3

Experimental Procedure

Cataract is opacity of the crystalline lens that usually increases with age and is the leading cause of blindness (Asbell *et al*., 2005). It is a protein aggregation ophthalmic disease where the insolubilization of modified proteins in the lens occurs (Harrington *et al*., 2004) due to a lack of turnover (Lynnerup *et al*., 2008). This implied that the analysis of biochemical changes and their characterisation in ocular lens can have clinical relevance for understanding the molecular basis of nuclear cataract (Truscott, 2005). Oxidative stress is the major cause of cataractogensis (Fletcher, 2010) and under this condition, the crystallins undergo post-translational modifications that tend to accumulate throughout the lifespan (Bloemendal *et al.,* 2004). Due to tissue density and structure, the characteristics of ocular lens changes according to density of cataract (Tabandeh *et al.,* 2000). Subsequently, the assessment of biochemical profile and characterisation of cataractous lens among assorted cataractous subjects were essential to understand the development of cataract and also to study the correlation of biochemical parameters with cataract development.

The experimental procedure pertaining to the study entitled "Biochemical Profile and Characterisation of Proteins in the Lens Extracted from Human Cataractous subjects" was performed under the following phases:

Phase I

3.1 Selection and Grouping of Subjects

3.1.1. Selection of Subjects

3.1.2. Grouping of Subjects

3.1.3. Collection of Eye Lens Samples

Phase II

3.2. Anthropometric Measurement and Lifestyle of Subjects

Phase III

3.3. Biochemical Assessment in Lens and Their Correlation with Cataract Development

3.3.1. Characteristics of Lens Tissue

3.3.2. Proteins

3.3.3. Antioxidants

3.3.3.1. Enzymatic Antioxidants

3.3.3.2. Glutathione System Components

3.3.3.3. Non-Enzymatic Antioxidants

3.3.4. Lipid Peroxidation Status

3.3.5. Nitrite Levels

3.3.6. Protein Carbonyl and Protein Sulphydryl

3.3.7. Enzymes of Polyol Pathway

3.3.8. Membrane Bound Enzymes

3.3.9. Sugars, Cholesterol and Nucleic acids

3.3.10. Glycoproteins

3.4. Statistical Analysis

Phase I

3.1. Selection and Grouping of Subjects

3.1.1. Selection of Subjects

The investigation commenced with the pursuit for ophthalmic centres in and around Coimbatore for the availability of cataractous lenses that fall under the framed inclusion and exclusion criteria. The samples were selected from Aravind Eye Hospital, Coimbatore, Tamil Nadu and Eye Foundation, Coimbatore, Tamil Nadu. This study was approved by Institutional Human Ethical Committee (HEC.2011.26). The informed consent (Appendix 1) was obtained from the subjects selected for the study. They were simultaneously administered with questionnaire (Appendix 2) to document the personal details, family history,

medical history and personal habits. All these details helped to identify few of the possible risk factors associated with cataract development.

