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Molecular epidemiology of cataract

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1. Introduction

Cataract or the loss of transparency of the eye lens due to opacification and accumulation of pigments is the major cause of blindness. Of the 50 million blind people in the world, cataract afflicts over 17 million. It is an age-related progressive affliction which arises due to a variety of causes. Of the many causative factors, genetic predisposition, diabetic condition, malnutrition, intense sunlight (and in particular ultraviolet radiation), environmental toxins and even behavioural habits are some of the more prominent. It is not a fatal disease but only a minor affliction which can be easily taken care of by means of a relatively simple surgical operation. However, considering the numbers involved, cataract is a major drain on the nation's exchequer. It has been estimated that by the turn of the century, there will be an annual addition of about 4 million bilateral cataract patients every year in India alone. Even at the minimal cost of Rs. 200 per operation, the economics involved are staggeringly high. Thus, any effort to prevent or even delay the onset of cataract, even by a few years, would be a major step towards human welfare. Being an age-related disease, there is no cure for cataract. Much of the current research is thus focussed on the understanding of the biochemical and molecular biological bases of cataract, and towards finding ways and means to counteract events leading to lens opacification. Our own research in the area over the last eight years has focussed on the biophysical and the epidemiological features of the disease. In many ways, the lens is an ideal tissue for such studies as, unlike most of the other tissues in the body, the lens metabolises very sluggishly and does not turn over. As a result, even minor modification or low-level damage, if chronic, tends to accumulate over the years leading ultimately to lenticular malfunction. We have focussed our attention on the following:

1. *In situ* spectral analysis of the normal human eye lens in an effort to try and understand the age-dependent accumulation of coloured compounds therein. We have also attempted to understand whether these compounds are benign or whether they can further damage the lens.

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2. We have attempted to work out the changes that occur in the covalent chemistry of the lens proteins as a function of age, oxidation and similar damage.

3. We have attempted to understand the molecular epidemiological connection between the sunlight–cataract risk factor, as well as the smoke–cataract connection. In these efforts, we have tied up with the opthalmological group at the L.V. Prasad Eye Institute in Hyderabad in a closely collaborative fashion.

2. *In situ* spectral analysis of the components of isolated intact eye lenses

The eye lens (see Fig. 1) is a remarkable tissue in that it has the most concentrated solutions of dissolved proteins that go to make its structure and function. Over 35% by weight of the lens is the family of proteins called crystallins—which are organized spatially in a graded fashion so as to offer a smooth refractive index gradient from the cortex of the lens nucleus. The *in situ* organization and intermolecular interactions of the crystallins among themselves and with other lens components are thus vital for lenticular function, which need to be studied as is; we would miss monitoring these supramolecular features if we adopt the conventional approach of isolating them and studying them in dilute solution.

It is in this context that we have adopted a direct fluorescence and absorption spectral analytic approach on isolated lenses themselves. We have fabricated a solid-state holder that holds the isolated lens in the sample chamber of a conventional spectrofluorimeter. Monochromatic light of chosen wavelengths fall on the lens at an angle of 30° to the discal plane and the emission beam is picked up at an angle of 60°, so that the fluorescence spectrum *in situ* is obtained without any complications arising from the inner filter effect.

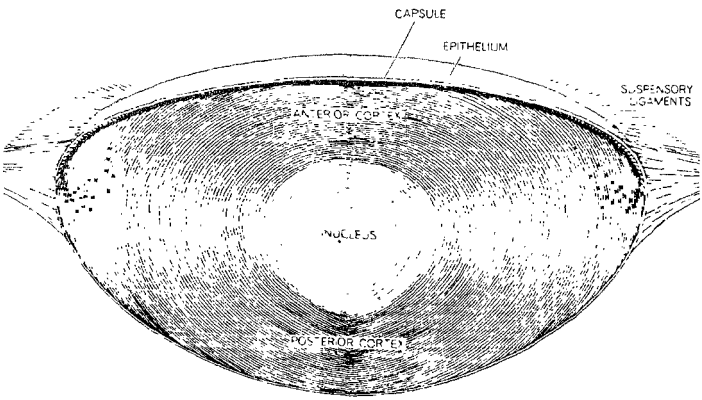

FIG. 1. The human eye lens. The anterior faces the iris while the posterior abuts the vitreous gel.

