CHAPTER 2 : OUTLINE OF WORK

Homocysteine has been recognized as a risk factor for a number of human diseases, including cardiovascular diseases, stroke, peripheral occlusive disease, venous thrombosis, neural tube defects and neurodegenerative diseases. Recent clinical trials on Homocysteine lowering treatment did not prove to give beneficial results in protecting people from cardiac risk. Intermediary amino acids of Homocysteine metabolism, namely Cysteine, Glutamic acid, Glycine, Methionine and Taurine have been reported to play a critical role in disease mechanism at the level of transcription factors. The proposed study will focus on the mechanistic aspects of Homocysteine metabolism related retinal vascular diseases. Understanding of these pathways may pave the way for development of novel treatments to people who suffer from vascular eye diseases.

In this study, first, standardization and optimization of the method for simultaneous determination of Homocysteine related amino acids were established. Then secondly, the focus was on the protein modifications due to hyperhomocysteinemia in retinal vascular diseases. Eales' disease and Age related macular degeneration were selected as models since both are associated with retinal neovascularization. Further association of trace element iron and its transporters have been studied. Using peripheral blood mononuclear cells, ARPE-19 and HUVEC, GSH synthesis and turnover have been studied.

Objectives:

Objective 1: To determine the levels of amino acids involved in homocysteine metabolism and the protein bound homocysteine modification in retinal vascular diseases.

1.1 The estimation of homocysteine and the amino acids involved in homocysteine metabolism, namely methionine, cysteine, glutamic acid, glycine and taurine by Reverse phase high performance liquid chromatography using OPA derivatization method with fluorescent detector.

1.2 The levels of the above listed amino acids as in objective 1.1 in the plasma samples of patient with Eales' disease (ED) and Age related macular degeneration (ARMD) and to compare the results with respective controls.

1.3 The protein-Cys bound homocysteine and protein-Lys bound homocysteine in plasma samples of patients with ED and ARMD by Reverse phase high performance liquid chromatography OPA derivatization with fluorescent detection.

Objective 2: To study the associations of Elevated homocysteine with the levels of trace element iron *in vivo* using ED as a model of retinal vascular disease.

2.1 By estimating the serum and blood haemoglobin levels by spectrophotometer.

2.2 By estimating the serum iron and total iron binding capacity by auto analyzer.

2.3 Estimating the activity of aminolevulinic acid synthase (ALAS) and heme oxygenase levels in serum and PBMC.

2.4 Estimating the heme levels in serum and peripheral blood mononuclear cells (PBMC).

2.5 Estimating the ferritin, transferrin and serum transferrin receptor levels in serum and PBMC.

2.6 Estimating the vascular endothelial growth factor level in serum and PBMC.

2.7 By detecting the mRNA and Protein expression level of iron transporters hepcidin and ferroportin by real time PCR in PBMC.

2.8 By detecting the gene expression level of hypoxia inducible factor – HIF1α and HIF 2 by real time PCR in PBMC.

Objective 3: To correlate the level of homocysteine with glutathione synthesis and turnover using ED as a model of retinal vascular diseases (*In vivo*).

3.1 By estimating the glutathione levels by spectrofluorometer in plasma.

3.2 By estimating the ratio between reduced and oxidized glutathione levels using high performance liquid chromatography and electrochemical detector in plasma samples of ED.

3.3 By estimating the rate limiting enzyme ϒ-glutamate-cysteine ligase activity for the synthesis of glutathione by spectrofluorometer in plasma.

3.4 By detecting the gene expression level of ϒ-glutamate-cysteine ligase by real time PCR in human peripheral blood mononuclear cells.

3.5 By detecting the gene expression level of transcription factor – nuclear related factor (Nrf2) by real time PCR in human peripheral blood mononuclear cells.

Objective 4: To understand the effect of homocysteine in ϒ- glutamate-cysteine ligase by molecular dynamics simulation study using bioinformatics tool.

4.1 The Structural comparison of GCLC-Cys and GCLC-CSO complex.

4.2 The molecular dynamics simulations studies of glutamate-cysteine-ligase with cysteine and Homocysteine.

4.3 And binding pose analysis of glutamate-cysteine ligase with Homocysteine and cysteine.

Objective 5a: To understand the effect of homocysteine in their expression of glutamate-cysteine ligase, glutathione and nuclear related factor (Nrf2) in human ARPE-19 cells a cell culture model system for ARMD.

5.1 The cell viability by MTT assay in ARPE-19 cells exposed to homocysteine by spectrophotometer.

5.2 The glutathione levels in ARPE-19 cells exposed to homocysteine by spectrofluorometer.

5.3 The levels of the homocysteine related amino acids in ARPE-19 cells exposed to homocysteine by HPLC using OPA derivatization with FLD detector.

5.4 The mRNA expression level of ϒ-glutamate-cysteine ligase by real time PCR in ARPE-19 cells exposed to homocysteine.

5.5 The mRNA expression level of transcription factor – nuclear related factor (Nrf2) by real time PCR in ARPE-19 cells exposed to homocysteine.

Objective 5b: To understand the effect of homocysteine in their expression of ϒ-glutamate-cysteine ligase and nuclear related factor (Nrf2) in human umbilical vein endothelial cells (HUVEC) as a cell culture model system for vascular diseases.

5.6 The cell viability by MTT assay in HUVECs exposed to homocysteine by spectrophotometer.

5.7 The level of ϒ-glutamate-cysteine ligase by real time PCR in HUVECs exposed to homocysteine.

5.8 The mRNA expression level of transcription factor –nuclear related factor (Nrf2) by real time PCR in HUVECs exposed to homocysteine.