Beneficial Effect of Amino Acids in Advanced Glycation End Product Induced Endothelial Dysfunction in Bovine Retina

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Under the supervision of

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CERTIFICATE

This is to certify that the thesis entitled "Beneficial Effect of Amino Acids in Advanced Glycation End Product Induced Endothelial Dysfunction in Bovine Retina" submitted by Ms. S.Bharathi, ID. No. 2005PHXF418 for award of Ph.D. Degree of the Institute embodies original work done by her under my supervision.

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ABBREVIATIONS

AAM	Amino acid mixture
ABCAI	ATP-Binding Cassette Transporter A1
AChE	Acetyl choline Esterase
aFGF	Acidic Fibroblast growth factor
AGE	Advanced Glycation End products
ALE	Advanced Lipoxiation End Products.
ALR	Aldose Reductase
Ang	Angiopoietin
API	Atmospheric Pressure Ionization
AMD	Age-related Macular Degeneration
AR	Aldose Reductase
ASC	Alanine-Serine-Cysteine
BBB	Blood-Brain-Barrier
BCE	Bovine Capillary Endothelial cells
bFGF	Basic Fibroblast growth factor
BLH	Bleomycin Hydrolase
BM	Basement membrane
BREC	Bovine retinal Capillary endothelial cells
BRP	Bovine retinal pericytes
BSA	Bovine Serum Albumin
Ca-1	Upper calcium atom
CAD	Coronary artery disease
CEL	Né-(1-Carboxy ethyl Lysine)
CEP	CarboxyEthylPyruvate
COPD	Chronic obstructive pulmonary disease
CTGF	Connective Tissue Growth Factor
DAG	DiAcylGlycerol
DHP	Dihydropyridine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide

DR	Diabetic Retinopathy
DRVS	Diabetic Retinopathy Virectomy Study
DTNB	[5,5'-dithiobis(2-nitrobenzoic acid)]
DVL	Delta Valero Lactone
EC	Endothelial Cell
ECGF	Endothelial cell growth factor
ECM	Endothelial Cell Matrix
EDTA	Ethylene diamine tetra acetate
EHS	Engelbreth Holm-Swarm
EI	Electron Impact ionization
ELISA	Enzyme Linked ImmunoSorbent Assay
eNOS	Endothelial Nitric oxide Synthase
ER	Endoplasmic Reticulum
esRAGE	Endogenous secretary -Advanced Glycation End products Receptors
ESI	Electron spray ionization
ET	Endothelin
ETDRS	Early Diabetic Retinopathy Study
FACS	Fluorescence-activated cell sorter
FDP	anacrolein-lysine adduct
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
fMLP	Formyl MethionylLeucyl-Phenylalanine
FSC	Forward Scatter
FVP	Fibro Vascular proliferation
GFAT	Glucose Fructose-6-Phosphate Amido Reductase
GLUT	Glucose transporter facilitator
GSH	Glutathione
GTBL	Gamma thiobutyrolactone
HBr	Homotropine
HBSS	Hams balanced salt solution
HC	Homo Cysteine

HCTL	HomoCysteine Thiolactone
HCTLase	HomoCysteine Thiolactonase
HDL	High Density Lipoprotein
HNE-Lys	4-Hydroxynonenal-Lysine
HPLC	High Performance Liquid Chromatography
HSCs	Hepatic Stellate Cells
HSP	Hexose amine shunt pathway
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM	Intercellular Cell adhesion molecule
IDDM	Insulin Dependent Diabetic Mellitus
IEJ	Inter Endothelial Junctions
IF	Immunofluorescence
IHC	Immuno HistoChemistry
IMDM	Iscove's Modified Dulbecco's medium
iNOS	Isoform of Nitric oxide Synthase
IOP	Intra-Ocular Pressure
IRMAs	IntraRetinal Microvascular Abnormalities
IVTA	Intra vitreal triamcinolone
LC-MS	Liquid Chromatography Mass spectrometry
LDL	Low Density Lipoprotein
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemo attractant protein
MDA-lys	Malandialdehyde- Lysine
MEC	Microvascular endothelial cell
MG	Meningoma-Expressed
MGEA5	Meningoma-Expressed Antigen 5
MH	Macular Hole
MMP	Matrix metalloproteinase
MRM	Multiple Reaction Monitoring
mTOR	mammalian target of rapamycin
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	Nuclear facto k B

NG 2	Neuro-Glial 2
NIDDM	Non Insulin Dependent Diabetic Mellitus
NO	Nitric Oxide
NOS	Nitric oxide Synthase
NPDR	Non Proliferative Diabetic Retinopathy
NVD	Neovascularization Disc
NVE	Neovascularization
OGA	O- Glycoseamime
O-GlcNac	O-linked β-N-acetylglucosamine
8-OHDG	8-Hydroxy-2-DeoxyGuanosine
PA	Phenyl Acetate
PAF	Plasmi activation factor
PAI-1	Plasmin activator inhibitor
PAR1	Protease-Activated Receptor-1
PC	Pericyte
PD ECGF	Platelet Endothelial cell growth factor
PDGF-β	Platelet-Derived Growth Factor- Beta
PDGFR-β	Platelet-Derived Growth Factor Receptor-Beta
PDR	Proliferative Diabetic Retinopathy
PG12	Prostacyclin
PI3-Kinase	Phosphatidyl Inositol-3-Kinase
PKC	Protein Kinase C
PKC-DRS2	Protein Kinase C Retinopathy Study
PM	Pyridoxamine
PON	Paraoxonase
PON-AREase	Paraoxonase Aryl Esterase
ProQ	Protein quality Predictor
RAGE	Advanced Glycation End products Receptors
RAS	Resin-angiotensin system
RCT	Random Clinical Trial
RO	Reactive Oxygen
ROS	Reactive Oxygen Species

RT-PCR	Reverse Transcriptase Polymerase Chain reaction
SDS-PAGE	Sodium Dodecyl Sulphate- Polyacrylamide gel electrophoresis
SGLT	Sodium coupled glucose transporters
sICAM	Soluble Intercellular adhesion molecule
SMVC	Smooth Muscular vacular cells
SNP	Single nucleotide polymorhism
SPARC	Secreted Protein Acidic and Rich in Cysteine
sRAGE	Soluble form of Advanced Glycation End products Receptors
SSC	Side Scatter
STZ	Streptozidin
sVCAM	Soluble Vascular cell adhesion molecule
TAFI	Thrombin Activatable Fibrinolysis Inhibitor
TEMED	Tetra ethylene methylene diamine
TF	Tissue Factor
TGF- β	Transforming Growth Factor
THP-1	Human acute monocytic leukemia cell line
TIMP	Tissue Inhibitor of Metalloproteinases
TNB	5-thio-2-nitrobenzoic acid
TNF-α	Tumor Necrosis Factor-Alpha
TSP	Thrombospondin
UFF	Universal Force field
WHO	World Health Organization
Symbols	
α	alpha
β	Beta
γ	Gamma
Δ	delta
ϵ	Epsilon
κ	Kappa

ABSTRACT

Diabetic retinopathy (DR) remains one of the leading causes for blindness worldwide. Although there are many reports based on *in vivo* and *in vitro* studies on the pathophysiology of diabetic retinopathy there are still lacunae in the understanding of the cellular mechanisms deranged in the retinal capillary ECs and pericyte due to high glucose environment and the accumulation of advanced glycation end products. Hence the objective of this study is to establish the primary cell culture models of bovine retinal ECs (EC) and pericytes (PC) and look into the effect of Advanced Glycation End products (AGE) on the glucose uptake in retinal ECs and pericytes. This study revealed a decrease in the expression of GLUT-1 by immunofluorescence in the AGE-BSA treated cells at the end of 6 days exposure, compared to BSA alone and untreated cells both in BREC and BRP cells. However labeled Deoxy glucose and GLUT-1 expression by RT-PCR and FACS revealed no change that was statistically change. In addition, *in vitro, in vivo* as well as *in silico* studies were done to see the effect of AGE, homocysteine (Hcys) and Homocysteine thiolactone (HCTL) on the novel antioxidant enzyme paraoxonase (PON) which exhibits both HCTLase and arylesterae (AREase) activity depending on the substrates. The vitreous levels of HCTL and the HCTLase activitywere reported for the first time in this study. The PON-HCTLase activity (spectrophotometry) in the vitreous of proliferative diabetic retinopathy cases (PDR) was found to be significantly elevated with a mean of 176.7 ± 16.2 U/L compared to a non diabetic disease control namely Macular Hole (MH) which showed a mean activity of 94.74 ± 13.7 U/L (p=0.00).The mean level of HCTL levels as detected by Mass spectrometry was found to be 1.37 nM ± 0.51 nM in PDR compared to 0.65 nM ± 0.18 in MH There was a significant decrease in the AREase activity of PON detected spectrophotometrically, *in vivo* as seen in PDR vs MH vitreous.. A significant positive correlation was observed between vitreous HCTL levels and the corresponding HCTLase activity, in PDR (r =0.77, p= 0.033). Amongst the isoforms of PON, PON 2 isoform was identified by western blot in the vitreous. The *in vivo*

findings were evaluated by *in vitro* experiments which showed a dose and time dependent increase of HCTLase activity, as well as significant (p=0.00) decrease in the AREase activity in response to added HCTL in the BREC cultures. A similar response was also seen for Hcys. In addition the expression of PON 2 was found to be significantly increased in cells treated with Hcys as well as HCTL and the effect was much pronounced when treated with Hcys. In order to understand if Hcys has a regulatory role on PON 2, *in silico* analysis was done for which the PON 2 protein was modeled based on PON 1 template using modeler 9V7. . It was observed that the PON 2 had lowest binding energy and highest affinity for the physiological substrate HCTL (-6.63Kcal/mol) than PON1 which showed a binding energy of (-5.72Kcal/mol). This is indicative of stronger lactonase activity of PON2 when compared to its esterase activity. The semi-flexible blind docking of Hcys with protein PON 2 reveals that the ligand Hcys is involved in the same hydrophobic interactions at the active site as for the HCTL and phenyl acetate. Therefore it is quite possible that Hcys can affect the HCTLase and AREase activity of PON 2. With respect to the effect of AGE on the PON activities, in vitro studies in BREC cultures showed dose dependent decrease in the activity of HCTLase with increasing concentration of AGE. This effect of AGE on PON activity was found to be retrieved by all the amino acid tested in the order of leucine (0.018), lysine (0.013) and cysteine(0.019), glutamic acid (0.042) and glycine (0.029). The effect of amino acids on the tube formation assay in BREC was tested in the presence of high glucose (30mM) as a pro-angiogenic factor, It was found that Leucine, cysteine and lysine were not effective but, glycine and glutamic acid were anti angiogenic dose dependently (0. 5 to 5 mM) with as much as 70% inhibitions in the concentrations studied. A similar effect on tube formation was observed when advanced glycation end products was used as the pro-angiogenic factor (100microgram AGE) and it was found to be inhibited by amino acids similar to the effect seen in earlier high glucose condition. There was an inhibition of migration induced by AGE (as evaluated by scratch assay in BREC culture) by both the amino acids glycine and glutamic acids and it was observed to be more effective with glutamic acid. The effect of amino acids in inhibiting the adhesion of monocytes as induced by AGE was also tested. It

was observed that glycine had a relatively inhibitory effect on adhesion of monocytes on the cells while glutamic acid had a mild effect. The effect of amino acids on vascular endothelial growth factor, a marker of angiogenesis was also studied by immunofluorescence (IF) and RT-PCR and it was found Glycine had a dose dependent decrease of VEGF mRNA expression showing a 50% inhibition as seen in BREC, 80 % in BRP and 70% drop in BRPE at a concentration of 1mM. The RAGE protein expression (receptor of AGE) as seen by IF was found to be deceased in all the three cells studied. However glutamic acid showed more of the decrease than Glycine. The actin stress fibers were also stained with phalloidin and there was an inhibition in the formation of the same by both the amino acids as seen by IF. All these observations were highly indicative of amino acids, especially glycine and glutamic acid as anti-angiogenic agents

CHAPTER 1: REVIEW OF LITERATURE

INTRODUCTION

Diabetes mellitus is increasing at an alarming rate, 171 million people were affected with diabetes in 2000 and WHO predicts that 366 million people world wide would be affected with diabetes by the year 2030. Coming to the South East Asian population it is predicted that the diabetes rate would increase to 119,541 million (2030) out of which the Indians affected would be 79,441 million. The WHO calculations indicate that worldwide almost 3 million deaths per year are attributable to diabetes. There are 2 major types of diabetes Type 1 or insulin-dependent diabetes (IDDM) and Type 2 or Non insulin-dependent diabetes (NIDDM). The complications of NIDDM and IDDM represent the major cause of morbidity and mortality. Together, macro- and microvascular diabetic complications are an ever-increasing burden to the healthcare authorities of the developed nations and therefore warrant immediate attention.

Retinopathy is one of the most common microvascular complications of diabetes and is still the leading cause of blindness in the working population of developed countries. Vision threatening retinopathy is rare in type 1 diabetes patients in the first initial (3-5 years) of diabetes but they soon develop retinopathy with in the next decade.In the case of Type 2 diabetes, 21% of the patients exhibit retinopathy at the first diagnosis (2003). In the South Indian Type 2 diabetic subjects, duration of diabetes, glycosylated HbA1c, male gender, postprandial hyperglycaemia and insulin therapy were found to be independent risk factors affecting the severity of Diabetic retinopathy. (DR) (Pradeepa, Anitha et al. 2008).

Retinopathy has been classified as follows (Mala et al 2003)

Nonproliferative Diabetic Retinopathy (NPDR)

A. Mild NPDR

- Atleast one microaneursym

B. Moderate NPDR

- ❖ Hemorrhages or Microaneurysms (H/Ma)
- ❖ Soft exudates, Venous beading (VB), and Intraretinal microvascular abnormalities (IRMAs) definitely present.

C. Severe NPDR

- ❖ H/Ma in all 4 quadrants
- ❖ VB in 2 or more quadrants
- ❖ IRMA in at least 1 quadrant

D. Very Severe NPDR

- ❖ Any two or more of severe NPDR

Proliferative Diabetic Retinopathy (PDR)

A. Early PDR

- ❖ New vessels growth on the retina

B. High-Risk PDR

- ❖ New vessels on the disc (NVD) of 1/4 to 1/3 or more within the disc area
- ❖ Any neovascularisation or pre retinal vitreous hemorrhage

Clinically Significant Macular Edema (any one of the following)

- ❖ Thickening of the retina located 500 μm or less from the center of the macula
- ❖ Hard exudates at 500 μm or less from the center of the macula with thickening of the adjacent retina
- ❖ A zone of retinal thickening, one disc area or larger in size, any portion of which is one disc diameter or less from the center of the macula

All these factors are detectable ophthalmoscopically, because the pigments in the blood and the lipid exudates stands out in contrast to the otherwise transparent retina. The pathological manifestation of DR commences with an initial and progressive decay of retinal vasculature with increased vascular permeability gradually yielding to diabetic macular edema and PDR. Important characteristics include microaneurysms, pericyte loss and basement membrane thickening (Engerman and Kern 1995).

Although advanced glycation end-products, Protein Kinase C activation and the initial onset of acute intensive insulin therapy have been associated with aggravation of DR (1995), the mechanisms linking this disease to its vascular phenotype remain ill-defined. The vascular cells which are most extensively studied to understand the pathophysiology of DR include the retinal ECs and the pericytes.