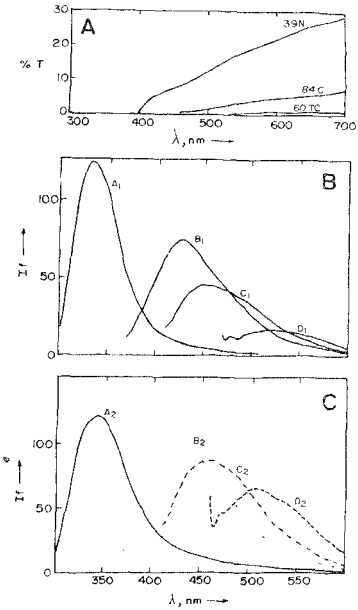

FIG 2 *Panel A:* Transmission spectra of human lenses obtained from the eye bank. The numbers 39, 84 and 60 refer to the ages of the lenses, and the characters N, C and TC denote normal, cataractous and totally cataractous states of the lenses. *Panels B and C.* Fluorescence emission spectra of normal (39-yr old) and cataractous (84-yr old) human eye lens, respectively. A1, B1, C1 and D1 refer to emission from wavelengths 295, 353, 394 and 460 nm, respectively, which correspond to the excitation maxima of the fluorophores shown. A2, B2, C2 and D2 refer to emission from wavelengths 295, 351, 395 and 453 nm, corresponding to the excitation maxima of the fluorophores shown. Human lenses were obtained by the courtesy of the L.V. Prasad Eye Institute, Hyderabad.

This approach has allowed us to routinely scan normal, aged and cataractous human lenses, and to assess their opacity (90° scattering at 700 nm), protein components and the presence of new coloured compounds that accumulate with time, due to oxidative damage, glycation reactions and due to deposition in the lens from systemic absorption (*e.g.*, xenobiotics, drugs). Figure 2 shows how the differences between a normal human lens and a brunescent cataract lens are easily monitored using our *in situ* spectral analysis[1,2]. The levels of normal, unmodified crystallins (assayed by the 355-nm band) have reduced in the cataract lens, giving rise to blue (435-nm band), yellow (520 nm) and brown (580–600 nm) colours. The presence of kynurenines, glycation products, anthranilates and other pigments that arise due to

oxidative damage (photochemical and radical reactions) and sugar-mediated modifications is easily monitored and quantified (see Fig.3).

This novel spectral approach has enabled us to routinely record and characterize a number of human lenses, and to attempt to answer some questions raised from epidemiological findings. We have embarked on this project in collaboration with colleagues at the L. V. Prasad Eye Institute, who provide us with the lens samples, and their clinical and pathological details. We also have had access to some lenses form the US, thanks to help from Dr Sam Zigler of the National Eye Institute (NEI), so that we are able to compare tropical lenses with those from temperate zones. Some of the specific questions that we are addressing our attention to are:

(i) the nature and levels of various coloured compounds that accumulate in the lenses with time.

(ii) the role that each of these compounds may have on the health or deterioration of lens structure and function. Some of these may act as protecting agents (*e.g.*, filters, antioxidants) while others may mediate photochemical damage (photodynamic agents).

(iii) the reason why lenses from tropical regions get brown with age, while those from temperate zones are paler.

