenses, mainly by immunochemical methods using both polyclonal and monoclonal antibodies [17-21] in normal, aged and cataractous lenses. The identified fluorescent AGEs species in lens include pentosidine[22], pyralline[24], crossaline[23], vesperlysine, and argpyrimidine[24]. Additionally, Franke et al., [17] have reported the presence of pentosidine and imidazolone, in cataractous lenses. Methylglyoxal-derived hyroimidiazolone AGEs are the major glycation adducts found in lens proteins compare to argpyrimidine and pentosidine [25]. Undoubtedly, there are a number of AGE-like fluorophore that are formed in human lenses during the course of aging. The direct relationship between the amount of AGEs and increased yellowing of lens was reported earlier by Das et al., [26] using synchronous fluorescence (SF) method. The SF spectra of human lens sample closely resembled those of in vitro AGEs derived from incubation of BSA with glucose.

In addition, post-translational modifications by kynurenine (tryptophan-metabolite), 3-hydroxykynurenine glucoside (3-OHKG) with lens crystallins [27-29] as aging fluorophores, have been considered as biomarker for aging of lens. However, no spectroscopic studies have been carried out on the aging of human lenses, which particularly deals with the early onset of cataractogenesis in terms of AGE-like fluorophores formation, which can be correlated with biochemical and photo-biological events occurring during our lifetime.

The present experimental investigation was designed to assess AGE-like fluorophores with properties comparable to those of advanced glyated end products (AGEs) in relation to age in human lenses by spectroscopic approach. This study was undertaken to address the early onset of cataractogenesis in terms of AGE-like fluorophore formation in human lens.

### Table 1

| Age group (years) | Number lenses (n) |
|------------------|-------------------|
| 2                | 2                 |
| 15–17            | 5                 |
| 20–23            | 5                 |
| 30–35            | 5                 |
| 45–47            | 5                 |
| 57–60            | 5                 |
| 65–70            | 3                 |
| 75 – 85          | 15                |

### Table 2: Relative change in the ratio of absorbance observed in the region of 511–520 nm versus absorbance recorded at blue-end cut-off region.

| Age of lens (year) | Absorbance# in the region of λ511–520 nm | Ratio of Absorbance# Absorbance at λ511–520 nm λmax at blue-end cut-off |
|--------------------|------------------------------------------|----------------------------------------------------------------------------|
| 2                  | 0.25                                     | 0.25/1.5 = 0.16                                                          |
| 15                 | 0.6                                      | 0.6/3.12 = 0.192                                                         |
| 20                 | 1.0                                      | 1.0/3.5 = 0.28                                                           |
| 30                 | 1.2                                      | 1.2/3.8 = 0.315                                                          |
| 45                 | 1.3                                      | 1.3/4 = 0.325                                                            |
| 60                 | 1.35                                     | 1.35/> 4*                                                                |
| 65                 | 2.6                                      | 2.6/> 4*                                                                 |
| 75                 | 1.18                                     | 1.18/3.5 = 2.96                                                         |
| 80                 | 2.3                                      | 2.3/> 4*                                                                 |
| 85                 | 2.75                                     | 2.75/> 4*                                                                |

# Mean value
* Values not calculated as absorbance was > 4.0
**Methods**

Human lenses (2–85 years) were obtained from Ramayamma International Eye Bank (Member of the International Federation of Eye Banks & Eye Bank Association of India) L V Prasad Eye Institute, Hyderabad, India. This study was undertaken with the approval of the ethics committee of L V Prasad Eye Institute, Hyderabad, India. The informed consent was also obtained for collecting those cadaver lenses through the Eye Bank of L V Prasad. They were stored at -80°C, until further use.

**Chemicals**

Bovine serum albumin \{ (BSA) Essential fatty acid & globulin free \}, Glucose (Dextrose; corn sugar), L-arginine were purchased from Sigma Chem. Co. St. Louis, USA. Crystaline D-ribose from Hi Media, India. Amino acids, L-tyrosine and L-histidine were from Eastman Kodak Company, Rochester, N.Y and L-lysine monohydrochloride from M/s Sarabhai M. Chemicals, Baroda, India. All other reagents used were of analytical grade.