1.1: ENDOTHELIAL CELL PHYSIOLOGY

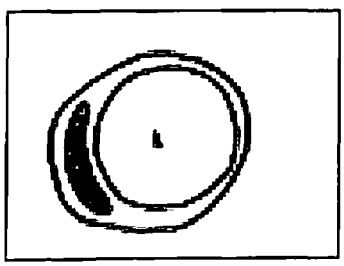
Shortly after the first description of circulating blood by William Harvey in 1628, the existence of a network of blood vessels arose from the studies of Malphigi, who described the physical separation between blood and tissue. In the 1800s, Von Reckingausen established that vessels were not merely tunnels bored through tissues but were lined by cells. The EC surface in an adult human is composed of approximately 1-6X10 ^{13} cells, weighs approximately 1 kg, and covers a surface area of approximately 1 to 7 m^2. ECs line the vessels in every organ system and regulate the flow of nutrients diverse biomolecules, and the blood cells themselves. The endothelium also plays a pivotal role in regulating blood flow, this results from the capacity of quiescent ECs to generate an active anti thrombotic surface that facilitates the transit of plasma and cellular constituents throughout the vasculature. (Cines, Pollak et al. 1998)

1.1.1. Quiescent and activated endothelium

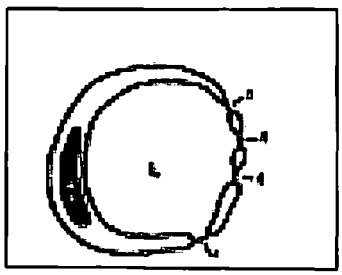
Endothelium although being metabolically active, is considered to be quiescent because the turnover of these cells are very low (Engerman, Pfaffenbach et al. 1967). Migrating ECs seem to assume a different phenotype (Kaprielian and Patterson 1994) and proteolytic activity seems to be necessary for the endothelial invasion into tissues. Extracellular matrix molecules and their receptors on endothelium probably play an important role in these processes and may differ in quiescent VS proliferating or migrating ECs. For example, fibronectin and its receptors are required for normal vascular development (Yang, Rayburn et al. 1993) (Brooks, Clark et al. 1994).

1.1.2: The concept of heterogeneity of endothelium

On the basis of morphology, microvascular endothehium in the normal adult organism has been divided into different phenotypes (Bennett, Luft et al. 1959)



- **Continuous**



- **Fenestrated**
- **Discontinuous**

Table 1.1: Location of Continuous and Discontinuous Endothelial Cell and Its Function.

Tissue or Organ	Properties	Functions
Continuous		
CNS	**Low no. of vesicles, complex tight junctions**	**Blood- brain barrier**
Lymph nodes	High endothelial venules HEV	Lymphocyte homing
Muscles	High no. of vesicles	Exchange/Transport
Discontinuous		
Endocrine glands	Fenestrate	Secretion
Gastrointestinal tract	Fenestrate	Absorption
Choroid Plexus	Fenestrate	Absorption
Kidney glomeruli	Pores	Filtration
Liver	Large gaps	Exchange of particles
Bone marrow	Marrow sinus	Hemopoiesis,delivery of blood cells
Spleen	Splenic sinus of red pulp	Blood cell processing

Adapted from: Werner risau et.al.

The morphological differences correlate with vascular permeability. In continuous capillaries, the endothelial cytoplasm is continuous and there is no fusion of luminal and abluminal plasma membranes except at the cell junctions. The only difference between fenestrated and discontinuous capillaries is the diameter of the pore and the presence or

absence of a diaphragm. Discontinuous ECs usually have clustered holes with a diameter of 80-200 nm each at the tapering edges of the cell. Functionally, fenestrated capillaries are more permeable to low molecular weight hydrophillic molecules, which is consistent with their presence at sites of filtration, secretion, and absorption (Larson, Carson et al. 1987). Because discontinuous, fenestrated endothelium occurs in close proximity to epithelium, interaction between the two cell types has been thought to be important for the differentiation and maintenance of fenestrae.

The continuous endothelium (as found in pulmonary, coronary, skeletal muscle and splanchnic vascular beds) is described as being restrictive because solutes with molecular radii of up to 3 nm move passively across the barrier via the paracellular route. The transcellular vesicular pathway is responsible for the active transport of macromolecules as shown for albumin (Minshall, Tiruppathi et al. 2000). Paracellular permeability is regulated by a complex interplay of cellular adhesive forces balanced against counter adhesive forces generated by actinomyosin molecular motors. The unperturbed endothelial barrier has restrictive properties that are primarily due to closed inter endothelial junctions (IEJ). Evidence now suggests that integrin receptor binding to the extracellular matrix (ECM) can also contribute to the barrier function by stabilizing the closed configuration of IEJs. The inflammatory mediator's thrombin, bradykinin, histamine, vascular endothelial growth factor (VEGF) and others upon binding to their receptors, disrupt the organization of IEJs and integrin- ECM complexes, thereby opening the junctional barrier (Dudek and Garcia 2001). Thus the formation of minute intercellular gaps allows passage of plasma proteins including albumin and liquid across the endothelial barrier in an unrestricted manner. The signaling pathways regulating opening and closing of junctions are of great interest as they relate to the regulation of fluid balance.

1.1.3: Glycocalyx

The glycocalyx is a negatively charged, surface coat of proteoglycans, glycosamino-glycans and adsorbed plasma proteins lining the luminal surface of the endothelium.The negative charge repels red blood cells (Damiano 1998), suggesting

that the glycocalyx can modulate oxygen delivery in a charge-dependent manner. Glycocalyx shields the endothelium from leukocyte attachment (Mulivor and Lipowsky 2002).

1.1.4: Extracellular Matrix

The ECM consists of collagen IV, fibronectin, entactin, laminin, chondroitin sulfate, and heparan sulfates, perlecan and syndecan. It appears in cross-section as a fuzzy band 40–60 nm thick. Matricellular proteins like thrombospondin (TSP) and secreted protein acidic and rich in cysteine (SPARC) are also present in the ECM. The first step in ECM assembly is the secretion by ECs of laminin polymers, which bind predominantly to α1-integrins (Albelda, Daise et al. 1989). Collagen IV polymers then interact with laminin polymers in the ECM space to form a scaffold onto which other ECM proteins are assembled to produce a basement membrane of unusually high elasticity and tensile strength (Kalluri 2003). ECs synthesize and secrete these ECM constituents during angiogenesis and vasculogenesis (Pratt, Form et al. 1985; Vlodavsky, Folkman et al. 1987) and have the capacity to continuously remodel ECM in mature vessels (Form, Pratt et al. 1986). The interaction of the ECM with EC surface integrins generates signals that inhibit EC proliferation and migration, but stimulate cell-cell and cell-ECM adhesion (Iruela-Arispe, Diglio et al. 1991). The ECM also plays an important role in remodeling the endothelium. This is achieved by the "counteradhesive" proteins such as SPARC and MMPs (Zucker, Mirza et al. 1998), VEGF (Partridge, Jeffrey et al. 1993; Kato, Lewalle et al. 2001) and TNF-α stimulates the production of MMP-2 and -9 and SPARC in ECs (Li, Zhang et al. 2003).

Endothelial functions

1.1.5: Vasoregulation

ECs contribute to the regulation of blood pressure and blood flow by releasing vasodilators such as nitric oxide (NO) and prostacyclin (PGI2), as well as vasoconstrictors, including endothelin (ET) and platelet-activating factor (PAF). NO is constitutively secreted by ECs, but its production is modulated by a number of

exogenous chemical and physical stimuli. Receptor-dependent agonists that stimulate eNOS include thrombin, adenosine 5'-diphosphate, bradykinin, substance P and muscarinic agonists, in addition to shear stress (Awolesi, Widmann et al. 1994). In addition to eNOS, cytokines have aslo been shown to stimulate inducible NOS (iNOS), or the Nos 2 gene product (Loscalzo and Welch 1995). NO has several important functions, it maintains basal tone by relaxing vascular smooth muscle cells (Mendelsohn, O'Neill et al. 1990). NO also inhibits platelet adhesion, activation, secretion and aggregation, and promotes platelet disaggregation, in part through a cyclic GMP dependent mechanism (Kubes, Suzuki et al. 1991). In addition to these effects on the vasculature, endothelial derived NO inhibits leukocyte adhesion to the endothelium (Garg and Hassid 1989) and inhibits smooth muscle cell migration and proliferation (Levin 1995). This suggests that NO helps to sustain vascular repair mechanisms. ECs produce not only the potent vasodilator NO, but also synthesize endothelin-1 (ET) (Marmur, Rossikhina et al. 1993), the most potent vasoconstrictor identified to date. ET-1 is not stored in granules but is formed after transcription of the gene encoding preproendothelin-1, the inactive precursor of ET-1, after stimulation by hypoxia, shear stress and ischemia. ET-1 released from ECs binds to the abundant G-protein coupled ET-A receptor expressed on vascular smooth muscle cells, which results in an increased intracellular calcium concentration and in turn, increases vascular smooth muscle cell tone. In states of endothelial dysfunction, such as atherosclerosis, in which concentrations of bioactive NO are reduced, the relatively unopposed actions of ET-1 promote vasoconstriction and smooth muscle proliferation.

1.1.6: Coagulation

The pivotal step in transforming the EC membrane from an anticoagulant to a procoagulant surface is the induction of tissue factor (TF). TF dramatically accelerates factor VIIa-dependent activation of factors X and IX. TF expression is rapidly induced after vascular injury (Hatakeyama, Asada et al. 1997), and TF is found associated with ECs within atherosclerotic plaque (Bajzar, Morser et al. 1996). TF may also contribute to the regulation of angiogenesis and tumor metastases through mechanisms independent of coagulation. The most thoroughly characterized EC binding site for a

coagulation protein is the thrombin receptor, also termed the protease-activated receptor-1 (PAR1). Binding of thrombin leads to a wide array of changes in expression of prothrombotic and antithrombotic molecules in cultured ECs, including TF, PAI-1, NO, PAF, ET, and PGI2, and disruption of cell-cell contacts. Thrombin is also mitogenic for ECs, fibroblasts, and smooth muscle cells and is chemotactic for monocytes. Binding of thrombin to thrombomodulin accelerates its capacity to activate a protein known as thrombin activatable fibrinolysis inhibitor (TAFI). TAFI is a procarboxypeptidase-B like molecule that, when activated, cleaves basic carboxyterminal residues within fibrin and other proteins. This results in the loss of plasminogen/plasmin and t-PA binding sites on fibrin such that fibrinolysis is retarded. Thus, through the regulated expression of thrombomodulin, ECs serve as potent templates to decrease the rate of intravascular fibrinolysis (McEver, Moore et al. 1995).

1.1.7 : Leukocyte adhesion during inflammation

During inflammation, leukocytes tether to and roll on the EC surface. The cells that are arrested, spread, and finally squeezes between ECs to reach the underlying tissues. Unlike platelets, which typically attach to the subendothelium of arteries under high shear stresses, leukocytes usually attach to the ECs, where shear stresses are lowest. In most circumstances, interactions with selectins, transmembrane glycoproteins that recognize cell-surface carbohydrate ligands found on leukocytes, initiate and mediate anchoring and rolling of leukocytes on the EC surface (Diacovo, Puri et al. 1996).

Selectin ligands expressed on high endothelial venules also mediate rolling of activated platelets and enhance accumulation of lymphocytes in lymph nodes (Sims 1991). Thus, selectins initiate inflammatory, immune, and hemostatic responses by promoting transient multicellular interactions under conditions of shear stress.

1.2 : PERICYTES

Broadly defined, pericytes are polymorphic, elongated, multibranched periECs that vary in their degree of envelopment of ECs in the microvasculature. Unlike smooth muscle cells, pericytes are covered by the same basement membrane of ECs except where two mural cells are in contact. Contact sites between pericytes and ECs are characteristically peg-and-socket or adhesion types of varying degrees of tightness (Larson, Carson et al. 1987) . Pericytes and ECs also form gap junctions (Nehls and Drenckhahn 1991). Based on microvascular location and histological characteristics, at least three types of pericytes exist: precapillary, capillary and postcapillary. Data obtained by immunostaining of mesenteric and retinal microvessels; pre- and postcapillary vessels stain positively for the smooth muscle isoform of cr-actin whereas in midcapillaries this pericyte protein is not present. Hence, microvessel pericytes located proximally and distally to the "true" capillary are thought to be a smooth muscle transitional type (Tilton, Kilo et al. 1979).

1. Pericytes are morphologically, biochemically and physiologically heterogeneous.
2. The *in situ* ratio of pericytes to ECs as well as the endothelial area covered by pericytes to a degree appears to be related to the degree of tightness of the interendothelial junctions and to the level of microvascular blood pressure. The greater the pericyte number and coverage, the better the microvascular barrier and in tissues it can be in the order of: retina, brain > lung > skeletal muscle > cardiac muscle > adrenal gland, and so on relating to the barrier function.
3. Pericytes regulate endothelial proliferation and differentiation.
4. Pericytes are contractile and the contraction may either exacerbate or stem endothelial junctional inflammatory leakage.
5. Pericytes function as a progenitor cell.
6. Pericytes synthesize and secrete a wide variety of vasoactive autoregulating agonists.
7. Pericytes synthesize and release structural constituents of the basement membrane and ECM.

8. Pericytes are involved in specific microvascular diseases.

Protruding from the cell body are long processes which parallel the long axis of the capillary and taper to smaller processes which encircle the capillary wall. Pericytes are embedded within a basement membrane which surrounds the capillary tubes. Their processes penetrate the basement membrane to directly contact the underlying endothelium and, in a reciprocal manner, endothelial processes penetrate into the pericytes (Williamson, Chang et al. 1993). *In vitro* evidence suggests that both EC and pericytes contribute to the formation of the basement membrane (Ballabh, Braun et al. 2004).

The differences in distribution and structure among pericytes suggest that they may have vessel- or tissue specific roles. Hence, pericytes have a variety of proposed functions including: regulation of capillary blood flow, as multipotent mesenchymal cells and specific precursors to vascular SMC, phagocytosis, and regulation of new capillary growth. Neuron-glial 2 (NG2), a chondroitin sulfate proteoglycan, and platelet-derived growth factor receptor beta (PDGFR), a tyrosine-kinase receptor, are cell-surface proteins. Antibodies against these proteins are commonly used to identify pericytes in tissue sections.

1.2.1 : Functions of Pericytes in Brain

The highest density of pericytes in the body is found in vessels of the neural tissues, such as the brain and the retinas. The reason for this is that ECs in the brain form a continuous endothelium with complex, tight junctions and they interact with astrocytic pedicels and with numerous pericytes to create the blood-brain barrier (BBB), which protects brain cells from potentially toxic blood-derived factors (Verbeek, de Waal et al. 1997). Pericytes play an essential role in the integrity of structural vessels and the BBB. Vessel degeneration is observed in hereditary cerebral hemorrhage with amyloidosis (Hayashi, Nakao et al. 2004), and pericytes have been shown to protect hypoxia-induced BBB disruption in vitro (Thomas 1999). Most interesting is that pericytes in the brain can perform macrophage-like activities, thus providing an immunological defense. This phenotype has raised the hypothesis that pericytes can

act as precursor cells of macrophages in the brain, and there are several observations to support this idea (Balabanov, Washington et al. 1996). Pericytes display several types of phagocytotic activity. They express scavenger receptors, which have broad ligand binding specificity, and are crucial in routine scavenging of many different molecules. Pericytes in culture can ingest various macromolecules, including polystyrene beads. In addition, pericytes express Fc receptors, which are essential for antibody-antigen complex recognition to trigger antibody-dependent phagocytosis (Suematsu and Aiso 2001).

1.2.2 : Functions of pericytes in Liver

Pericytes also have specialized functions in the liver. Hepatic stellate cells (HSCs), also called Itoh cells (Sato, Suzuki et al. 2003) are the pericyte equivalent in the liver (Abbott 2002). They are located between the parenchymal cell plates and the sinusoidal ECs. In contrast to the continuous brain endothelium, liver ECs are highly fenestrated and discontinuous. They line the hepatic sinusoids and mediate the exchange of metabolites between the portal blood, Kupffer cells, and hepatocytes and the processing of toxins (Sato, Suzuki et al. 2003). Although a dense basement structure between hepatic epithelial cells and ECs does not exist, HSCs have close contact with ECs through incomplete basement-membrane components and interstitial collagen fibers. HSCs regulate the remodeling of the ECM by producing both ECM components and matrix metalloproteinases. HSCs are also involved in vitamin A metabolism and contain more than 80% of the total vitamin A in the body (Knittel, Dinter et al. 1999). Finally, also HSCs are involved in the recruitment of inflammatory cells during hepatic-tissue repair and in fibrotic responses to liver diseases (Betsholtz, Karlsson et al. 2001).