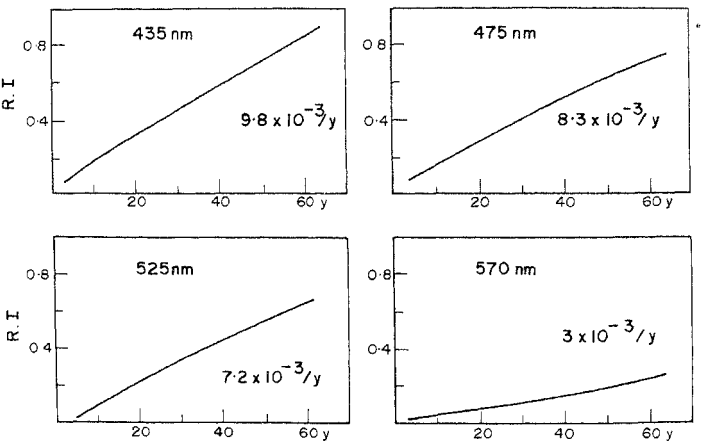

FIG. 3. The age-dependent accumulation of coloured compounds in the normal human lens. The compounds have been monitored by *in situ* fluorescence emission bands occurring at 435, 475, 525 and 570 nm. The intensities of these bands are relative to that of the protein tryptophan emission around 340 nm (hence the units R.I. in the ordinate). The x axis indicates the age of the human donor from whom the lens was obtained. Lenses were obtained through the courtesy of Ms Shaila of the Ramayamma International Eye Bank of the L.V. Prasad Eye Institute, Hyderabad. The rate of accumulation of each of the fluorophore is indicated against each panel, and was obtained from the slope of the curve in each case.

3. Analysis of the coloured substances in the lens and their photodynamic properties

We have been analysing isolated human lenses by *in situ* spectroscopy in order to identify the structures and quantify the levels of coloured compounds that accumulate in the lens with time. In the next step, we collect spectrally matched lenses, homogenize and digest them with proteolytic enzymes, and fractionate the low molecular weight substances by HPLC. The various coloured compounds that have accumulated in the lenses are thus isolated and purified, and their chemical structures elucidated by us, using spectral techniques.

We have been able to identify the following compounds: N-formylkynurenine (NFK), kynurenine(Ky), 3-hydroxykynurenine (3HK), kynurenic acid(KUA), 3-hydroxyanthranilic acid (3HAA), anthranilic acid (AA) and some flavins (F). Many of these absorb light in the near UV (or UVA, in the 320–400 nm region) and thus extend the absorption range of the lens. Absorption of UV light can often prove damaging to the system, leading to chemical reactions and structural changes. Therefore, it became necessary for us to study whether each of these coloured compounds behaves as a protecting agent (filtering UVA light from entering the vitreous and retina) or as a damaging agent, by absorbing light and photodynamically generating reactive radicals that cause covalent chemical damage in the membranes and proteins.

Our measurements of the sensitization quantum yields of the coloured compounds suggest that NFK, KUA, AA and F are capable of generating radicals and thus act as potentially harmful agents in the lens. On the other hand, Ky, 3HK and 3HAA are not just benign filters but are active antioxidant defence chemicals that inhibit lenticular damage[3].

It is interesting to note that the human lens contains 3HK and its glucoside 3HKG, the levels of which go down with age. With our findings, we believe we now are able to explain the endogenous role of 3HK and 3HKG (as antioxidants)[4].

In related experiments, we have also been able to show that such photodynamic (or radical-mediated damage) occurs to the lens cell membranes through the peroxidation of the constituent lipids. In addition, lens crystallins are oxidized and covalently crosslinked to produce high molecular weight products that precipitate and cause opacity of the solution. We find that amino acid side chains trp, tyr, cys, met and his are oxidized. his is oxidized and the subsequent product crosslinks with the lys side chain[5,6].

4. The smoking–cataract connection

Of the multifaceted etiology of cataract, two striking epidemiological findings are that (i) the use of cheap smoke in cooking fuel such as firewood is linked to an increased risk of cataract, and (ii) cigarette smoking leads to an earlier onset of cataract, while stopping smoking correspondingly reduces the risk. In the light of the fact that significant fraction of the population in India (and in the third world countries) uses smoky cooking fuel, the risk factor of cataract can be expected