**Spectral measurements**

Human lenses (2–85 years, n = 45) with varying degree of yellow-brown coloration were randomly selected for the spectral analysis and grouped as shown in Table 1.

Intact lenses were subjected to fiber optic based digital UV-Vis-NIR spectrophotometer (Ocean Optics, Netherlands) analysis. This system uses the continuous output of Xenon lamp (200–1000 nm) and is based on 1024 diode array detector system. This detector is capable of collecting full wavelength spectra with good signal to noise ratio at an integration on time of 1 milli sec. The excitation light is led into the sample chamber through a fiber optic bundle, and the transmitted light is then collected by a second fiber optic bundle positioned at an angle of 180° to the excitation source. The excitation and transmitted light are collimated by a set of focusing lens on the either side of the sample chamber. The spectra from the intact human lenses were taken against the dark (0% transmittance) and the net fluorescence intensity (density), which was measured by the method of Lowry et al. [30] using BSA as reference standard. For fluorescent studied 1 mg/mL of 0.1 N NaOH.

**Fluorescence studies**

Forty lenses (20–80 years) were selected for fluorescent studies. Fluorescent intensity (density) in the intact human lenses were measured peak volume at both UV-B and UV-A (λ_ex312 nm & λ_em365 nm) respectively with an emission in the range of 380 nm to 470 nm, using highly sensitive charged coupled device (CCD) based digital image analyzer (UVItect) Cambridge, U.K. Volume of the digital image due to fluorescent intensity (density) was analyzed by using UVI Image Acquisition and Analysis Software.

**Digital Image Analysis System**

This system consists of a high-resolution CCD-based (charged coupled device) camera (monochrome type) with absorbance and fluorescence facility. The CCD-camera has an optical zoom lens (12.5 × 75 mm/f1.8) along with 49-mm+1 dioptres close-up lens. The sensitivity of the camera is 10^(-5) lux, with negligible signal to noise ratio (< 30 db). The CCD camera is housed in a light-tight compact cabinet over a trans-illuminator. The camera is equipped with UV & IR interference filter. The acquired image is displayed on an in-built LCD screen (resolution ~ 8 bit, 256 gray level images). The image acquisition is base on real time integration in the range of 0.04 to 10 seconds. The image acquisition system is linked to Intel Pentium 4 processor base computer, loaded with UVi-tech image processing and store software (64-bit data software). The digitized image has a resolution of 752(H) × 582 (V) pixels. The trans-illuminator consists of dual wavelength (312 and 365 nm) UV lamps with output light intensities of 8 mW/cm^2.

The digital image acquisition was achieved by placing the intact lens on flat surface of the trans-illuminator, housed in the light-tight cabinet. For florescence studied, the sample was excited individually from both UV-B & UV-A (λ_ex312 nm & λ_em365 nm) region of the electromagnetic spectrum. Lens image formed due to emission (range 380 nm to 470 nm) was acquired by real time integration at 0.2 seconds for 312 nm and 0.04 seconds for 365 nm. Annotated images were saved in PC compatible file format (tiff file) in a floppy. Later, the digital images of the intact lens were analyzed by the software for determining the net fluorescence intensity (density), which was measured as peak volume.

**Processing of human lens samples**

Lenses (20–80 years) were homogenized individually in 20 mM Phosphate buffer pH 7.4 (10% w/v), centrifuged at 10,000 × g for 30 min at 4°C, and separated into supernatant and precipitate. The supernatant referred to as the "water soluble" fraction and precipitate as the "water insoluble" fraction. An aliquot of the insoluble protein (1–3 mg) was solubilized in 200–500 µL of 0.1 N NaOH. Both fractions were used for fluorescent studies for the estimation of AGE-like fluorophores. Protein was estimated by the method of Lowry et al., [30] using BSA as reference standard. For fluorescent studied 1 mg/mL of protein was taken.