1.2.3 : Functions of pericytes in Kidney

Pericytes of the glomerular capillaries in the kidney are called mesangial cells and account for approximately 30% of the glomerular cells. The cells are instrumental in the intussusceptive branching or splitting of a single invading vascular loop into

several glomerular capillaries, which creates a significantly, increased capillary surface area for blood ultrafiltration (Hellstrom, Kalen et al. 1999). Pericytes have a complex ontogeny, because they can develop from various cells, as a function of their location in the embryo.

1.2.4 : Pluripotent nature of pericytes

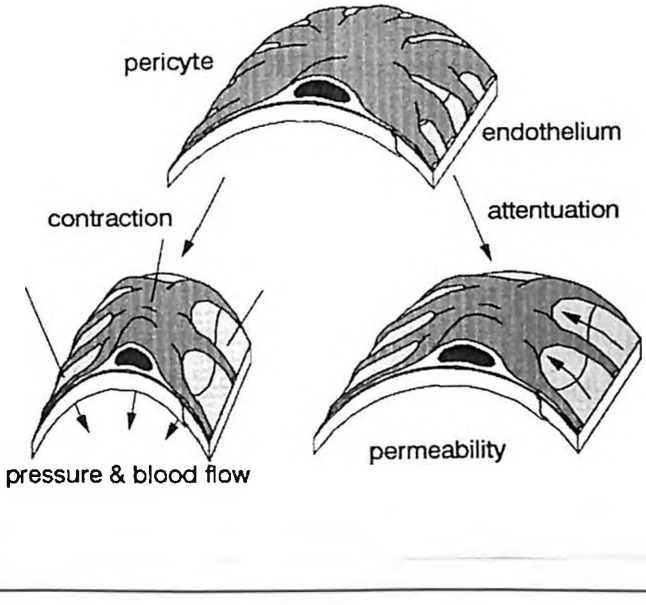
Pericytes have a complex ontogeny, because they can develop from various cells, as a function of their location in the embryo. TGF- β1 appears to initiate differentiation of PDGFR + pericyte progenitor cells that are then chemotactically attracted by PDGF-B- secreting ECs in the capillary plexus (Chen and Lechleider 2004). TGF- β 1 also appears to drive the differentiation of neurocrest or mesenchyme derived progenitors into smooth muscle like cells (Carmeliet 2004). Further more, *in vitro* analyses revealed that there exists a common VEGFR2 + vascular-progenitor cell type, derived from embryonic stem cells, which has the ability to differentiate into ECs in the presence of VEGF or into vascular smooth-muscle cells when PDGF-B is added (Gittenberger-de Groot, DeRuiter et al. 1999). Finally, there have been reports that pericytes can be generated from ECs by transdifferentiation in a TGF β dependent manner, as has been described in the dorsal aorta (Nakajima, Mironov et al. 1997), and cardiac valves (Canfield, Allen et al. 1990).

1.2.5 : Functions of pericytes

Retinal pericyte synthesize most of the structural and adhesive proteins associated with the ECM (Canfield, Schor et al. 1989). Sparse cultures of pericytes from the retina and the brain produce an ECM in collagen I and III, fibronectin, and thrombospondin. Since cultured pericytes express phenotypic traits of bone cells *in vitro,* pericytes may be osteoblast progenitor cells. Pericytes synthesize plasminogen activator inhibitor (type I) and in larger amounts than smooth muscle cells or ECs (Wakui 1992). Epidermal growth factor receptors (EGF-R) are localized in immature capillaries on the pericyte membranes at the cytoplasmic tips where they interdigitate with ECs (Takahashi, Brooks et al. 1989). A smooth muscle-like function for pericytes is emphasized by the identification of ET-1 receptors (Lee, Hu et al. 1989).

Diacylglycerol content is increased 24% when pericytes are stimulated with ET-1 (Dodge, Hechtman et al. 1991). In co-culture with ECs, pericytes contract when either agonist is added to the media. The mechanism is believed to be by the way of stimulating the secretion of endothelial-derived ET-1, which in turn binds to pericyte receptors to induce contraction (Orlidge and D'Amore 1987). Substantive studies document that endothelial proliferation is in part controlled by pericytes (Antonelli-Orlidge, Saunders et al. 1989). In cocultures pericytes secrete an inhibitor, the active form of transforming growth factor beta (TGF-β), that controls endothelial growth at certain cell densities (Goumans, Valdimarsdottir et al. 2002). Observations *in vivo* as well as *in vitro* indicate that pericytes modulate endothelial proliferation and vice versa. The following figure shows the EC pericyte interaction of how they exist in the vasculature figure 1.1

Figure 1.1: Endothelial cell and pericyte interaction



1.3 : ENDOTHELIAL PERICYTE INTERACTION

1.3.1 : TGF beta signalling

Studies highlight the intimate interactions between ECs and pericytes, indicating their functional interdependence. TGF-β has context-dependent effects on ECs, and different endothelial responses are mediated by signaling through ALK1/Smad1/5

(proliferation) and ALK5/Smad2/3 (differentiation) (Lebrin, Goumans et al. 2004). Endoglin promotes ALK1 signaling, thereby shifting the TGF- β response toward proliferation (Carvalho, Jonker et al. 2004). Endoglin knockouts and endothelium-specific knockouts of *tβrII* and *alk5* found that the disrupted TGF- β signaling in ECs also impaired the TGF- β /ALK5 signaling in adjacent mesenchymal cells, inhibiting their differentiation into VSMC and association with the endothelial tubes. Therefore, TGF- β signaling in ECs promotes TGF- β synthesis, expression and release by these cells, which in turn induces differentiation of VSMC from surrounding mesenchymal cells thus reinforces TGF- β expression in ECs as an autoregulatory loop (Vikkula, Boon et al. 1996). Figure 1.2

1.3.2 Angiopoietin – Tie2 Signaling in the Vascular Wall

Numerous studies suggest that the angiopoietin–Tie2 signaling pathway is also involved in the reciprocal communication between ECs and pericytes. Activating mutations in *TIE2* causes human venous malformations associated with abnormal VSMC (Sato, Tozawa et al. 1995). The Tie2 receptor is generally held as being endothelial specific, whereas its agonistic ligand Angl, appears to be expressed mainly by perivascular and mural cells (Sundberg, Kowanetz et al. 2002).

Angl- or *tie2*-null mice die at mid gestation from cardiovascular failure. These embryos show defective angiogenesis and their blood vessels have poorly organized Basement membrane.They show reduced coverage and detachment of pericytes (Dumont, Gradwohl et al. 1994). Conversely, the overexpression of Angl leads to an expanded and stabilized, leakage resistant vasculature (Suri, McClain et al. 1998). The importance of Angl as a pericyte-derived, microvessel-stabilizing signal was also demonstrated by the ability of recombinant Angl to partially rescue the vascular defects in the retina attributable to pericyte loss (Uemura, Ogawa et al. 2002). Together, these studies point to Angl as a pericyte-derived signal that mediates maturation and quiescence of the microvascular endothelium.

Ang2 is an antagonistic ligand for Tie2 in ECs (Maisonpierre, Suri et al. 1997) but possibly an agonistic ligand for Tie2 in mesenchymal cells (Maisonpierre, Suri et al. 1997; Witzenbichler, Maisonpierre et al. 1998). Ang2 over expression mimics Angl

or Tie2 deficiency, and Ang2 deficiency does not disturb prenatal vascular development but leads to defects in the eye vasculature (hyaloid vessel persistence) and intestinal lymphatics postnatally (Gale, Thurston et al. 2002). Ang2 is expressed mainly in ECs and upregulated endothelial-derived Ang2 marks the onset of angiogenic sprouting in tumors (Zhang, Yang et al. 2003). In these cells, Ang2 is stored in specialized secretory vesicles and can be rapidly released on stimulation (Fiedler, Reiss et al. 2006). Figure 1.3

Figure 1.2: TGF beta signalling

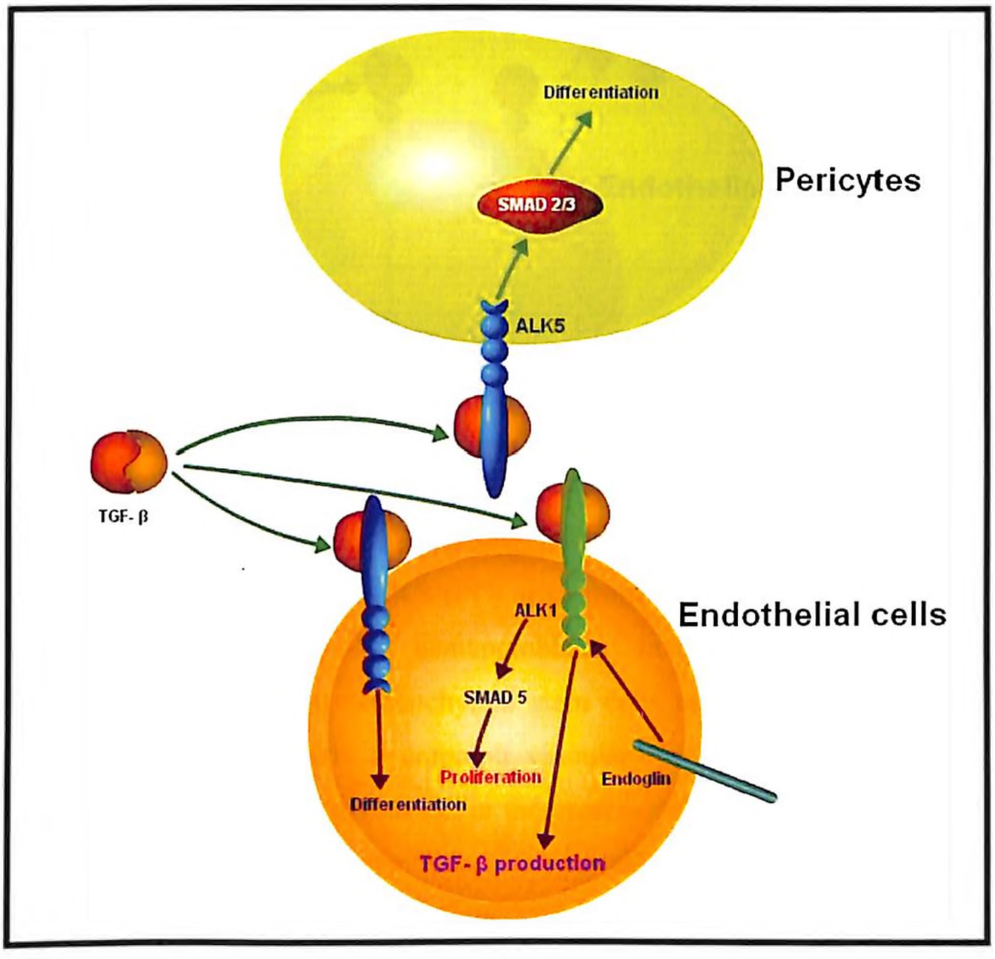
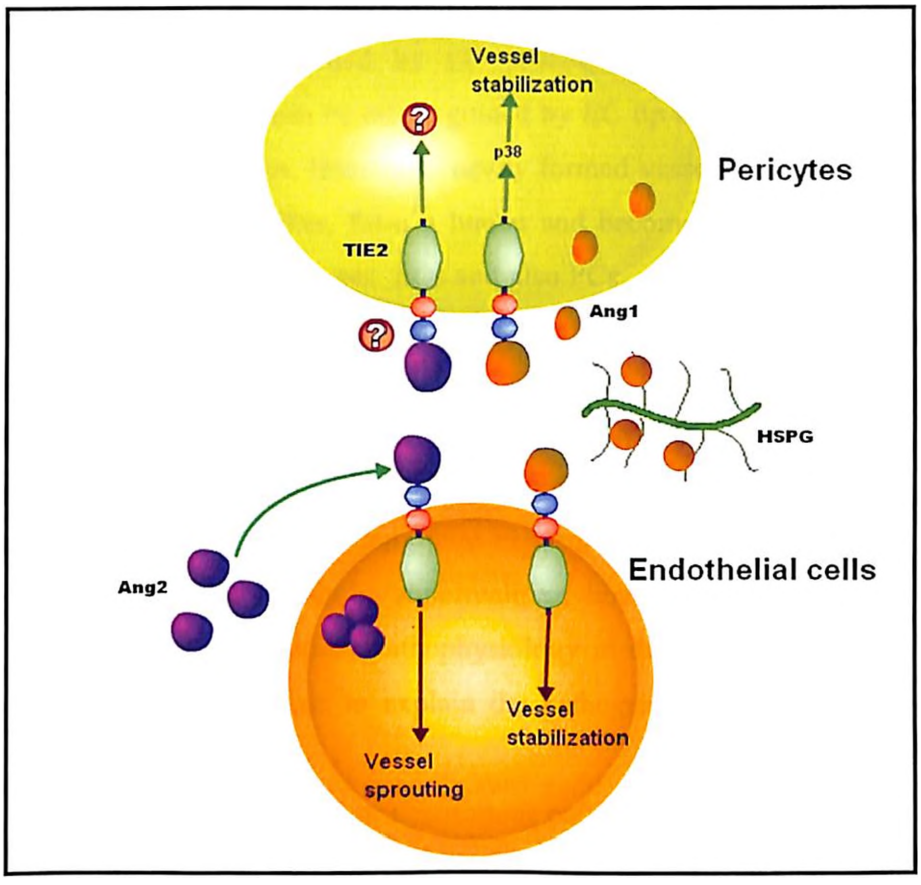


Figure 1.3: Angiopoietin signaling



1.3.3 : Pericytes and endothelial cells in vasculogenesis and angiogenesis

Vasculogenesis: ECs and pericytes/vSMCs (PC) arise from different precursor cells. ECs develop from angioblasts or hemangioblasts in the embryo, while pericytes/vSMCs are derived form mesenchymal stem cells or neurocrest cells. *In vitro* data indicate that there exists a common vascular progenitor derived from embryonic stem cells that can give rise to EC in the presence of VEGF, and to PC in the presence of PDGF-B. In the embryo, ECs first assemble into a simple capillary network. Vessels then sprout and prune become stabilized by pericytes/vSMCs that are recruited by PDGF-B-secreting.

Angiogenesis : New vessels are formed from existing blood vessels by EC bridging, intussusceptions, and/or sprouting. This is in general preceded by pericyte detachment from the vessel wall and subsequent vessel hyperdilation. When vessels form new

sprouts, the vascular basement membrane is first degraded to enable EC to move into the ECM. This is accompanied by EC proliferation and migration toward an angiogenic stimulus. ECs can be either guided by EC tip cells expressing high levels of PDGF-B or by pericytes. Immature, newly formed vessels cease the proliferation and ECs adhere to each other, form a lumen and become encircled by a basement membrane with recruited pericytes. ECs and also PCs, can be recruited from the bone marrow, specifically in tumor angiogenesis.