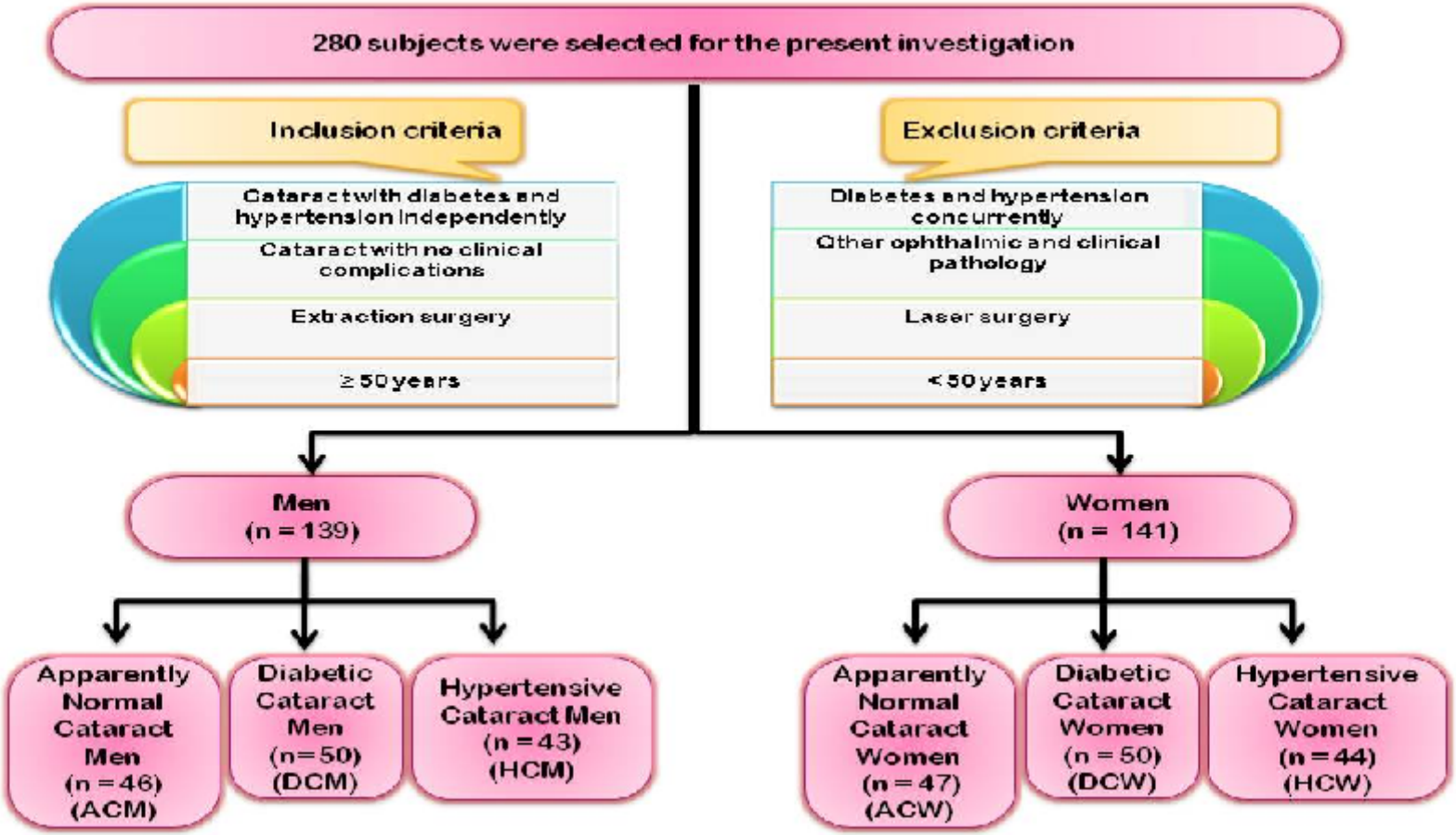
3.1.2. Grouping of Subjects

Subjects of interest were grouped based on their clinical complications that included diabetes mellitus and hypertension. The study was carried out in 280 cataractous subjects who satisfied the desired criteria and were grouped as in figure 13. Out of 280 subjects, 139 were men and 141 were women. Cataract subjects with co-morbidities of diabetes and hypertension occurring independently and those with cataract alone were selected for the research provided their age were ≥ 50 years. Cataract individuals who had diabetes and hypertension occurring concurrently were excluded from the investigation. People who had poor vision due to other ophthalmic pathology were disqualified from the study. The study was carried out in six groups as listed below:

- Apparently Normal Cataract Men (ACM) with n= 46
- Apparently Normal Cataract Women(ACW) with n= 47
- Diabetic Cataract Men (DCM) with n= 50
- Diabetic Cataract Women (DCW) with n= 50
- Hypertensive Cataract Men (HCM) with n= 43
- Hypertensive Cataract Women (HCW) with n= 44

Figure 13

Grouping of subjects for the study



The lens required for the study was obtained exclusively from the conventional surgery rather than the laser treatment. The cataractous lenses removed by laser surgery were avoided for the present investigation as they would break the lens completely and will be in emulsified form with the interference of some chemicals causing hindrance to the research.