**Chemical synthesis of AGE-like fluorophores**

Synthetic AGE-like fluorophores were prepared for comparison with lens AGE-like fluorophores [31].

(i) BSA (1 mg/mL of 0.1 N NaOH) was used as control blank for fluorescent studies. (ii) BSA-AGE was prepared...
by modified method of Nakagawat, et al., [31]. It was prepared by incubating 1.5 µ mole BSA with 1.6 mmole glucose, 0.7 mmole ribose in 5 mL of 0.4 M phosphate buffer, pH 7.4. The samples were processed under sterile condition using the Laminar flow hood. The vials were sealed and placed in an air-circulating incubator, at 37°C for 10 weeks. After incubation, mixture was dialyzed and concentrated using spin column (Ultrafree-MC filters, molecular weight cut-off limit – 10 kDa, Sigma Chem. Co. St. Louis, USA.). The degree of glycation was checked by trinitobenzene sulfonic acid method [32]:

Fluorescence measurements
Fluorescent measurements were performed in total as well as both fractions of human lens samples, using a spectrophotometer (Perkin-Elmer, LS-3B, Norwalk, NJ, USA). AGE-like fluorophores were measured as described earlier [33] in sample, experimentally synthesized AGE-like fluorophores as well as in control blank (BSA /1 mg/mL in 0.1 N NaOH). These AGE-like fluorophores were measured at their respective excitation and emission wavelength, in the following order:

AGE (λEx347 nm/λEm415 nm); pentosidine (λEx335 nm/λEm385 nm); pentosidine (λEx366 nm/λEm440 nm); crossline (λEx379 nm/λEm463 nm); pyropridine (λEx370 nm/λEm455 nm); argpyrimidine(λEx320 nm/λEm382 nm). Results are expressed as fluorescence intensity/mg of lens protein.

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
The absorption spectra of intact human lenses from various age groups (2–85 years) are given in Figure 1. There was a progressive shift of absorbance in the region of λ210 nm to λ470 nm was observed. An increase in absorbance was recorded (range 0.25 to = 3.0 - not shown on y - axis) at λ511–520 nm with age.

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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
The absorption spectra of intact human lenses from various age groups (2–85 years) are given in Figure 1. There was a progressive shift of absorbance in the region of λ210 nm to λ470 nm in relation to age (exception 75 year old lens). Table 2 clearly indicates a relative increase in the absorbivity at λ(511–520 nm), with age. The ratio of absorbivity at λ(511–520 nm) versus the maximum absorbance recorded at blue-end cut-off (210–470 nm) was also found to increase, with age.

The Digital image analysis of the intact lens (20 and 60 years) is depicted in figure 2. A significant increase in the net fluorescence intensity was observed in the 60-year-old lens as compared to 20-year-old lens, which was used as a control. Figure 3, 4 depicts the net fluorescence intensity recorded in the intact lenses at λEx312 nm and λEx365 nm from the different age groups. The fluorescence intensity (density) in the intact lenses shows an increasing trend up to the age of 50 years (r2 = 0.9 at λEx312 nm, and r2 = 0.94 at λEx365 nm, Confidence interval 95%). There was a statistically
cally significant (p < 0.001) increase in fluorescence intensity (density) at $\lambda_{\text{Ex}312\ \text{nm}}$ as compared to $\lambda_{\text{Ex}365\ \text{nm}}$ for all age groups (figure 5). The quantitative estimation of AGE-like fluorophores is shown in Figure 6a, b, c and 6d for the processed lens samples and Figure 7a & b for in vitro synthesized AGE-like fluorophores. Distribution of AGE-like fluorophores show a similar trend in processed lens samples as well as in vitro synthesized AGE-like fluorophores (figure 6 & 7). There was a 2.5 fold increase in fluorescence intensity at 40 years as compared to 20-year-old lenses, with an increasing trend up to 60 years in total lens homogenate (figure 6a & 6b). Similar changes were also observed in soluble/insoluble protein fractions of lenses. A threefold difference in fluorescence intensity was observed at the age of 60 years as compared to 20-year-old human lenses (figure 6c & 6d).