1.4 : PATHOGENESIS OF DIABETIC RETINOPATHY

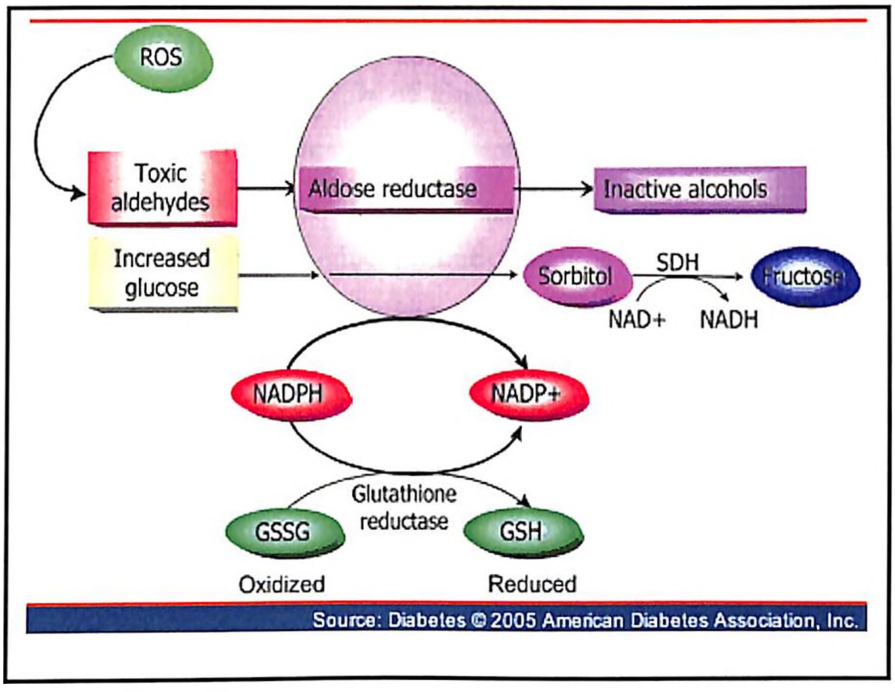
The pathogenesis of DR is highly complex and multifactorial in nature. Mechanistic studies have shown that short or long-term exposure to the diabetic milieu result in a host of biochemical and metabolic abnormalities; however it remains uncertain how much each contributes to retinal pathophysiology in diabetes. Multiple biochemical pathways have been proposed to explain the pathogenesis of DR which are listed below

1. Hyperglycaemia , polyol or hexosamine pathways
2. Diacylglycerol (DAG) leading to the over-activation protein kinase C
3. Oxidative stress , growth factors and adhesion molecules
4. Advanced glycation end products

1.4.1 Polyol pathway

When there is hyperglycemia, the excess glucose is metabolized to sorbitol and fructose by aldose reductase and sorbitol dehydrogenase, which is accompanied by increased oxidation of NADPH to $NADP^+$ and increased reduction of NAD^+ to NADH (Gabbay 1975; Oates 2002) as shown in figure 1.4.

Figure 1.4: Shows the conversion of Glucose to sorbitol and fructose



High concentrations of glucose increase flux through the polyol pathway with the enzymatic activity of aldose reductase, leading to an elevation of intracellular sorbitol concentrations. Sorbitol accumulation decreases other osmolytes such as *myo*-inositol and taurine. The polyol accumulation leads to basement thickening, pericyte loss, and microaneurysm formation (Duby, Campbell et al. 2004; Winer and Sowers 2004). Secondly, the increase in the cytosolic NADH/NAD^+ ratio results in a redox imbalance and resembles tissue hypoxia and therefore is termed hyperglycemic pseudohypoxia (Williamson, Chang et al. 1993). The redox imbalance favours the accumulation of triose phosphates which increases the formation of methyl glyoxal and AGEs and enhances oxidative stress. Recent report shows that deletion of aldose reductase leads to less severe brain and retinal ischemic injuries in the diabetic db/db mouse (Yeung, Lo et al.). Fidarestat treatment which is an inhibitor of AR has been shown to significantly decrease the concentrations of sorbitol and fructose in the retinas of streptozidin-induced diabetic rats. It also reduced the leukocyte accumulation in the retina (Hattori, Matsubara et al.).

1.4.2 : Hexose amine pathway

During normal physiology only 3 % of glucose is channeled into the hexose amine pathway. The glutamine fructose-6-phosphate amido transferase (GFAT) catalysis the conversion of fructose-6–phosphate to glucosamine-6-phosphate which is metabolilized to UDP-N-acetyl-glucosamine which is used as a substrate for the synthesis of glycoproteins, proteoglycans, gangliosides and glycolipids. Nuclear and cytosolic proteins are glycosylated on serine or threonine residues by O-linked beta-N-acetylglucosamine (O-GlcNAc). O-GlcNAc modification is one of the various posttranslational modifications and seems to be involved in the modulation of transcription and signal transduction. Accumulating data suggest a role for O-GlcNAc-modified proteins in diabetes, acting as a glucose sensor. It has been suggested that the hexosamine biosynthetic pathway is involved in the mechanism causing insulin resistance and diabetic complications (Akimoto, Hart et al. 2005). Studies have shown that, although both high glucose and glucosamine treatments lead to increase in UDP-GlcNAc and *O*-GlcNAc levels, they also can lead to oxidative and endoplasmic reticulum stress (Werstuck, Khan et al. 2006), both of which have been shown to cause chronic inflammation and insulin resistance (Gregor and Hotamisligil 2007). Genetic studies have linked a mutation in the OGA gene [meningioma-expressed antigen 5 (*MGEA5*)] to the susceptibility of diabetes in the Mexican American population (Lehman, Fu et al. 2005).

The mode of action appears to be transcriptional regulation, likely modulated by O-GlcNAc modification of transcription factors (Buse 2006). There is strong evidence supporting a role for the hexose shunt pathway in the physiopathology of the vascular/ renal complications of diabetes. There are more than 75 proteins which have been identified as *O*-GlcNAc modified.

1.4.3 : Protein Kinase C pathway

Protein kinase C (PKC), a family of large group of structurally related enzymes that require for their activation phosphotidylserine/diacylglycerol (DAG)/free fatty acids and Ca^{2+} ions in addition to Mg^{2+}. Although the activity of multiple PKC isoforms (ά, β1, β2, and έ) is increased in vascular tissues in the diabetic state, studies suggest that

the PKC- β 2 isoform preferentially mediates the pathologic complications associated with hyperglycemia (Inoguchi, Battan et al. 1992). Moreover, PKC- β has been shown to be an integral component of cellular signaling by vascular endothelial growth factors (VEGFs) (Xia, Aiello et al. 1996), which are important mediators of ocular neovascularization. Ruboxistaurin is a specific inhibitor of PKC-β and is the first oral pharmacologic agent shown to reduce visual loss in diabetic patients over an extended period. The results from the PKC-β inhibitor Retinopathy Study 2 (PKC-DRS2) showed that Ruboxistaurin reduces the occurrence of sustained moderate visual loss by 40% in patients with moderately severe to very severe NPDR, simultaneously increasing the likelihood of visual improvement (Danis and Sheetz 2009). The second PKC inhibitor, PKC412, inhibits multiple PKC isoforms and at least two other receptor kinases (Fabbro, Reitz et.al, 2000).

1.4.4 : Oxidative stress in Diabetic Retinopathy

Oxidative stress, defined as an imbalance between oxidants and antioxidants, leads to modifications in biochemical properties of biomolecules and to the formation of reactive oxygen species (ROS) in the cells, resulting in physiological dysfunction (Lum and Roebuck 2001). Acute and chronic oxidant stress to the vascular endothelium is a serious causative agent for endothelial dysfunction. The production of ROS is increased by a variety of pathophysiological stimuli, including cytokines, hypertension, hyperglycemia, hyperhomocystenemia, AGE and altered mechanical forces. It is reported that the detachment of EC results in a rapid and dramatic rise in intracellular ROS levels which leads to EC anoikis which occurs through a redox sensitive pathway (Li, Ito et al. 1999). ROS are reported to elicit specific growth and induce the expression of growth-related genes, including c-fos, c-myc, and c-jun and also shown to block the internalization of insulin (Bertelsen, Anggard et al. 2001).

1.4.5 : Cytokines and oxidative stress

In ECs, cytokine-induced expression of vascular cell adhesion molecule-1 has been reported. The expression of vascular cell adhesion molecule-1 promotes monocyte adhesion to ECs and may be an important event in the development of atherosclerosis (Esposito, Nappo et al. 2002). ECs treated *in-vitro* with interleukin-1 and interferon

results in a dose and time dependent increase in $O_2^{\cdot-}$. Exposure of ECs to hypoxia followed by reoxygenation induces the production of 10-50 nmol $O_2^{\cdot-}$/h (Lum, Barr et al. 1992). Once the super oxide radical is produced more reactive intermediates like H_2O_2 and hydroxyl radical are produced through the enzymatic catalysis and also by Fenton's reaction. Several enzymes such as NOS and NADPH oxidase generate the small molecules involved in redox signaling pathways in a controlled manner within the cell (Lambeth 2004). A recent report suggests that the endothelial dysfunction occurring in type 2 diabetes is the result of the effects of the inflammatory cytokine TNF-alpha and its signaling, which up regulates the expression of MCP-1 and adhesion molecules, which further exacerbates vessel inflammation and oxidative stress (Yang, Park et al. 2009).

1.4.6 : Hyperglycemia and oxidative stress

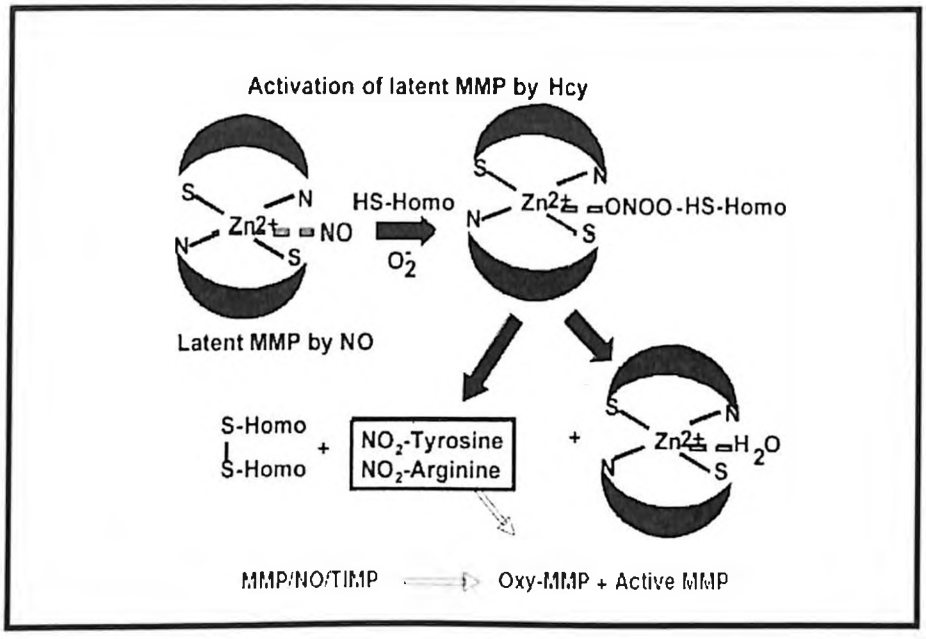
In vitro, high glucose has been demonstrated to affect endothelial and other vascular cells at the cellular level *(Lorenzi and Cagliero 1991),* delay EC replication (Ono, Umeda et al. 1988) and cause excessive cell death *(Lorenzi, Cagliero et al. 1985).* High glucose induces apoptosis of human umbilical vein ECs, by activating ROS which inturn involves in JNK activation, which leads to triggering of caspase-3 and facilitation of apoptosis(Ho, Liu et al. 2000). It is reported that 8-hydroxy-2-deoxyguanosine (8-OHdG), which is an indicator of oxidative damage of DNA, increases in patients with type 2 diabetes mellitus (Dandona, Thusu et al. 1996). Incubation of adipocytes with high glucose shows increased oxidative stress (Lu, Ennis et al. 2001). Oxidative stress induced by high glucose is also strongly suspected to be involved in chronic hyperglycemia-induced insulin resistance (Eriksson 2007). In DR elevated glucose blocks the prosurvival effect of VEGF and causes accelerated EC apoptosis via the action of peroxynitrate in causing tyrosine nitration of PI 3 kinase, inhibiting activity of Akt-1 kinase and increasing the activity of p38 MAP kinase. Generation of ROS in diabetes seems to be directly linked to chronic hyperglycemia which may contribute to the onset or development of diabetic micro- or macro vascular complications. Such a mechanism may be a new target of antioxidative therapy for preventing diabetic vascular complications (Kawahito, Kitahata et al. 2009).

1.4.7 : Homocysteine and endothelial cell dysfunction

Homocysteine (Hcy) is a highly reactive thiol intermediate in amino acid metabolism, which can modify the function of ECs in a myriad of ways. It has been reported that ECs can detoxify homocysteine by stimulating the release of nitric oxide, with an accompanying increase in *Nos3* mRNA levels (Stamler, Osborne et al. 1993). Therefore, from the oxidation of Hcy with nitric oxide, S-nitroso-Hcy formation ensues. Thus Hcy can actually reduce NO• bioavailability. However, S-nitroso-homocysteine has also vasodilatory and platelet antiaggregation properties and does not support hydrogen peroxide generation. This represents a protective mechanism against the adverse effects of Hcy. However, this scavenging effect of NO is eventually overcome during chronic exposure to high Hcy, due to a reduced availability of NO• leading to unopposed oxidative-injury mediated by Hcy and by formation of peroxynitrite (De Groote, Testerman et al. 1996). Homocysteine produces an indirect suppression of endothelial NO• synthase eNOS activity, without affecting its expression. Folates, superoxide ions and peroxynitrite scavengers restore the NO• generating activity of eNOS (Zhang, Li et al. 2000).

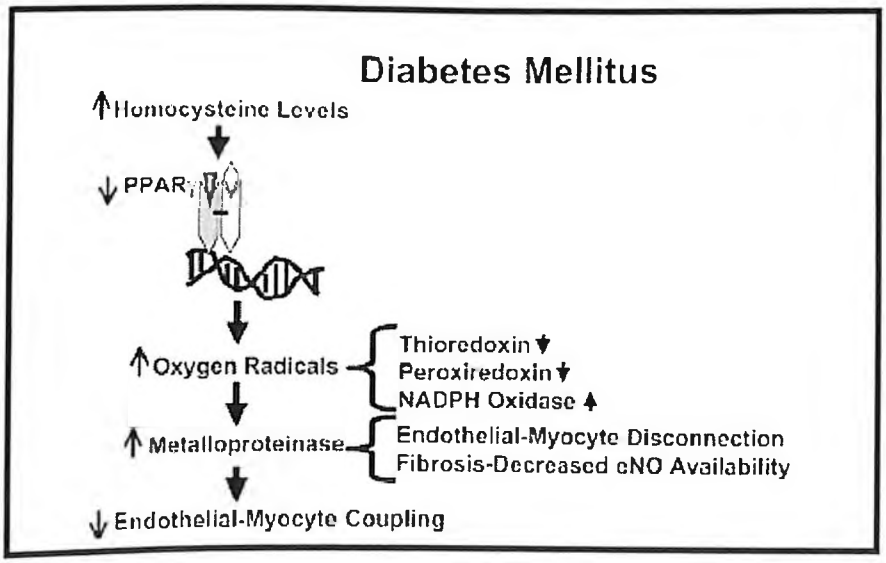
Homocysteine transport into the cells is an essential step of Hcy signaling that leads to its downstream biological effects. Studies with human umbilical vein ECs (HUVECs) found that 2 transporter systems, alanine-serine-cysteine (ASC) and large branched-chain neutral amino acids (L), mediate Hcy uptake, and that the neutral amino acids cysteine and serine inhibit Hcy transport (Jiang, Yang et al. 2007). Homocysteine is the only thiol that suppresses the generation of other thiols, activates the MMPs and inactivates the TIMPs, causing a decrease in endothelial NO bioavailability (figure 1.5) (Mujumdar, Aru et al. 2001).

Figure 1.5: The role of Homocysteine in regulation of MMP:



The activated MMP generate nitro-derivatives of tyrosine and arginine. The nitro-derivatives of arginine, in turn become competitive inhibitors of endothelial NO synthase and therefore, decrease bio-availability of NO. The nitro-derivatives in tissue inhibitor of metalloproteinases (TIMPs) abrogate their ability to inhibit MMP (figure 1,6).