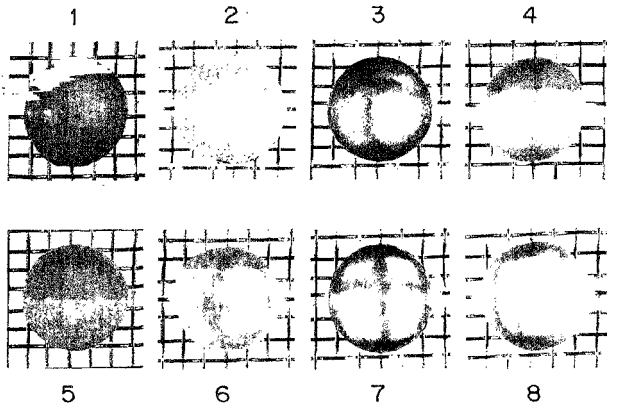

FIG. 4. FSC/CSC-induced colouration and opacification of rat lenses: effect of light and antioxidants. Lenses (1) and (5); incubated with FSC in the presence and absence of light, respectively; lenses (2) and (6): incubated with FSC and antioxidant mixture, in the presence and absence of light, respectively; lenses (3) and (7): incubated with CSC in the presence and absence of light, respectively; and lenses (4) and (8): incubated with CSC + antioxidant mixture, in the presence and absence of light, respectively. FSC and CSC were in phosphate-buffered saline, pH 7.4, with OD values at 270 nm of 26. Incubation was at ambient temp. for 24 h, with both the condensates and the antioxidants replenished at 4, 8, and 20 h. The light used was a 60-watt incandescent white light bulb placed about 2 feet above the radiation-sterilized 24-well Costar # 3424 Mark II tissue culture plate used for incubation. A large beaker filled with water was placed on top of the plate as a heat insulator. The antioxidant mixture contained 1 mM each of D-mannitol, Na benzoate, thiourea and 10 μM of *n*-propyl gallate, all in PBS

to be large[7]. In addition, this risk factor will further get enhanced in individuals who are active or passive smokers. In an effort to understand the molecular aspects of this smoke–cataract connection, we undertook this project.

There has been no reported work on this problem other than the epidemiological findings. However, the increased risk of carcinogenesis with smoking has been investigated in some detail. The damaging factors here are the polycyclic aromatic hydrocarbons (PAH) present in the smoke, as well as reactive oxygen species (ROS) that have been reported to be generated in cigarette smoke condensates (CSC). With regard to cheap smoky cooking fuel, extensive work has been done by the group of Dr Leela Srinivas of the Central Food Technological Research Institute (CFTRI), Mysore, on the cellular and molecular damage caused by firewood smoke condensate (FSC). They have shown that FSC is able to rupture red-blood cells, elicit membrane-lipid peroxidation and also cause single strand breaks in human lymphocyte DNA.

In collaboration with Drs Shalini and Leela Srinivas, we argued that the cataractogenic action of cigarette smoke and of fuel smoke might have a common basis and that this action could not be due to direct topical incidence since the eye lens is protected by the capsule, the aqueous humor, the cornea and the eyelid. Thus, we investigated the more likely probability of substances in the inhaled smoke being carried within the body to all tissues including the lens and causing damage there. In that event, FSC and CSC can be expected to operate in a similar fashion. Accordingly, we prepared FSC and CSC by smouldering twigs and dry leaves, or cigarettes, in a flask fitted inside with an air vent, and a long closed tube on the top. The smoke collected on the tube was condensed in phosphate-buffered saline (PBS) solution, filtered with glasswool and used as is, or after serial dilution. The optical density of the solutions at 271 nm were used as indices of their concentrations.

Incubation of isolated, intact encapsulated rat lenses in FSC or CSC was done for extended periods of time both in laboratory light and in darkness. The condensates were able to permeate the capsule, impart colour and opacity to the lens, and to leach out the lens and generate particulate debris upon incubation for several hours. We found that: (a) more damage occurs in the presence of visible light than in the dark, and (b) the addition of ROS–scavenging antioxidants to the incubate inhibits the damage, indicating that the process is oxidative in nature (see Fig. 4). The fact that it takes several hours of incubation in order for gross lenticular damage to occur and the fact that the ROS are short-lived species implies the continuous generation and a steady-state supply of ROS occurs in the condensates, lasting many hours.