3.1.3. Collection of Eye Lens Samples

Cataractous lenses were collected after cataract surgery from Aravind Eye Hospital and Eye Foundation, Coimbatore, Tamil Nadu. They were transported in an ice box to the place of research where all analysis were carried out. The extracted lenses were preserved in saline and stored in deep freezer (-40^{0}C) until further analysis. Lens samples were ground in motor and pestle and 10% homogenate was prepared in 0.1M sodium phosphate buffer. They were centrifuged at 10,000rpm for 10 minutes in cooling centrifuge and the supernatant was used for majority of further estimations except for those which needed different sample preparation that are mentioned in the corresponding appendices.

Phase II

3.2 Anthropometric Measurement and Lifestyle of Subjects

The height and weight of the subjects were recorded along with their blood pressure and according to World Health Organisation (WHO), body mass index (BMI) was calculated using the below given formula as given below. The individuals were categorised as underweight who had a BMI < 18.5kg/m^2, normal whose BMI ranged between 18.5kg/m^2-25kg/m^2, overweight with a range 25kg/m^2–30kg/m^2 and obese for whom BMI was > 30kg/m^2 (WHO, 2004).

$$BMI = \frac{\text{Weight [Kg]}}{(\text{Height})^2 \text{ [Sq m]}}$$

Several studies have suggested that body mass index was related to cataract (Caulfield *et al.,* 1999; Schaumberg *et al.,* 2000). The details of the subjects with regard to their age, sex, socioeconomic status, marital status and

employment status were collected at the time of administering the questionnaires to them. Age of subjects was recorded to know whether they fall into the desired inclusion criteria set up. Aging is the major risk factor in all types of cataracts where the cataract development is significantly increased with increasing age (McGwin *et al.,* 2003; Congdon *et al.,* 2005).

Phase III

3.3 Biochemical Assessment in Lens and their Correlation with Cataract Development

3.3.1 Characteristics of Lens Tissue

The weight, diameter and thickness of cataractous lenses of all subjects under investigation were recorded. The lenses obtained after surgery was preserved in saline and were dried using filter paper which were weighed in a butter paper using a digital weighing balance. Diameter and thickness of the lenses were measured using a divider and ruler in two different directions and their average was taken as the thickness of ocular lens capsule was found to be increased in diabetic lenses in earlier studies (Laurent *et al.,* 1981).

3.3.2 Proteins

The accumulation of insoluble proteins and oxidative damage in human lens, as they are more susceptible to age related degenerative changes leads to cataract formation especially the senile cataract form (Ponce *et al.,* 2006; Takemoto and Sorensen, 2008). It is believed that a precursor complex, water soluble high molecular weight (HMW) proteins mediate the water insolubilization of lens proteins (Srivastava *et al.,* 1996; Roy and Spector, 1976). The exposure of eyes to heat, chemicals and other environmental stresses causes perturbation on lens crystalline protein structure leading to opacification of lens due to spontaneous precipitation or crystallization of disturbed or denatured crystalline molecules as observed by Pande *et al.* (2001). Truscott (2010) opined that the progressive damage to these long lived proteins may contribute to the age related decline in function and that cumulative denaturation of proteins ultimately limits human life span.

Total protein in lens of all subjects was assessed by Lowry *et al.* (1951) as explained in Appendix 3. As age increases soluble protein is converted to insoluble protein causing lens opacity. The insoluble protein and soluble protein of all subjects were estimated in the pellet and supernatant respectively by the same method as described in Appendix 3.