Figure 1.6: Deleterious effects of Homocysteine and link to diabetes



A preponderance of biochemical and genetic data suggest that elevated Hcy promotes a proatherothrombotic phenotype. Potential mechanisms include modification of proteins by homocysteinylation, oxidative stress, inflammation, endothelial dysfunction, and thrombosis (Jakubowski 2004; Jakubowski 2007). Fasting Hcy levels of adults with type I diabetes with retinopathy was increased when compared to patients with no retinopathy (Saeed, Nixon et al. 2004).A recent study reports when the levels of hcys, folate, B_{12} between patients with macular edema and without edema with diabetes were compared only hcys was increased in patients with macular edema.Another study has reported that plasma Hcy concentration may be a useful biomarker and a novel risk factor for increased risk of DR with type 2 diabetes (Brazionis, Rowley et al. 2008).

Hcy-thiolactone hypothesis, was originally formulated in 1997 (Jakubowski 1997) and states that a pathway initiated by Hcy conversion to Hcy-thiolactone contributes to Hcy Pathobiology. Hcy-thiolactone induces apoptotic death in cultured human vascular endothelial (Mercie, Garnier et al. 2000; Kerkeni, Tnani et al. 2006) and inhibits insulin signaling in rat hepatoma cells (Najib and Sanchez-Margalet 2005). Hcy-thiolactone also induces endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in retinal epithelial cells. Furthermore, Hcy-thiolactone is more toxic to cultured cells than Hcy itself (Roybal, Yang et al. 2004).Fibrinogen is known to undergo *N*-homocysteinylation by Homocysteinethiolactone *in vitro* (Jakubowski 1999; Jakubowski 2000) and *N*-Hcy-fibrinogen is present *in vivo* in humans (Jakubowski 2002). Sauls *et al.* showed that clots formed from Hcy-thiolactone-treated normal human plasma or fibrinogen lyse slower than clots from untreated controls. Some of lysine residues susceptible to *N*-homocysteinylation are close to tissue plasminogen activator and plasminogen binding or plasmin cleavage or sites, which can explain abnormal characteristics of clots formed from *N*-Hcy-fibrinogen (Sauls, Lockhart et al. 2006).

The involvement of an autoimmune response against *N*-Hcy-protein in cardiac artery disease is supported by the findings that lowering plasma Hcy by folic acid supplementation lowers anti-*N*-Hcy-protein autoantibodies levels in control subjects but not in patients with Cardiac artery disease. These findings suggest that once

accumulated, the antigens causing the antibody response, i.e. *N*-Hcy proteins persist and that chronic protein damage caused by N-homocysteinylation cannot be easily reversed in CAD patients. Furthermore, these findings also suggest that while primary Hcy-lowering intervention by vitamin supplementation is beneficial, secondary intervention may be ineffective and may explain at least in part the failure of vitamin therapy to lower cardiovascular events in myocardial infarction patients (Lonn, Yusuf et al. 2006).

Two enzymes are known that have the ability to hydrolyze the toxic metabolite Hcy-thiolactone: extracellular (serum) Hcy-thiolactonase/paraoxonase 1 (PON1) and intracellular Hcy-thiolactonase/bleomycin hydrolase (BLH) (Zimny, Sikora et al. 2006). PON1, named for its ability to hydrolyze the organophosphate paraoxon, is synthesized exclusively in the liver and carried on HDL in the circulation. PON1 transgenic mice (carrying 3 copies of the human *PON1*) are less susceptible to atherosclerosis (Tward, Xia et al. 2002). *In vitro* studies indicate that HDL from PON1 deficient animals does not prevent LDL oxidation, whereas HDL from PON1 transgenic animals protects LDL against oxidation more effectively than HDL from wild type mice. PON1 is an Hcy-thiolactonase which is able to protect proteins against N-homocysteinylation, at least in vitro (Jakubowski, Zhang et al. 2000; Jakubowski, Ambrosius et al. 2001). Human *PON1* has genetic polymorphisms, e.g. PON1-M55L, PON1-R192Q, which affect PON1 function (Jarvik, Rozek et al. 2000), including Hcy-thiolactonase activity. In mice, Hcy is a negative regulator of PON1 expression (Jarvik, Rozek et al. 2000). In humans, Hcy-thiolactonase activity of PON1 is negatively correlated with tHcy (Lacinski, Skorupski et al. 2004) and predicts cardiovascular disease (Domagala, Lacinski et al. 2006).

1.5 : ADVANCED GLYCATION END PRODUCTS

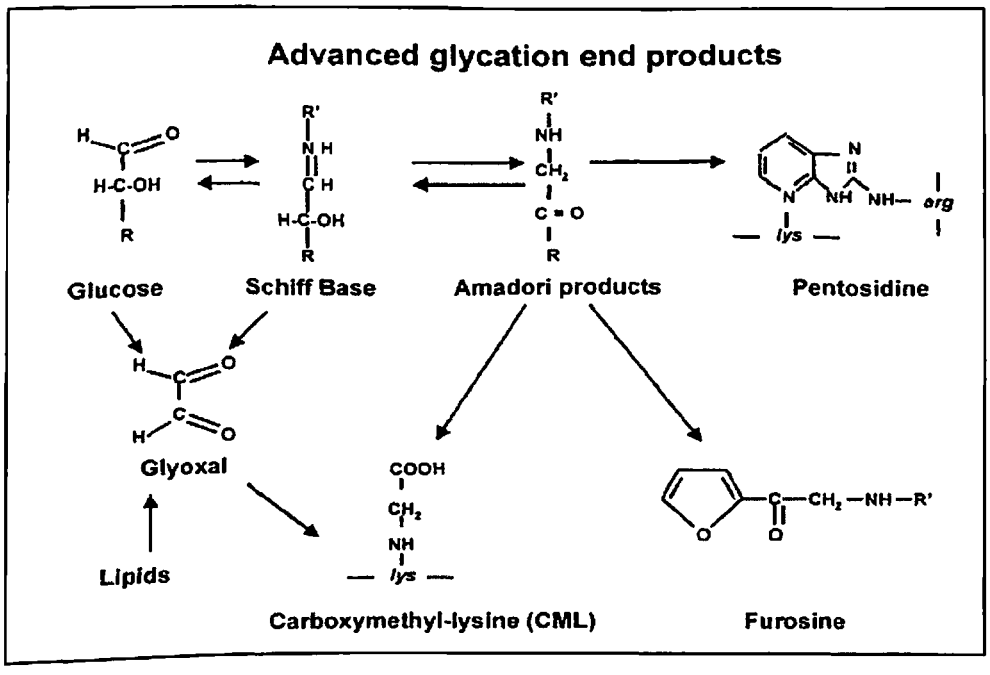
1.5.1 : Formation of advanced glycation end products

The AGEs are a heterogeneous group of structures formed as both cross-linking and noncross-linking adducts on proteins. The nonenzymatic reaction of the amino groups of amino acids, peptides and proteins with reducing sugars, ultimately result in the formation of complex brown pigments and protein-protein crosslinks. This was first

studied under defined conditions by L.C. Maillard in the early 1900s. In the 1920s, Amadori showed that glucose-aniline Schiff bases could be converted to isomeric products that were not glycosylamine anomers but were subsequently found to be 1 -aniline-1-deoxyfructose derivatives that Amadori products can form from aliphatic amines such as amino acids, not just from aromatic amines, and that the Amadori rearrangement was a key early step in the Maillard reaction (Hodge 1955). The Amadori rearrangement of the Schiff base to the Amadori product is believed to occur via an intermediate, open-chain enol form.

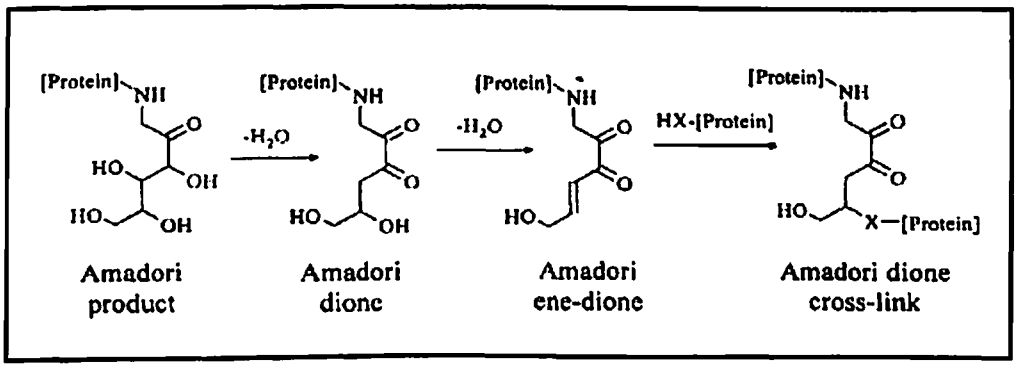
Formation of the Schiff base from sugar and amine is relatively fast and highly reversible. Formation of Amadori product from the Schiff base is slower but much faster than the reverse reaction, so that the Amadori glycation product tends to accumulate on proteins. The Amadori rearrangement of a lysine-glucose Schiff base is thought to be facilitated if there is a histidine side-chain or lysine amino group, near to the amino group on which the Schiff base has formed, due to localized acid-base catalysis (Acosta, Hettinga et al. 2000). The Amadori product can break down via its enol form to reactive, free α-dicarbonyl glyoxal compounds such as 3-deoxyglucosone, methylglyoxal and glyoxal (Figure 1.7).

Figure 1.7: The formation of advanced glycation end products



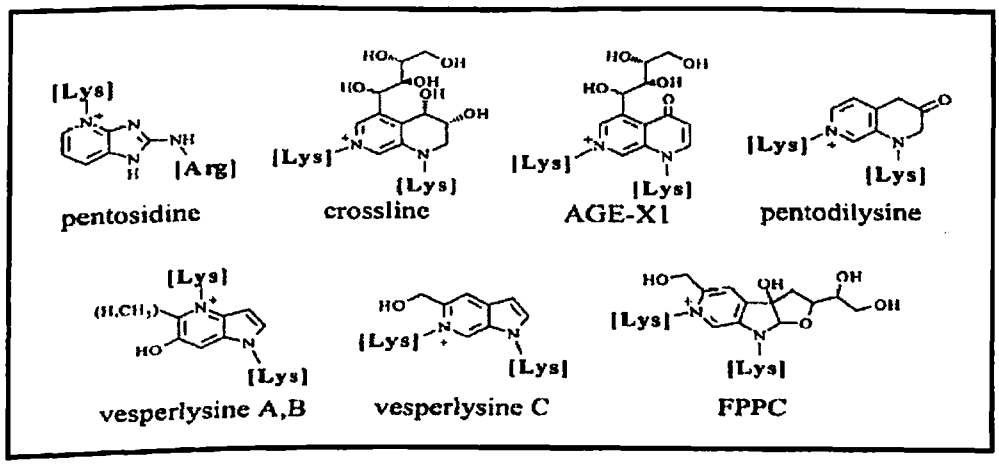
The Amadori product also can dehydrate at the 4-position to yield a 1-amino-4-deoxy-2,3-dione (Amadori dione). This substance can further dehydrate at the 5-position to yield an unsaturated dione, the Amadori ene-dione (Estendorfer et al., 1990). Figure 1.8.

Figure 1.8: The Amodori Dione formation



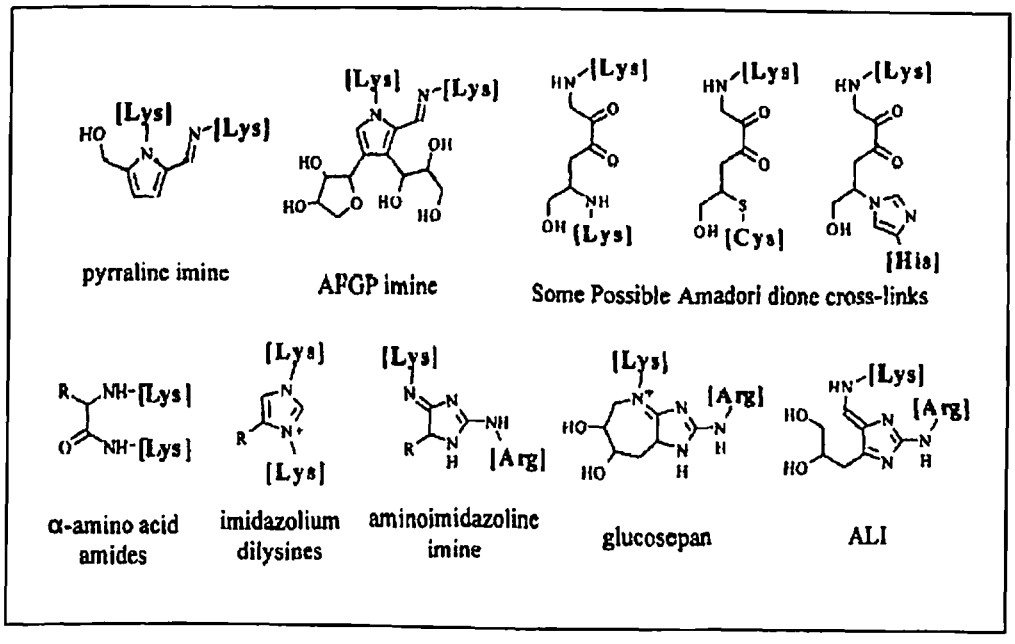
The fluorescent AGE crosslink pentosidine was first isolated and identified from duramater collagen and has since been identified in many tissues (Sell, Nagaraj et al. 1991). Pentosidine is unusual in that it can be formed by the reaction of lysine and arginine with any of several carbohydrate precursors, including glucose, ribose, ascorbic acid, and 3-deoxyglucosone. Workers in a number of laboratories have isolated a variety of other fluorescent crosslinks in model reactions under physiological conditions: crosslines A and B: vesperlysines A, B and C; AGE-Xl; pentodilysine and pyrropyridine or FPPC. Of these, the crosslines (Obayashi, Nakano et al. 1996) and vesperlysines (Tessier, Obrenovich et al. 1999) have been detected in vivo.Figure 1.9

Figure 1.9: The fluorescent AGE's



Although their ease of detection makes them useful markers of AGE formation, the fluorescent AGE crosslinks are thought to account for only one percent or less of the total crosslinking structures formed under physiological conditions (Dyer et al., 1991). Thus, the major AGE structures responsible for protein-protein crosslinking *in vivo* are non fluorescent structures that have not yet been conclusively identified. Recently, criteria other than fluorescence have been utilized to find new, non fluorescent crosslinking AGE candidates that might represent the major *in vivo* AGE crosslink Figure 1.10

Figure 1.10: The Non fluorescent AGE's



In addition to glucose, a number of other sugars and sugar metabolites are known to initiate the Maillard reaction. Among them methylglyoxal (MG) has attracted considerable attention recently. MG is formed by enzymatic and nonenzymatic routes from glycolytic intermediates as well as from autoxidation of sugars (Phillips and Thornalley 1993). It has been shown that in diabetes the concentration of MG increases in the lens, blood and kidney. In Type I diabetic patients, the blood MG levels increased 5–6 fold and in Type II 2–3 fold compared with normal control subjects (McLellan, Thornalley et al. 1994) The high reactivity of MG with proteins and its relatively high concentration (0.1 mM) in the plasma suggest that it is one of the major glycating agents present *in vivo*. Effective defense systems may exist

intracellularly to reduce MG toxicity through the Maillard reaction. The GSH-dependent glyoxalase converts it to D-lactate (Thornalley 1993). It is therefore conceivable that in tissues where the GSH level is decreased in aging and diabetes, MG concentration would increase. In fact, the MG concentration is reported to be elevated in lens, red blood cells and kidney in diabetes, where GSH levels have been determined to decrease (Grunewald, Weber et al. 1993). The observation that nonenzymatic glycation of proteins results in covalent cross-linking and insolubilization led to the suggestion that AGEs may in part be responsible for age- and diabetes-associated protein cross-linking. The long-lived proteins in the body like lens crystallins and collagen accumulate AGEs because of their negligible or slow turnover. Immunochemical and chemical methods have clearly shown the progressive accumulation of AGEs in tissue proteins in aging (Horiuchi and Araki 1994).