Chemically, BSA-AGE and amino acid based AGE was successfully synthesized and tested by fluorimetric analysis. The degree of BSA-glycation was monitored by TNBS assay and was observed to be 86% at the end of 10 weeks of incubation. The BSA-AGE and amino acid mixture-AGE showed typical fluorescence similar to that of lens AGEs, while the BSA and amino acid mixture control blank, showed no fluorescence (figure 7a & 7b).

**Figure 2**
Digital based photographic analysis of a typical intact human lens (A)- 20 year (control) and (B) 60 year cataractous, at $\lambda_{\text{Ex}312\ \text{nm}}$ / $\lambda_{\text{Em}380-470\ \text{nm}}$. The fluorescence intensity (density) increased with age.

**Figure 3**
Relationship between age (20–80 years) and fluorescence intensity at $\lambda_{\text{Ex}312\ \text{nm}}$ of intact human lenses, using digital based image analysis. ($y$-axis values in exponential; Values, mean ± S.E).
because no significant change with age at longer wavelength, we chose to use the red-end tail of the UV-absorption peak of the intact lens for calculating the ratio of absorptivity at $\lambda_{321-520}$ nm versus the maximum absorbance recorded at blue-end cut-off (210–470 nm), during the course of ageing. Possibly, these changes indirectly reflect the AGE-like complex formation from the age of 45 years onwards (Table 2). Lerman and Borkman [4] observed two-age related fluorescent compounds, which develop in the lens nucleus. The first showed an excitation at 340–360 nm with emission at 420–440 nm. The second, which appeared to be a secondary product of the former being detectable only after the first decade of life, absorbing light at 415 nm–435 nm with emission at 500–520 nm. It remained relatively at low level until the fourth or fifth decade. The presence of a second type of fluorophore as reported by Lerman & Borkman [4], which remained at low level (absorbance value < 1.0) until the third decade (age 30 years) of life. We observed no significant spectral changes below 20-year-old lenses, thus the fluorescence studies were carried out on lenses from 20 years onwards.

It is contentious as to whether fluorescence studies are true representative of absorption by specific fluorescent compounds present, particularly in the aging lenses. Thus, a study of intact lens, preserving all its condensed phase features, would be of interest. To quantify this age related changes, the fluorescence studies were carried out at two wavelengths - $\lambda_{EX312}$ nm and $\lambda_{EX365}$ nm (figure 2,3,4). The results obtained clearly demonstrate that as the human lens ages, there were significant changes in the non-tryptophan (or "blue") fluorescence [22] because both selected wave length are for blue fluorophore compounds like argpyrimidine[18] and pentodilysine[35]. There are undoubtedly a number of post-translational modifications that occur in lenses with age. The change of AGE-like fluorophore in lenses were found to increase until the age of 50 years, there after, fluorescence begins to drop. A probable explanation for this observation is that a method of external fluorescence measurement on the intact lens cannot follow the increasing concentration of fluorophores because it is affected by increasing self absorption (figure 3 & 4) at $\lambda_{EX312}$ nm and $\lambda_{EX365}$ nm, respectively with age. These observations indirectly decipher the characteristic trough observed at $\lambda_{467-556}$ nm (figure 1) in aging lenses. Previous studies have shown that lens auto-fluorescence increases quantitatively with age [21,22] and in diabetic condition [22]. The remarkable similarities between diabetic and the non-diabetic lenses suggest that the mechanisms of fluorophore formation are alike in diabetic and non-diabetic condition. Increased glycation of lens crystallins in senile and diabetic cataractous lenses have been investigated by immunochemical and fluorescence studies [19,26]. These, findings indicate fluorescent AGEs species including pentosidine [22], pyralline [24], crossaline [23], vespertilysine, and argpyrimidine [24] in lens. Undoubtedly, there are a number of AGE-like fluorophore that are formed in human lenses during the course of aging. The rate of change of $\lambda_{EX312}$ nm/$\lambda_{EX365}$ nm may be considered to be a qualitative estimate of argpyrimidine ($\lambda_{EX320}$ nm) and pentodilysine ($\lambda_{EX366}$ nm), which may reflect their relative level during ageing. The level of argpyrimidine ($\lambda_{EX320}$ nm) was found to be significantly higher than any other fluorophore (Figure 5). This inference has been further supported by the observations of Kessel et al.,
[22], wherein they reported higher concentration of argpyrimidine in cataractous lenses. It is pertinent to note that the distribution of these fluorophores in processed lens samples, did not show any distinct pattern at their respective excitation (Figure 6c & 6d). This suggests that one of the dominating fluorophore or very closely related Determination of AGE-like fluorophores in processed lens (total, soluble, & and insoluble) fractions from different age group (20–80 years).