3.3.3 Antioxidants

3.3.3.1 Enzymatic Antioxidants

Antioxidant enzymes synthesized by all aerobic organisms scavenge and inactivate the reactive oxygen intermediates (ROI) (Rumley and Paterson, 1998). The important antioxidant enzymes that protect the eyes from free radicals and hydrogen peroxide include catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), thus preventing the eye from oxidative damage (Bhagyalaxmi *et al.,* 2009; Lou, 2003). The production of superoxide dismutase and other repair systems within mitochondria helps in scavenging the reactive oxygen species (ROS) such as hydroxyl radical, hydrogen peroxide and peroxynitrite (Brennan *et al.,* 2012; McCord and Fridovich, 1969).

The enzymatic antioxidants viz superoxide dismutase and catalase in the eye lens tissue among the selected cataractous subjects were assessed as given in table 1.

Table 1

Methods of analysis of enzymatic antioxidants

Parameters	Method	Reference	Appendix
Superoxide dismutase	Spectrophotometry	Beauchamp and Fridovich (1971)	4
Catalase	-do-	Luck (1974)	5

3.3.3.2 Glutathione System Components

The major antioxidant in the lens is glutathione which maintains protein in a reduced form (Head, 2001) and prevents protein cross linking, crystalline aggregation and light scattering (Giblin, 2000). Antioxidant system of

glutathione includes reduced glutathione, GPx, GR and $NADPH^{+}$+ H^{+} in which the latter two are required for the reduction of oxidized glutathione and GSH regeneration. GSH/GSSG ratio is normally high in the lens and this glutathione redox cycle is localized in the lens epithelium and superficial cortex. The presence of glutathione with ascorbic acid is a requisite for the function of GPx which neutralizes reactive oxygen species and in detoxification of hydrophobic substances in reactions that are catalysed by GST (Lou, 2000).

The glutathione system include the components represented in table 2 and were analysed as mentioned in the same.

Table 2

Methods of analysis of glutathione system components

Parameters	Method	Reference	Appendix
Glutathione peroxidase	Spectrophotometry	Rotruck *et al*. (1973)	6
Glutathione-S-transferase	-do-	Habig *et al*. (1974)	7
Glutathione reductase	-do-	Beutler(1984)	8
Reduced Glutathione	-do-	Moron *et al*. (1979)	9

3.3.3.3 Non-Enzymatic Antioxidants

Vitamin E and A are lipid soluble antioxidants and vitamin C is water soluble antioxidant. The ocular structures contain vitamin C that functions through ascorbate-dehydroascorbate redox system and maintains the reduced forms of pyridine nucleotides and glutathione. The damage of cation pumps which are induced by UV radiation and photoperoxidation in the lens are curbed by ascorbic acid (Garland, 1991). Vitamin C may function as an antioxidant and protect lens proteases from photooxdiative destruction which is present in aqueous compartments of lens membranes (Blondin *et al.,* 1986). In tissues such as lens, where there is low partial pressure, carotenoids can be an effective

antioxidant (Spector *et al.,* 1993a; Woodall *et al.,* 1997). The concentration of vitamin E in ocular lens fibres and membranes may inhibit cataract formation by decreasing photo peroxidation of lens lipids and stabilising lens cell membranes (Varma *et al.,* 1982; Libondi *et al.,* 1985; Ohta *et al.,* 1996).

The levels of vitamin A, vitamin E and vitamin C were estimated in the lens by the methods given in the table 3.

Table 3

Methods of analysis of non enzymatic antioxidants

Parameters	Method	Reference	Appendix
Vitamin A	Spectrophotometry	Bayfield and Cole (1980)	10
Vitamin C	-do-	Omaye *et al*. (1979)	11
Vitamin E	-do-	Quaife *et al.* (1949)	12

3.3.4 Lipid Peroxidation Status

The impairment in lipid-lipid and protein-lipid interactions in lenticular fibre membranes and the change in lipid bilayer geometry occurs due to the accumulation of modified oxygen and phospholipid molecules resulting from lipid peroxidation, a detrimental factor for cataract (Babizhayev, 2012). Lipid peroxidation which is an indicator of oxidative stress in cells and tissues was estimated by Niehaus and Samuelsson (1968) as described in Appendix 13.