In long-term diabetes, the vascular basement membrane (BM) thicken significantly, reaching thicknesses that are typically two to three times that of normal vascular BMs. The BM thickening associated with diabetes and hyperglycemia is irreversible due to AGE formation and cross-linking. These effects of AGE contribute not only to the thickening of the capillary BM, but also to the endothelial dysfunction seen early on in DR and promote progressive deterioration (Roy, Trudeau et al.).

1.5.2 : Non cross linking, protein-bound AGE structures of importance *in vivo*:

1-Carboxyalkyllysines:

AGEs in which a 1-carboxyalkyl group is attached to a free amino group of an amino acid residue, such as Nϵ-(carboxymethyl)lysine (CML) and Nϵ-(1-carboxyethyl) lysine (CEL), are found in proteins and in free form *in vivo* (Ahmed, Brinkmann Frye et al. 1997).They may arise either from reaction of lysine residues with glyoxal derivatives (Glomb and Monnier 1995) or from autoxidation of early-stage AGEs such as the Amadori product (Frye, Degenhardt et al. 1998). Carboxymethyl derivatives of α-amino groups of N-terminal amino acids are also possible, such as N-carboxymethylvaline in hemoglobin. CML formation seems to be a major pathway *in vivo*, as urinary levels of CML in humans average 30 to 40 percent of levels of the lysine Amadori product (Knecht, Dunn et al. 1991).

1.5.3: Effects of AGE formation on connective tissue

During formation of normal collagen fibrils, some collagen lysine residues and hydroxylysine residues are oxidized by lysyl oxidase, which converts their ϵ-amino groups to aldehydes. These aldehydes then undergo crosslinking reactions with lysine or hydroxylysine residues in adjacent collagen molecules in the fibrils. This process is self-limiting in that lysines in closely crosslinked collagen molecules are no longer sterically accessible to lysyl oxidase. AGE-mediated over crosslinking of connective tissue molecules (e.g., collagen molecules) to each other causes loss of bulk elasticity and flexibility and increased brittleness. Another example of damage to connective tissue by AGEs is the crosslinking of exogenous molecules to collagen, which can lead to pathological surface changes and over activation of tissue repair mechanisms.

1.5.4 : Effects of AGE Formation on Lens Proteins

Human lens crystallins are very long lived, lasting an entire life time. Although crystallins have evolved to retain optical clarity for an extended period, yellow-brown pigments with the spectral and fluorescent properties of AGEs do form in the lens as a function of age (Monnier and Cerami 1983). These pigments can become dark as to form cataracts, leading to blindness.

1.5.5 : Effects of AGE Formation on Lipoproteins

AGE modification of LDL can occur both on amino groups of the apoprotein component and on aminolipid components such as phosphatidylethanolamine. AGE formation on the apoprotein componentcan lead to crosslinking of LDL to the collagen layer of the blood vessel wall (Brownlee, Pongor et al. 1983) and can increase the half-life of LDL in serum by blocking the recognition site for its receptor-mediated uptake (Bucala, Mitchell et al. 1995). This increases the probability of autoxidation of the lipid component (Bucala, Makita et al. 1993).

Protein bound adducts (ALEs) that are uniquely derived from lipids include MDA-lysine, 4-hydroxynonenal-lysine (HNE-lysine), an anacrolein-lysine adduct (FDP-lysine), hexanoyllysine. A recently described adduct, carboxyethylpyrrole-lysine (CEP) uniquely produced from the oxidation of docasahexaenoate, a fatty acid

enriched in rod outer segments and the retinal pigment epithelium has been shown to accumulate in the retina. MDA-, HNE-, FDP- and hexanoyl-adducts has all been shown by immunohistochemical techniques to be increased at sites of vascular pathology in a variety of diseases (Stitt, Frizzell et al. 2004).

1.5.6: Receptor for Advanced glycation end products

Receptor for AGE (RAGE) is a multi-ligand cell surface receptor initially isolated from bovine lung by the group of Stern (Neeper, Schmidt et al. 1992). Endogenous ligands, such as amphoterin, calgranulin, amyloid β proteins, and transthyretin, have also been identified. Tanaka *et al.* identified three inducers of RAGE gene transcription: AGE ligands themselves, TNF-α and 17β-estradiol (Tanaka, Yonekura et al. 2000). The former two shared the same *cis*-element for induction, which was located around nucleotide 671 in the human RAGE 5'-flanking sequence, whereas estradiol-responsible elements resided at 189 and at 172. The finding that AGES themselves can activate the RAGE gene seems to be consistent with the observation that the AGE-rich vasculature exhibits enhanced RAGE immunoreactivity (Soulis, Thallas et al. 1997). Such a positive feedback loop may exacerbate diabetic vasculopathy. The RAGE gene activation by estradiol may provide a biochemical basis for the well-known fact that pregnancy worsens diabetic complications (Moloney and Drury 1982).

Endogenous Secretory RAGE : Yonekura *et al.* analyzed poly $(A)^+$RNA isolated from polysomes of human EC and pericytes and isolated previously undescribed splice variants of RAGE mRNA (Yonekura, Yamamoto et al. 2003). Three major variants were identified: the known full-length membrane bound form, a novel N-terminally truncated membrane-bound form and a novel C-terminally truncated soluble form. The ratio of expression of these variants differed from one cell type to another; C-truncated (38 %) > full-length (31 %) > N-truncated (31 %) in EC; full-length > N-truncated > C-truncated in EC. In contrast, the fulllength type was the predominant form in pericytes (61%) followed by the N-truncated (33%) and then the C-truncated (6%) types. Each cDNA directed synthesis of the protein product in COS cells showed both

the full-length and the N truncated forms mainly resided on the plasma membrane, whereas the C-truncated form was liberated into the media. Furthermore, the full-length and C-truncated forms bound to an AGE-immobilized column, whereas the N-truncated form was recovered in the pass-through fractions, thus confirming that the ligand-binding site is located in the amino-terminal V region–like domain of RAGE proteins.

The C-terminal truncated form of RAGE lacks the exon 10 sequences encoding the transmembrane and intracytoplasmic domains. This spliced variant mRNA of RAGE encodes a protein consisting of 347 amino acids with a 22–amino-acid signal sequence and is released from cells. The C truncated form endogenous secretory RAGE (esRAGE). The es- RAGE would be cytoprotective, because it is able to capture AGE outside cells. In effect, this variant was found to neutralize effectively the AGE action on EC and does exist in human circulation (Chavakis, Bierhaus et al. 2003). An ELISA system for esRAGE has been developed and with it, diabetic subjects with or without complications is now being screened.

1.5.7 : Removal of AGE The receptor-mediated removal of senescent, highly modified molecules from the circulation is an important function of many cells and tissues, including the vascular endothelium. It is thought that AGE receptor systems have evolved to provide specific removal pathways for these molecules, which are subsequently destroyed by lysosomal degradation. In diabetes, where AGEs occur at markedly elevated levels, the enhanced receptor-mediated removal systems may result in sequestration of high levels of AGEs within intracellular compartments. The receptor-mediated sequestration of highly reactive species within intracellular compartments is likely to have a serious effect on biochemistry and physiology. Caveolae appear to be the surface organelle where exogenous AGEs interact with their receptors but little is known about the signal transduction ensuing from these receptor-ligand interactions. In retinal microvascular endothelium, AGEs are endocytosed and eventually trafficked to the lysosomal system where they may be proteolytically cleaved and released, compartmentalised within the cell or transcytosed beyond the endothelial barrier in a similar manner to other proteins (Stitt, Burke et al. 2000).

1.5.8 : Methods to detect AGE

The advanced glycation end products which are formed are highly complex and there is no single method to measure its level. There are various forms of AGEs as discussed above. The fluorescent AGEs that can be measured easily are crosslines A and B, vesperlysines A, B, and C, AGE-Xl, pentodilysine and pyrropyridine or FPPC. The non fluorescent AGEs were initially detected using immunohistochemistry (Nakayama, Mitsuhashi et al. 1993) and to quantify their levels ELISA is used (Swamy-Mruthinti, Miriam et al. 2002). Recently mass spectrophotometry is also being used to measure these AGEs (Mittelmaier, Funfrocken et al.).

1.5.9 : Effects of AGE in retinal Endothelial cells

There have been *in vivo* and *in vitro* studies, which indicate that elevated intracellular and extracellular AGEs during diabetes, may be an important factor in retinopathy development and progression. Preformed AGEs can initiate abnormal proliferative responses in both retinal microvascular ECs and human vascular ECs. The work of Hammes et al demonstrating, (i) the presence of AGE products in retinal capillaries of 26-week diabetic rats, and (ii) significant reduction in the number of diabetes-induced a cellular capillaries and pericyte loss when the formation of AGE is inhibited with aminoguanidine, seems to suggest a possible link between excessive accumulation of AGE and the pathogenesis of DR (Hammes, Martin et al. 1991). The ability of AGE-Alb to induce substantial activation of ICAM-1 in the retinal microvascular endothelium, possibly through increased transcription of NFκB, is also reported which may be a mechanism for retinal leukostasis (Moore, Moore et al. 2003).

More recently, Stitt et al.(Stitt, Burke et al. 2000) has also demonstrated that AGE-modified albumin co-localizes with the p60 and p90 component of the AGE-receptors in the retinal vasculature of both diabetic rats and AGE-infused rats suggesting that progressive accumulation of AGE may well be the underlying mechanism for the loss of pericytes and proliferation of ECs in DR. This phenomenon can be explained, at least in part, by the acute AGE-mediated induction of the potent endothelial mitogen VEGF which can initially stimulate cells through short-lived autocrine stimulation of VEGF-receptor-2 (VEGF-R2) (Yamagishi, Yonekura et al. 1997).

1.5.10 : Effect of AGE in retinal pericytes

Di acyl glycerol is an intracellular mediator that could be implicated either in proliferation pathways through PKC activation (Kishimoto, Takai et al. 1980) or in apoptosis as an activator of the acidic form of sphingomyelinase which forms ceramides (Schutze, Potthoff et al. 1992; Wiegmann, Schutze et al. 1994; Genestier, Prigent et al. 1998). AGE is associated with a 2-fold increase of cellular DAG and ceramide levels was reported by Denis U et al (Denis, Lecomte et al. 2002)

The same group reported that the caspases 10 is the initiator of apoptosis in the early phase of AGE induced apoptosis of pericytes. Time-course analysis of caspase activation in retinal homogenates of diabetic mice revealed that caspase-3 induction was associated with diabetes duration at which microvascular cells undergo apoptosis (Lecomte, Denis et al. 2004).

AGE-BSA can decrease the activities of catalase and SOD and increase the MDA production in BRP, which is similar to the antioxidant results in patients with DR (Gupta and Chari 2005). Under a long cultured condition (15 days), the chronic increase of catalase and SOD activities suggests a compensatory feedback mechanism against superoxide radical and H_2O_2 production (Paget, Lecomte et al. 1998). Decreased protein ratio of Bcl-2 to Bax is associated with increased caspase-3 activities and oxidative stress, resulting from an imbalance between oxygen free radical production and destruction by antioxidant scavenger systems (Chen, Jiang et al. 2006).

1.5.11 : AGE and angiogenesis

AGE-BSA caused a consistent increase in cell number and in DNA synthesis in both umbilical and microvasular ECs, at a concentration of (50 mg/ml) giving the maximal effect in both cases (Yamagishi, Yamamoto et al. 1996). This concentration of AGE was comparable with that of the *in vivo* situation in diabetes. Makita et al. reported (Makita, Vlassara et al. 1992) that human serum AGE levels were elevated more than 2-fold in diabetic patients (about 25 mg/ml) and almost 8-fold in diabetic patients on hemodialysis (about 80 mg/ml) in comparison with normal patients.mRNAs for VEGF121 and VEGF165 are present in microvascular ECs and their levels are up-

regulated by AGE in both dose- and time-dependent manner. AGE act on pericytes, the microvascular constituent that encircles the endothelium.Through interactions with RAGE, AGE decrease the number of this cell type (Yamagishi, Hsu et al. 1995), leading to pericyte dropout, which would in turn relieve the restriction on EC replication and facilitate angiogenesis. The resultant cessation of pericyte-EC interactions would impair prostacyclin production (Yamagishi, Kobayashi et al. 1993), which would cause thrombogenesis (Yamagishi, Yonekura et al. 1997).

In an established co-culture model of *in vitro* angiogenesis it was (Korff, Kimmina et al. 2001) demonstrated that the AGEs inhibit the cooperation of angiogenesis (sprouting of new capillaries from preexisting vascular structures) and vasculogenesis (homing of progenitor cells into ischemic regions and de novo formation of vascular structures) by attenuating the incorporation of progenitor cells into sprouting capillaries (Scheubel, Kahrstedt et al. 2006) by concentrations of AGEs observed in extra cellular fluids of diabetic patients (Xu, Ji et al. 2005). However, both mechanisms require incorporation into or nearby sprouting endothelium as an initial step, which is efficiently inhibited by AGEs. This important aspect of the AGE-mediated vascular pathology has been overlooked so far. Recently, it was shown that aging is associated with a reduced number of circulating progenitor cells (Scheubel, Zorn et al. 2003) and with lower survival, migration and proliferation of EPC, implicating functional impairment of EPCs from old subjects (Heiss, Keymel et al. 2005). Similar functional alterations have been observed in diabetes. EPCs isolated from patients with type II diabetes showed decreased proliferation and adherence to HUVECs with reduced capability to participate in tube formation (Tepper, Galiano et al. 2002). These observations stimulated the expectation that EPCs from young healthy donors might serve as a better therapeutic tool for improvement of impaired vasculogenesis in aging and diabetes.

Several studies on different animal models have now established that dietary AGE could play a significant role in the pathogenesis of various pathologic conditions and their complications. Wound healing is typically delayed in diabetes mellitus and angiopathy seems to contribute significantly (Fong, Aiello et al. 2003), (Goova, Li et al. 2001). In diabetes, delayed differentiation of fibroblasts into myofibroblasts in

concert with the cellular fatigue may contribute to the poor wound contraction and tissue remodeling (Desmouliere, Redard et al. 1995), (Grinnell 1994). Experimental evidence using products of the Maillard reaction as well as anti-AGE agents suggests that AGE contribute to the impaired wound healing in various conditions including diabetes (Peppa and Vlassara 2005; Vlassara 2005; Peppa and Raptis 2008; Peppa, Uribarri et al. 2008). AGE accumulation in diabetic tissues including the wound tissue and possibly their interaction with specific cell surface receptors such as the RAGE have been related to the decreased trans endothelial migration of neutrophils leading to hypocellularity and delayed inflammatory response (Liu, Miyata et al. 1999; Schmidt, Yan et al. 2001; Collison, Parhar et al. 2002; Toth, Rong et al. 2008). RAGE receptor is a multiligand receptor expressed by diverse cell types that is up-regulated in environments rich in RAGE ligands such as CML adducts. Increased RAGE expression has been found in wound tissues from diabetic mice in parallel with increased AGE accumulation and increased inflammatory status. RAGE expression has been strongly linked to the expression of MMPs-1, -3, -9, mainly through RAGE engagement by AGE (Cipollone, Iezzi et al. 2003). In vitro work showed that in the presence of AGE, type I collagen synthesis from fibroblasts is not affected, while the synthesis of hyaluronic acid is significantly decreased (Alikhani, Alikhani et al. 2005). CML-collagen treated adult human fibroblasts exhibited a five-fold increase in caspase- 3, a 4.3-fold increase in caspase-8, and a 3.2-fold increase in caspase-9 activity, compared with control, while in the presence of pancaspase inhibitor they showed a 92% reduction in apoptosis (Alikhani, Alikhani et al. 2007). AGE seem to constitute an important pathogenetic factor for the impaired diabetic wound healing (Peppa, Stavroulakis et al. 2009).

1.5.12 : Genetics of Diabetic retinopathy

Genetic influence is the increased risk of severe DR among family members with diabetes, in siblings of affected individuals approximately 3-fold increased risk and the moderate heritability of DR risk (0.52). The promoter region of VEGF has several polymorphisms, some of which have been associated with DR. In Japanese (Awata, Inoue et al. 2002), and Indian (Suganthalakshmi, Anand et al. 2006; Uthra, Raman et

al. 2008) population, the C(634)G polymorphism is associated. Studies have also implicated other VEGF single-nucleotide polymorphisms (SNPS) and haplotypes in DR (Al-Kateb, Mirea et al. 2007; Buraczynska, Ksiazek et al. 2007). Several clinical trials are currently investigating the efficacy of anti-VEGF agents in treatment of DR.