**Figure 6**

Determination of AGE-like fluorophores in processed lens (total, soluble, & and insoluble) fractions from different age group (20–80 years). Values are mean of five determinations and their CV was < 8%. **Fig. 6a** shows AGE-like fluorophores in the total lens homogenate. **Fig. 6b** depicts the profile of various AGEs in the total lens homogenate. **Fig. 6c** illustrates the AGE-like fluorophores as a ratio of soluble to insoluble fraction of lens homogenate. **Fig. 6d** represents the profile of various AGEs as a ratio of soluble to insoluble fraction of the lens homogenate.
groups of fluorophores give rise to a typical pattern (Figure 6a & 6c) of the fluorescence spectra in the processed lens sample. Kessel et al., [22] observed a similar phenomenon. The changes in these fluorophores possibly commence from 30 years of age and peaks at 60 years, when total lens proteins are taken into account (figure 6a); while the trend is reversed when the ratio of soluble to insoluble lens protein fraction is considered (figure 6c).

AGE-like fluorophore concentration is found to increase in both total and insoluble fraction in the lens during the period of 40 – 60 years. (Figure 6a & 6c). This finding is similar to the one reported earlier by Das et al., [26], using synchronous fluorescence (SF) and immunochemical methods. These observations suggest that AGE-like fluorophores may contribute to protein insolubilization, which ultimately leads to the colouration, opacity and cataractogenesis along with the age.

Our present fluorescent data of human lens samples resemble those of chemically synthesized AGES, derived from incubation of BSA and amino acids mixture with sugars (figure 7), may suggest that such fluorophores contribute to the fluorescence of human lens. In addition to the spectroscopic observations, biochemical basis is put forth to explain the accumulation of AGE-like fluorophore in processed lens samples, which significantly contribute to the absorption of blue light and thereby appearing yellow in aging lenses.

**Conclusion**

The present experimental study clearly indicates that changes in AGE-like fluorophore in lens increases along the age and with major changes commencing from the age of 40–50 years onwards. This is a significant observation with respect to Indian human lenses as it may lead to early onset of senile cataract.

**Competing interests**

The author(s) declare that they have no competing interests.

**Authors’ contributions**

Mala Ranjan, carried out the experimental as well as data analysis under the supervision of Dr. B. Sashidhar Rao. Both authors read and approved the final manuscript.

**Acknowledgements**

Department of Biotechnology (DBT), Government of India New Delhi, India is duly acknowledged for providing the research grant (Grant # BT/PR/2360/Med/09/340/2001) and Apollo Hospital, Hyderabad, India for research fellowship to Ms. Mala Ranjan. We also acknowledge, Ramayamma International Eye Bank (L V Prasad Eye Institute, Hyderabad, India) for providing human lenses samples.

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Pre-publication history

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