Spectral analysis of FSC and CSC reveals the presence of a mixture of polycyclic aromatics with absorption bands in the near UV and visible regions. This finding, and the fact that lenticular damage is greater in light than in darkness, led us to investigate the possible photodynamic properties of CSC and FSC. We found that both the condensates were photodynamically efficient in producing superoxide anion (O_2^-), upon visible radiation (445 nm). Extended irradiation led to a time-dependent drop on O_2^- production and a concurrent bleaching of the fluorescence spectrum of the condensate leading to photodynamically inert but coloured compounds.

Elemental analysis of FSC and CSC, using ICP atomic emission spectroscopy revealed that FSC contained Mn, Fe, Cu, Pt and Ni (12, 18, 24, 48, 94 and 294 ng/g of fuel, respectively) while CSC contained Mn, Fe, Cu, Pb, Ni and Pt (6, 9, 14, 61, and 150 ng/g fuel, respectively). The presence of these metals would enable the condensates to generate hydroxyl radicals in the dark through the Fenton reaction. In confirmation, we found using the TBARS assay that FSC and CSC indeed generate $OH^{\cdot}$, for as long as 20 hours in amounts comparable to that produced by micromolar concentrations of the classical Fenton reagent.

We next monitored the molecular damage that occurs to the lens constituents by FSC and CSC. We found that the condensates bring about the peroxidation of the lipids isolated from the membranes of bovine lenses, as monitored by the TBARS as well as the diene–triene spectral assay. At the concentrations chosen, the efficiencies of the condensates to oxidise membrane lipids are higher than that of the Fenton

reagent. This reaction is time dependent; when FSC and CSC were kept standing for 20 hours and then incubated with lens membranes, little peroxidation occurred.

The other readily observable damage is the ability of the condensates to bring about covalent non-disulphide crosslinks in the lens crystallins leading to high molecular weight products. We, thus, believe that FSC and CSC bring about oxidative damage to lens cell membrane lipids and to the crystallins and these might be responsible for the eventual opacification and lenticular damage.

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A preliminary account of some of this work was presented as a keynote address at the XIX Conference of the Association of Clinical Biochemists of India, held at Hyderabad, during January 27-31, 1993.

References

1. BALASUBRAMANIAN, D. — *In situ* optical spectroscopy of some systems of biological interest, *Biosci. Rep. (Lond.)*, 1988, **8**, 498–508.

2 LUTHRA, M., SIVAKAMA SUNDARI, C., GUPTASARMA, P. AND BALASUBRAMANIAN, D. — Crystallin structure and eye lens function. In *Molecular conformation and biological interactions*—G.N. Ramachandran *Festschrift*, 1992, pp. 281–290, Indian Academy of Sciences, Bangalore, India.

3. MURALI KRISHNA, C , UPPULURI, S., RIESZ, P., ZIGLER, J.S., JR AND BALASUBRAMANIAN, D. — A study of the photodynamic efficiencies of some eye lens constitutents, *Photochem. Photobiol.*, 1991, **54**, 51–58.

4 LUTHRA, M. AND BALASUBRAMANIAN, D. — 3-Hydroxykynurenine and 3-hydroxyanthranilic acid may act as endogenous antioxidants in the eye lens, *Exp. Eye Res.*, 1992, **55**, 641–643.

5. BALASUBRAMANIAN, D., DU, X. AND ZIGLER, J.S. — The reaction of singlet oxygen with proteins, with special reference to crystallins, *Photochem. Photobiol.*, 1990, **52**, 761–768.

6. GUPTASARMA, P., BALASUBRAMANIAN, D., MATSUGO, S AND SAITO, I. — Hydroxyl radical-mediated damage to proteins with special reference to the crystallins, *Biochemistry*, 1992, **31**, 4296–4303.

7. BALASUBRAMANIAN, D , RAO, G.N. AND BHAT, K.S. — The epidemiology of cataract in India; a commentary on the Indo–US case related study, *Curr. Sci.*, 1990, **59**, 498–505.