RAGE is a member of the immunoglobulin super family and the gene maps to chromosome 6p21.3. Activation of this receptor by high circulating levels of AGE leads to secretion of cytokines that hasten the progression of diabetic complications, partly by increasing endothelial permeability. The -374 T/A polymorphism in the RAGE gene has been associated with DR in a large Scandinavian study of 3,539 Caucasians, with a suggestion the effect may be dependent on glycosylated hemoglobin levels (Lindholm, Bakhtadze et al. 2006). This polymorphism has also been associated with DR in Asian Indians (Ramprasad, Radha et al. 2007). The Gly82Ser and -429T/C polymorphisms may also increase risk of DR in Asian Indian (Kumaramanickavel, Ramprasad et al. 2002) and Causcasian (Hudson, Stickland et al. 2001) populations respectively. Recent genome-wide association studies have reported a number of genetic variants that are consistently associated with risk of type2 diabetes. A genome linkage study has suggested that the genes for susceptibility to DR may be distinct from those for diabetes itself, highlighting the complexity of multifactorial interactions in the pathogenesis of diabetes and the development of its major complications. Despite these limitations, a few fairly consistent associations involving variants in the ALR2, VEGF and RAGE genes have been demonstrated. However, these associations have not been replicated in linkage analyses (Abhary, Hewitt et al. 2009; Liew, Klein et al. 2009).

Associations between MnSOD Val16Ala single nucleotide polymorphism (SNP) and DN (Mollsten, Marklund et al. 2007) or DR in type 2 diabetes (Mailaparambil, Krueger et al. 2008). The MnSOD Val/Val genotype was associated with a 2.49-fold higher risk for DR in a cohort of patients with type 1 diabete s. Val/Val genotype combined with smoking is associated with Diabetic nephropathy in type 1 diabetes. *In vitro* studies show that amino acid substitution of Ala with Val modifies helical

structure of the signal sequence and alters the import of MnSOD enzyme into the mitochondrial matrix (Sutton, Khoury et al. 2003). Studies on mice, treated with streptozotocin, show that MnSOD overexpression in mitochondria play a significant protective role in development of DR (Hovnik, Dolzan et al. 2009).

1.6: AGE - BLOCKERS

Several therapeutic strategies such as inhibition of AGE formation, blockade of the AGE–RAGE interaction and suppression of RAGE expression or its downstream pathways, may be promising for the treatment of vascular complications in diabetes.

Table1.2: Inhibitors of formation of Advanced Glycation End Products.

Agents	Company	Clinical outcome
Aminoguanidine (Pimagedine)	Synvista Therapeutics, Inc	Pimagedine reduced proteinuria and prevented the decrease in glomerular filtration rate in type1 diabetic patients. Comparing with those receiving placebo, fewer Pimagedine patients experienced a 3 step or greater progression of DR.
Pyridoxamine	Biostratum	Rate of rise in serum creatinine, a well-accepted marker for the progression of DR, was decreased in statistically significant manner in patients receiving pyridorin compared with those receiving Placebo.
Alagebnum	Synvista Therapeutics, Inc	Alagebnum reduced arterial pulse pressure and increased large artery compliance in patients with systolic hypertension. Patients with diastolic heart failure who received Alagebnum for 16 weeks experienced rapid remodeling of heart, resulting in a statistically significant reduction in left ventricular mass as well as a marked improvement in initial phase of left ventricular diastolic filling

Aminoguanidine prevented the formation of AGE modified apoA-1, thereby improving the capacity of apoA-1 to promote cholesterol efflux from human monocytes.

In addition, aminoguanidine not only stabilized ATP-binding cassette transporter A1 (ABCA1) in THP-1 macrophages but also decreased expression of CD11b in human monocytes, by inhibiting the AGE modification of apoA-1.(Hayashino, Fukuhara et al. 2007) These observations suggest that aminoguanidine may exert atheroprotective properties, at least in part, by improving reverse cholesterol transport in diabetic patients. Further, aminoguanidine also inhibited oxidative modification of LDL by binding reactive aldehydes formed during lipid peroxidation and preventing their subsequent conjugation of apoB (Picard, Parthasarathy et al. 1992).

In addition, AGE modification was reported to impair receptor-mediated clearance of LDL, thus contributing to elevated LDL levels in patients with diabetes (Hayashino, Fukuhara et al. 2007). Since administration of aminoguanidine to diabetic patients actually decreased circulating LDL levels by 28% (Bucala, Makita et al. 1994) amino guanidine is a promising therapeutic agent for the management of diabetic dyslipidemia.

1.6.1: Pyridoxamine (PM)

Recently, vitamin B complexes such as pyridoxamine and thiamine pyrophosphate have been found to inhibit the formation of AGEs both *in vitro* and *in vivo*. PM, originally described as a postAmadori inhibitor (so-called Amadorins) of AGE formation, also inhibits the formation of advanced lipoxidation end products (ALEs) on protein during lipid peroxidation reaction (Onorato, Jenkins et al. 2000; Price, Rhett et al. 2001). PM is effective at inhibiting AGE formation at three different levels by i) blocking oxidative degradation of the Amadori intermediate of the Maillard reaction; ii) scavenging of toxic carbonyl products of glucose and lipid degradation; and iii) trapping of reactive oxygen species (Voziyan and Hudson 2005). PM inhibited copper-catalyzed LDL oxidation and decreased high glucose-induced superoxide generation and lipid peroxidation in human red blood cells (Giannoukakis 2005). PM also protected against high glucose-induced DNA oxidative damage in ECs (Shimoi, Okitsu et al. 2001). Pyridoxal treatment also suppressed Hcy- and copper-induced apoptotic cell death of ECs by decreasing reactive oxygen species generation and lipid peroxide levels (Endo, Nishiyama et al. 2007). Further, PM was found to inhibit

AGE/ALE formation and hyperlipedemia and protected against wall thickening of the aorta and renal arterioles in non-diabetic obese rats (Alderson, Chachich et al. 2003). In addition, administration of pyridoxal phosphate, an active form of vitamin B_6, significantly inhibited albuminuria, glomerular hypertrophy, mesangial expansion, and interstitial fibrosis in association with the reduced expression of RAGE in the kidney (Nakamura, Li et al. 2007). Phase II trials are ongoing to evaluate the efficacy of Pyridoxin in inhibiting the progression of albuminuria in patients with early-stage diabetic kidney disease.

1.6.2: Metformin

Metformin (dimethylbiguanide) was introduced into clinical practice in 1957 as an oral antihyperglycemic agent for the management of type 2 diabetes (Bailey and Turner 1996). Metformin is a guanidine compound that is structurally related to aminoguanidine, suggesting that it may also have a potential effect on the inhibition of glycation reactions. Several groups reported inhibitory effects of metformin on protein glycation (Rahbar, Natarajan et al. 2000). A number of studies have shown that metformin is beneficial in reducing diabetes-associated vascular risks beyond the benefits expected from its antihyperglycemic effect. *In vitro*, metformin reduced the expression of RAGE and subsequently blocked the downstream signaling of AGEs in cultured ECs via a redox-sensitive nuclear factors such as NF- ķB (Ouslimani, Mahrouf et al. 2007).Further, metformin inhibited glycation of LDL and prevented the *in vitro* formation of foam cells (Brown, Mahroof et al. 2006). Chronic metformin treatment prevented functional and structural alterations of the diabetic myocardium associated with glycation as well (Regan, Jyothirmayi et al. 2001). These findings suggest that blockade of glycation reaction might provide one possible mechanism to explain the beneficial effects of metformin on diabetic vascular complications (Beisswenger and Ruggiero-Lopez 2003).

1.6.3: LR-90

LR-90, a methylene bis (4, 4-[2-chlorophenylureido phenoxyisobutyric acid]) is a new AGE inhibitor whose action is thought to be similar to those seen with

aminoguanidine and pyridoxamine. This agent has a strong metal chelation, leading to the inhibition of the formation of glycoxidative-AGEs and their interaction with ROS. Figarola and colleagues demonstrated that LR-90 prevented progression of diabetic nephropathy in streptozotocin induced diabetic rats (Figarola, Scott et al. 2003). LR-90 treatment also prevented acellular capillaries and pericyte dropout in experimental DR (Bhatwadekar, Glenn et al. 2008).

1.6.4 : Indirect inhibitors of the AGE–RAGE system

Administration of a recombinant soluble form of RAGE (sRAGE), consisting of the extracellular ligand-binding domain, has been recently shown not only to suppress the development of atherosclerosis but also to stabilize established atherosclerosis in diabetic apoE-null mice. The blockade of the AGE–RAGE axis by administration of sRAGE also ameliorates neuronal dysfunction and reduces the development of acellular capillaries and pericyte ghosts in hyperglycemic, hyperlipidemic mice (Barile, Pachydaki et al. 2005). Furthermore, recently, Kaji and colleagues have shown that attenuation of the RAGE axis with soluble RAGE inhibits retinal leukostasis and blood–retinal barrier breakdown in diabetic C57/BJ6 and RAGE-transgenic mice, which are accompanied by decreased expression of VEGF and ICAM-1 in the retina(Kaji, Usui et al. 2007). These observations suggest that exogenously administered sRAGE may capture and eliminate circulating AGEs, thus protecting against the AGE-elicited tissue damage by acting as a decoy. Antagonism of the RAGE axis by sRAGE is a novel therapeutic target for diabetic vascular complications.

1.6.5 : AGE-angiogenesis-Blockers

Nifedipine, one of the most widely used dihydropyridine-based calcium antagonists (DHPs) for treatments of patients with angina pectoris and hypertension, was found to inhibit RAGE over expression in AGE-exposed ECs by suppressing reactive oxygen species generation (Yamagishi and Takeuchi 2004). Thereby nifedipine may have therapeutic potentials in treatment of patients with various AGE-related disorders (Yamagishi, Nakamura et al. 2005). Glyceraldehydes and glycoaldehyde derived

AGEs which are formed at a faster rate were found to elicit angiogenesis through interaction with RAGE. They have found that cerivastatin an inhibitor of cholesterol biosynthesis completely prevented the AGE elicited angiogenesis in physiological range by inhibiting the transcriptional activation of NFkB and AP-1 and the resultant VEGF mRNA upregulation in EC (Okamoto, Yamagishi et al. 2002). PEDF has been shown to be versatile and the most potent endogenous inhibitor of angiogenesis, being more than twice as potent as angiostatin and more than seven times as potent as endostatin (Dawson, Volpert et al. 1999). PEDF occurs naturally in the eye, where it counteracts the stimulatory activity of pro-angiogenic factors, thus preventing ocular neovascularization under normal conditions (Dawson, Volpert et al. 1999) (Spranger, Osterhoff et al. 2001; Ren, Jie et al. 2005). PEDF inhibits the growth of microglial cells (Sugita, Becerra et al. 1997) and inhibits the proliferation of RAW264.7 cells (derived from macrophages) (Cohen, Sugita et al. 2000) and thus may also function as a neuroimmune modulator

PEDF inhibited the AGE-BSA-induced DNA synthesis and tube formation of microvascular ECs, by steps of suppressing VEGF gene induction. It was previously reported that PEDF generates the anti-angiogenic signal through the activation of the Fas/FasL death cascade by inducing its essential transducer, caspase-3, resulting in apoptosis in ECs (Volpert, Zaichuk et al. 2002) and opposing effects on ECs of different phenotypes (Hutchings, Maitre-Boube et al. 2002). PEDF (5 nM) alone significantly inhibits cell proliferation, migration and capillary tube formation, while the inhibition by PEDF is partial in the AGE-BSA-induced cell proliferation, migration and tube formation (Sheikpranbabu, Haribalaganesh et al. 2009).

These observations suggest the active participation of AGEs in PDR and that pharmacological upregulation or substitution of PEDF proteins may be a promising strategy in treatment of patients with DR. In support of this, PEDF levels in vitreous fluid are found to be decreased in angiogenic eye disease such as PDR (Spranger, Osterhoff et al. 2001). In addition, low content of PEDF in aqueous humor has been shown to predict the progression of DR as well (Yamagishi, Nakamura et al. 2007) .

Recently, PEDF has been shown to be a highly effective inhibitor of angiogenesis in cell cultures and animal models: PEDF inhibits the growth and migration of cultured

ECs, and it potently suppresses ischemia-induced retinal neovascularization (Tombran-Tink and Barnstable 2003; Yamagishi, Matsui et al. 2008).

In animal models, the administration of PEDF or pyridoxal phosphate, an AGE inhibitor, decreased the retinal levels of 8-hydroxydeoxyguanosine and suppressed intercellular adhesion molecule-1 (ICAM-1) gene expression and retinal leukostasis in diabetic rats by reducing RAGE expression (Yamagishi, Matsui et al. 2006; Yamagishi, Matsui et al. 2007). In vivo, overexpression of PEDF was found to alleviate microalbuminuria, prevent the expression of two major fibrogenic factors, TGF-β and connective tissue growth factor (CTGF) and significantly reduce the production of an ECM protein in the diabetic kidney (Wang, Zhang et al. 2006).

Moreover, PEDF up-regulated metalloproteinase-2 expression in the diabetic kidney, latter is responsible for ECM degradation. These findings suggest that PEDF functions as an endogenous anti-TGF-β and anti-fibrogenic factor in the kidney (Yamagishi 2009).

1.7 : CURRENT TREATMENT OF DIABETIC RETINOPATHY

1.7.1 : Laser photo coagulation

Laser photocoagulation restores useful vision in many cases of proliferative retinopathy and maculopathy. The blue-green light of the argon laser is maximally absorbed by vascular structures. It has a spot size of 50-500 μm and can be used to target discrete lesions such as clusters of leaking vessels identified by fluorescein angiography, but is usually employed to destroy large areas of generally diseased retina (Aiello, Pierce et al. 1995; Aiello, Bursell et al. 1997). Diabetic Retinopathy Study (DRS) (Sharp, Fallon et al. 1987) (Dowler 2003) and the Early Treatment Diabetic Retinopathy Study (ETDRS) (Mohamed, Gillies et al. 2007) have provided the strongest evidence to establish the place of panretinal photocoagulation as a standard technique for treating severe nonproliferative and proliferative DR and have shown that panretinal photocoagulation reduces the risk of moderate and severe visual loss by 50% in patients with severe nonproliferative and proliferative retinopathy.Laser treatment is also used to treat diabetic maculopathy either in the form of focal laser treatment or grid treatment. Focal treatment is required for focal

lesions (e.g microaneurysms, or short capillary segments) located between 500 and 3000μm from the centre of the macula, which causes the hard exudates and retinal thickening. For lesions which do not leak the fluorescein dye but which are located beyond 3000 μm from the center of the macula, focal laser photocoagulation is considered only if prominent leaks are present and are associated with retinal thickening that extend closer to the center. Photocoagulation may also be used in grid pattern to treat diffuse areas of leakage or non-perfused retinal thickening (1991). Properly conducted laser photocoagulation rarely causes serious complications.

1.7.2: Vitreo retinal surgery

Conventional laser treatment may fail in eyes with vitreous hemorrhage or in eyes with tractional retinal detachments and active progressive PDR. Early vitrectomy has been shown to improve visual recovery in patients with proliferative retinopathy and severe vitreous hemorrhage. The Diabetic Retinopathy Vitrectomy Study (DRVS) evaluated the risks and benefits of early surgical intervention versus conventional treatment for vitreous hemorrhage and very severe PDR (1988).The results of DRVS demonstrated that patients who underwent early vitreoretinal surgery had better outcome than those treated conservatively.