3.3.5 Nitrite Levels

Nitrite is the metabolite of nitric oxide (NO). Nitrite levels are estimated spectrophotometricallly as NO has a short half life (Ornek *et al.,* 2003). In animal studies, the cataractogensis was reported to be suppressed by an inhibitor aminoguanidine, which induces nitric oxide synthase (Inomata *et al.,* 2000; Paik and Dillon, 2000; Ito *et al.,* 2001). The nitrite levels in lens tissue among cataractous groups were assessed by Green *et al.* (1982) as explained in Appendix 14.

3.3.6 Protein Carbonyl and Protein Sulphydryl

The disulphide cross linking and molecular aggregates due to oxidation of sulphydryl content leads to protein precipitation and lens opacification (Kyselova *et al.,* 2005a). A valuable index to determine the protein redox status in the lens has been suggested to be the assessment of carbonyl and sulphydryl proteins (Altomare *et al.,* 1997). The estimation of levels of carbonyl proteins represents a direct measure of oxidative damage to these molecules (Stadtman, 1992) and that of sulphydryl levels represent an indirect measure of protein oxidation leading to protein aggregation (Boscia *et al.,* 2000).

The protein carbonyl and protein sulphydryl in cataractous lens tissue among selected subjects were estimated by the methods as mentioned in table 4

Table 4

Methods of analysis of protein carbonyl and protein sulphydryl

Parameters	Method	Reference	Appendix
Protein carbonyl	Spectrophotometry	Uchida *et al.* (1998)	15
Protein sulphydryl	-do-	Altomare *et al.* (1997)	16

3.3.7 Enzymes in Polyol Pathway

The polyol pathway includes two major enzymes viz aldose reductase (AR) and sorbitol dehydrogenase (SoDH). The primary mediator of diabetes induced oxidative stress in the lens has been illustrated by the polyol pathway (Chung *et al.,* 2003). The key rate limiting enzyme of the polyol pathway is AR that catalyses the conversion of glucose to sorbitol utilising NADPH as the cofactor and is found to be increased in diabetic patients (Ghahary *et al.,* 1989). The sorbitol formed from glucose is an osmolyte which is metabolized to fructose by SoDH (Kinoshita, 1974). The accumulation of sorbitol and other sugar alcohols within the lens leads to osmotic stress as these polyols are neither able to diffuse out easily nor metabolize rapidly consequently causing hypertonicity accountable for formation of cataract (Kinoshita *et al.,*1962).

Aldose reductase and sorbitol dehydrogenase activities were determined as given in table 5

Table 5

Methods of analysis of enzyme activities in polyol pathway

Parameters	Method	Reference	Appendix
Aldose reductase	Spectrophotometry	Hayman and Kinoshita (1965)	17
Sorbitol dehydrogenase	-do-	Gerlach and Hiby (1974)	18

3.3.8 Membrane Bound Enzymes

The normal cell permeability function of the lens epithelium by Na^+K^+ATPases is influenced by proteins containing thiol groups. Oxidised glutathione has low concentration in the lens as they are permaeable to lens membrane but lens membrane is impermeable to reduced glutathione (Lou, 2000). The alteration in Na^+K^+ATPases activity causes accumulation of Na^+ and loss of K^+ with hydration and swelling of the lens fibres leading to cataractogensis (Chylack and Kinoshita, 1969). Ca^{2+}ATPase counteract the inward passive diffusion of Ca^{2+}, which maintains the lenticular Ca^{2+} levels (Liu *et al.,* 2002). The oxidative damage caused to these enzymes as they are sensitive to oxidation may result in elevated levels of Ca^{2+} in the lens (Ahuja *et al.,* 1999) and are also sensitive to membrane lipid order which leads to their decreased activity and may be related to structural changes in membrane lipids (Borchman *et al.,* 1993).

Table 6

Methods of analysis of the activities of membrane bound enzymes

Parameters	Method	Reference	Appendix
Na^+K^+ATPase	Spectrophotometry	Bonting (1970)	19
Mg^{2+}ATPase	-do-	Ohnishi *et al.* (1982)	20
Ca^{2+}ATPase	-do-	Hjerten and Pan (1983)	21

3.3.9 Sugars, Cholesterol and Nucleic acids

The reaction of reducing sugars such as glucose with amino groups in proteins, lipids and nucleic acids occurs through Maillard reaction with the formation of a Schiff base which slowly reorganizes to form relatively stable amadori adducts (Glenn and Stitt, 2009). Cataract development occurs at higher levels of glucose due to osmotic and oxidative stresses in the lens (Harding, 1991a; Kinoshita, 1990).

Fructose forms advanced glycated end products (AGE) 10 times faster than glucose which is because of higher proportion of fructose existing in the acyclic form and fructose derived equivalent to the amadori product is more reactive than that of glucose derived (Kawasaki *et al.,* 1998). Fructose could contribute to oxidative damage in vivo as there is increased hydroxyl radical production due to the increased oxidation of proteins which occurs in the presence of fructose (Tagaki *et al.,* 1995).

The flexing of cell membrane during accommodation requires a certain degree of membrane fluidity which is dependent upon the lipid composition of the cell membranes (Truscott, 2009). An increase in the cholesterol content normally leads to alterations in membrane stiffness (Borchman *et al.,* 1996).

As nucleic acids are susceptible to oxidative damage by ROS, their continuous attack by the same results in unscheduled DNA synthesis in lens epithelium cells which undergo apoptosis (Jose and Yeilding, 1977).

The amount of sugars, cholesterol and nucleic acids in lens were estimated according to the methods represented in the table 7. The nucleic acids were extracted according to the procedure by Schneider (1957) with slight modifications as described in appendix 26. The extracted DNA and RNA from the mentioned procedure were used as sample for the estimation of DNA and RNA by diphenylamine and orcinol method that were determined spectrophotometrically.

Table 7

Methods of analysis of sugars, cholesterol and nucleic acids

Parameters	Method	Reference	Appendix
Glucose	Spectrophotometry	Trinder (1969)	23
Fructose	-do-	Ashwell (1957)	24
Total Cholesterol	-do-	Parekh and Jung (1970)	25
DNA	-do-	Burton (1956)	27
RNA	-do-	Bial (1902)	28

3.3.10 Glycoproteins

The complexes between carbohydrate and protein that are linked by covalent bond are defined as glycoproteins in which the carbohydrate moiety is referred to as glycan. The function of glycoproteins may be to assure the transparency of the lens at different stages of accommodation and growth by influencing the orientation of macromolecules at the fibrillary interfaces (Dische, 1965). The lens capsule contains approximately 11% carbohydrate (Fukushi and Spiro, 1969) and sugars in lens capsule includes galactose, glucose, mannose, sialic acids, hexosamines, fucose and hexuronic acid (Spiro and Fukushi, 1969).

The following glycoproteins given in table 8 were analysed in the lens among cataractous subjects.

Table 8

Methods of analysis of glycoproteins

Parameters	Method	Reference	Appendix
Hexose	Spectrophotometry	Niebes (1972)	29
Hexosamine	-do-	Wagner (1979)	30
Fucose	-do-	Dische and Shettles (1948)	31
Sialic Acid	-do-	Warren (1959)	32

3.4 Statistical Analysis

All the data obtained in the present investigation were subjected to statistical analysis using SPSS 16 version. Parametric and non parametric tests

were selected based on the normal distribution of the data by Shapiro Wilk test. One way ANOVA (parametric) and Kruskal Wallis (non parametric) was carried out for multiple group comparisons of cataractous subjects on normally and not normally distributed data respectively. But for a larger sample size of > 30 or 40 the data were analysed by parametric test albeit they were not normally distributed. The significance level was set at 5% interval. Correlation analysis was performed to identify the relationship between the biochemical parameters among cataractous subjects for which Pearson coefficient of correlation and Spearman's rank correlation analysis was followed on normal and non normal data respectively.