1.7.3: Role of Adjunctives and Other Pharmacotherapies

Although medical management (control of blood sugar, BP, and serum lipids) and ocular management (laser photocoagulation and vitrectomy) remain the mainstays of DR management, advances in pharmacotherapy have shown promise in the treatment of DR. The three major classes of medications currently being studied in this context are

- Corticosteroids,
- VEGFinhibitors/antagonists,
- Miscellaneous agents such as growth hormone inhibitors and other nutritional supplements.

1.7.4: Corticosteroids

Corticosteroids may produce their effects through multiple mechanisms of actions apart from their potent anti-inflammatory and VEGF downregulating effects. Intravitreal triamcinolone (IVTA) has shown significant improvements in DME and visual acuity in various Random clinical trails (RCTs) (Gillies, Sutter et al. 2006). However, steroid-induced elevation of intra-ocular pressure (IOP) and steroid-induced cataract are commonly reported as adverse effects of IVTA. Intravitreal or retinal implants have also been developed, allowing extended drug delivery. In this context, a surgically implanted intravitreal fluocinolone acetonide was shown to be associated with improvement in visual acuity in diabetic macular edema. An injectable, biodegradable intravitreal dexamethasone extended release implant was also evaluated in an RCT, with reported improvements in visual acuity and macular thickness.

1.7.5: VEGF Inhibitors

VEGF is an important mediator of angiogenesis and breakdown of blood-retinal barrier in the ischemic retina and its role in the pathogenesis of DR has already been described in earlier sections. Inhibition of VEGF activity may play a pivotal role in the prevention of DR. Three VEGF antagonists which are currently under investigation and are briefly discussed below.

Pegaptanib acts by targeting the 165 isoform of VEGF for treatment of neovascular age-related macular degeneration (AMD). In a RCT (n=172) patients with DME were randomized to repeated intravitreal Pegaptanib or sham injections. The results showed that Pegaptanib treated eyes were more likely to achieve improvements in visual acuity ($P = 0.03$), macular thickness ($P = 0.02$), and need for focal laser treatment ($P = 0.04$) at 36 weeks than those on sham injections (Cunningham, Adamis et al. 2005).

Another anti-VEGF agent, Ranibizumab, a recombinant humanised monoclonal antibody fragment with specificity for all isoforms of human VEGF, has been used for treatment of neovascular AMD and may also be useful for DR and DME (Brown, Kaiser et al. 2006; Chun, Heier et al. 2006; Rosenfeld, Brown et al. 2006). Similar to

ranibizumab, bevacizumab is a full-length humanised monoclonal antibody against VEGF which appears to produce similar efficacy for treatment of neovascular AMD and may also be effective for DME and proliferative DR.

Ranibizumab (Lucentis, Genentech, Inc.) and VEGF Trap-Eye (Regeneron Pharmaceuticals, Inc., Tarrytown, NY) are currently being evaluated for treatment of macular edema. All of the VEGF antagonists appear to require repeated intra vitreal injections to sustain benefits, increasing the likelihood of local complications including uveitis, cataract, retinal detachment, and endophthalmitis. Theoretical concerns about occlusion of native retinal vessels, hypertension, stroke, myocardial infarction, and thrombosis have not been demonstrated in large studies of these agents.

Table 1.3: Local (Ocular) therapies for diabetic retinopathy

Agent	Current status
corticosteroids	
Intravitreal triamcinolone acetonide	Published RCTs
Triamcinolone Intravitreal implant	Completed phase -I RCT
Fluocinolone acetonide Intravitreal implant	Completed phase - III RCT
Bioerodable deramethasone implant	Continuing phase -III RCT
VEGF antagonists	
Bevacizumab	Published RCTs
Ranibizumab	Continuing phase -III RCT
Pegaptanib	Published phase -IIRCT
Aflibercept(VEGF trap)	Published phase-I RCT
Serolimus rapamycin	Continuing phase -II RCT
Bevasiranib	Completed phase-II RCT
Vitreolytic agents	
Hyaluronidase	Published RCTs
Microplasmin	Continuing phase –III RCT
Other Anti-inflammatory agents	
Nepafenac	Published case series
Extanercept	Published pilot study
Infliximab	Continuing RCTs

RCT- Randomized Clinical Trial

1.7.6: Growth hormone inhibitors

Somatostatin analogs may inhibit angiogenesis directly through somatostatin receptors present on ECs and also indirectly through the inhibition of postreceptor signaling events of peptide growth factors such as insulin-like growth factor 1 and VEGF. In a randomized controlled study (n = 23) of patients with severe NPDR or early PDR, octreotide (a long-acting somatostatin analogue) along with conventional treatment reduced the requirement for laser photocoagulation and reduced the incidence of ocular disease progression compared to conventional treatment alone. A multicenter, randomized, placebo-controlled clinical trial of octreotide is currently under way in patients with severe NPDR and early PDR (Grant, Mames et al. 2000).

1.7.7: Beneficial effects of amino acids

Other than these, normal nutrients like amino acids have also been shown to play a role in diabetes. Amino acid availability is of considerable importance since amino acid deficiency could be an important factor in the etiology of some diabetic complications (Rosenlund 1993; Franconi, Di Leo et al. 2004). Insulin deficiency produced complex alterations in the amino acid level. The concentration of alanine, valine, leucine and isoleucine were increased while threonine, serine, lysine and histidine were decreased and some were unchanged. Amino acid content has been determined in plasma, erythrocytes, leucocytes and platelets of diabetic patients (Szabo, Kenesei et al. 1991; Franconi, Bennardini et al. 1995). Insulin has differential effects on protein and amino acid catabolism in type 1 and type 2 diabetic subjects. In addition, effects on protein metabolism in humans differ from those obtained from animal models of type 1 and type 2 diabetes. In animals, insulin seems to stimulate protein synthesis as well as prevent breakdown while human studies indicated that insulin deprivation is associated with very little protein synthesis and an increased rate of breakdown (Nair, Garrow et al. 1983; Nair, Ford et al. 1995).

Reports have shown that taurine administration at 1% to diabetic rats diminished diabetic nephropathy by reducing renal oxidant injury and AGEs accumulation and prevented nerve growth deficit (Obrosova, Fathallah et al. 2001). Studies in 1988

showed that taurine might interact/bind with insulin receptor and stimulate glucose and amino acids uptake (Maturo and Kulakowski 1988).

Subchronic administration of 4-OH isoleucine reduced basal hyperglycemia and improved glucose tolerance, attributed atleast partly, to a direct stimulatory effect on beta-cell function. Besides, its insulinotropic effect, the amino acid derivative activates phosphatidyl inositol-3-kinase (PI3-kinase) activity and reduces insulinemia in insulin resistance rat models (Broca, Breil et al. 2004). A mixture of ten nutritionally required amino acids were tested for insulin secretory response and arginine was found to be the most potent in stimulating insulin secretion (Floyd, Fajans et al. 1970). A mixture of eleven amino acids could improve glycaemic control and insulin sensitivity in elderly patients with poorly controlled type 2 diabetes mellitus and may be a promising antidiabetic formulation (Solerte, Gazzaruso et al. 2004). The varied effects of amino acids on insulin signaling were explained by Hinault et.al. in adipocytes (Hinault, Van Obberghen et al. 2006).They observed the potential benefits of amino acids in the pathophysiological situation of Insulin receptor, where signaling pathway is impaired at the level of PI3-kinase upstream of mTOR. Amino acids in combination with insulin are able to restore and activate Akt independently of PI3-kinase in the freshly isolated rat adipocytes and adipose tissue explants phosphorylating Thr 308 through PDK-1 and Ser 473 through mTOR–rictor (a rapamycin insensitive TOR; also PDK2).

Intervention of glycation may prove to be beneficial to patients suffering from diabetes mellitus. Free amino acids are known to mitigate the glycation of lens protein, delay cataractogenesis and bring down blood sugar levels in diabetic rats (Ramakrishnan and Sulochana 1993; Sulochana, Punitham et al. 1998). Further, free amino acids decrease the *in vitro* glycation of cytoskeletal actin. The amino acids lysine, arginine, alanine, aspartic acid and glutamic acid show varying degrees of antiglycating effect (Sulochana, Indra et al. 2001). Amino acids are shown to inhibit the binding of glucose with proteins, the first step in the pathway of glycation cascade, by competitive inhibition thereby offering protection (Manduteanu, Calb et al. 1992). Some amino acids can influence and mitigate other pathological pathways like oxidant stress and promote tissue sensitivity towards insulin. Besides being an insulin

secretagogue, glycine has antidiabetic effects. Supplementation studies with glycine have shown unequivocal results in both animals and patients. Administration of glycine attenuates the diabetic complications in the streptozotocin-induced diabetic rat model, probably due to inhibition of non-enzymatic glycation process (Alvarado-Vasquez, Lascurain et al. 2006). Glycine diminished hyperglycemia, hyper-cholesterolemia and glycated heamoglobin concentrations in diabetic rats (Alvarado-Vasquez, Zamudio et al. 2003). Glycine prevents non-enzymatic glycation of lens protein, decreases lipid peroxidation (Deters, Siegers et al. 1998) and H_2O_2-induced lethal cell injury (Weinberg, Varani et al. 1992).

To conclude what triggers EC Dysfunction in diabetes mellitus. Several arguments exist that link the formation of AGE to the EC dysfunction. In fact, infusion of AGE to non diabetic animals can reproduce many vascular complications of diabetes. Therefore the effect of AGE in DR has to be explored at the level of basal metabolism (like glucose uptake) which has not been studied so far, oxidative stress (at the enzyme level) and on angiogenesis and the role of amino acids in these conditions.

CHAPTER 2: OUTLINE OF THE WORK

Diabetic retinopathy remains one of the leading causes for blindness worldwide. Retinal microvascular dysfunction in diabetes is characterized clinically by hemorrhages, microaneurysms, cotton-wool spots, lipid exudates, macular edema, capillary occlusion, and ultimately, neovascularization. Animal studies have demonstrated that accumulation of AGE is associated with formation of microaneurysm, neovascularization and pericyte loss. AGE is formed from excess accumulation of glucose in the system due to the maillard reactions and is known to glycate proteins. AGE increases the RAGE expression which in turn induces the VEGF through ROS mediated mechanism. Recent reports have shown elevated levels of homocysteine in vitreous of DR. Hcys is a well known risk factor for cardiovascular disease and also for microvascular complications. Increased accumulation of Hcys inturn results in the formation of Homocysteinethiolactone (HCTL) which is more toxic than homocysteine and is removed from the system by the enzyme paraoxonase.

Although there are many reports *in vivo* and *in vitro* on the pathophysiology of DR associated with elevated Hcys, apart from hyperglycemia and AGE accumulation, there are still lacunae in the understanding of the disease mechanism as well as in treatment strategies. The proposed work is addressed to look into the effect of AGE on the glucose uptake in retinal ECs and pericytes and on the enzyme paraoxonase.To further explore the role of the enzyme paraoxonase both *in vitro* studies on the retinal capillary ECs and *in vivo* studies at the level of vitreous in DR were done. In addition, in silico analysis for structural elucidation of PON and its interaction with Hcys were done.

Although medical management (control of blood sugar, BP, and serum lipids) and ocular management (laser photocoagulation and vitrectomy) remain the mainstays of DR management, advances in pharmacotherapy have shown promise in the treatment of DR. However there is still a search for newer compounds which are cost effective with no side effects. Amino acids which are the essential nutrients for the system were found to have anti glycating effects and glucose lowering effects by our previous

studies. Therefore *in vitro* studies on bovine retinal EC, pericytes and retinal pigment epithelial cell were done to asses the role of amino acids as inhibitors of angiogenesis.

2.1: OBJECTIVES

1. To establish the primary cultures of bovine retinal ECs and pericytes and characterize them using specific markers.

2. To study the effect of AGE on Glucose uptake and GLUT-1 expression in bovine retinal ECs and pericytes.

3. *In vitro* studies on the effect of AGE, homocysteine, homocysteine thiolactone on paraoxanase in bovine retinal ECs and in *vivo* studies on the paraoxonase and HCTL levels in the vitreous of PDR cases

4. To study the effect of amino acids on the AGE induced angiogenesis in BREC cells

CHAPTER 3 : METHODOLOGY

3.1 : CELL VIABILITY ASSAY

Principle: Viability assays measure the percentage of a cell suspension that is viable. This is accomplished by a dye exclusion stain, where cells with an intact membrane are able to exclude the dye while cells without an intact membrane take up the coloring agent. The dye used for exclusion·stain is usually trypan blue but erythrosin B and naphthalene black can also been used. The dye is normally taken up by viable cells but not by the non-viable cells. The trypan blue dye exclusion is commonly used and a protocol for this procedure (Freshney 1987)

Method: The 0.4 % trypan blue dye exclusion test was carried out to find the number of viable cells in culture. A mixture of 1 volume of dye and 9 volumes of cell suspension was made in Phosphate buffered saline (pH 7.4). This was allowed to stand at room temperature for 10 minutes. Charged on to an improved neubaur chamber, the four corner 1mm sized squares were counted. Cells touching upper and left boundaries were included and those touching lower and right boundaries were excluded. Number of stained and unstained cells was separately noted. Calculations were done to express the number of viable cells as percentage. The viable cells do not take up the stain while the dead cells were stained. Cell count was carried out thrice to confirm results.

Calculation

The cell count (cells per ml) was determined as follows:

Cell count per ml = average cell count per square x dilution factor x 10^4

The cell viability (%) was determined as follows:

Cell viability (%) = cell count (viable) / total cell count (viable + non-viable) x 100

3.2 : IMMUNOFLOURESCENCE / IMMUNOHISTOCHEMISTRY

Immunofluorescence Method : Cultured BREC and BRP cells were grown on coverslips or in chambered slides and immunofluorescence (IF) or immuno-histochemistry (IHC) was done. The entire staining procedure was done in moist chamber. For immunoflourescence staining, the cells were fixed with cold acetone for 30 minutes. Primary antibody (refer following chapters) was added and incubated in moist chamber for 1 hour. After which the cells were washed thrice in PBST 5 minutes each. Secondary antibody conjugated to FITC was added and incubated for 45 minutes. Washed thrice in PBST, 5 minutes each. The cells were counter stained using Evan's Blue for 30 seconds. After washing in PBST, it was mounted in glycerol, and viewed under fluorescence microscope with blue filter (490 nm).

Immunohistochemistry Method: The slides were placed in an immunostaining workstation. The protocol was carried out with NovolinkTM Min polymer detection system. The cells grown on coverslips were fixed with 100 % methanol. They were blocked for peroxidase for 10 min provided in the kit and washed with TBS. This was followed by incubation with protein block for 30 min. After this blocking step, slides were incubated for 2 h with primary antibody. After washing with TBS, the slides were incubated for 45 minutes with post primary block. The slides were washed again and incubated for 45 minutes with polymer link. Finally, the slides were washed in TBS and rinsed with 0.1 M Acetate buffer (pH 5.0-5.2) and stained for 20 minutes with 0.8 % amino ethyl carbazone(AEC) in acetate buffer (pH 5). The sections were counterstained with haematoxylin for 15 seconds, rinsed for 10 minutes with water, and air dried. In negative control, the primary antibodies were skipped and the above protocol was followed.

3.3: WESTERN BLOTTING

The first step in a western blotting procedure is to seperate the macromolecules using gel electrophoresis. Following electrophoresis, the separated molecules are transferred or blotted onto a second matrix, generally a nitrocellulose membrane. Next, the membrane is blocked to prevent any nonspecific binding of antibodies to the surface

of the membrane. The transferred protein is complexed with an enzyme-labeled antibody as a probe. An appropriate substrate is then added to the enzyme and together they produce a detectable product such as a chromogenic or fluorogenic precipitate on the membrane for colorimetric or fluorometric detection, respectively. The most sensitive detection methods use a chemiluminescent substrate that, when combined with the enzyme, produces light as a by-product. The light output can be captured using film. Whatever substrate is used, the intensity of the signal should correlate with the abundance of the antigen on the blotting membrane.

3.3.1: Cell lysate preparation for Endothelial cells and Pericytes

The cultured cells once they reach the confluence were harvested by trypsinisation and the protein was extracted using 100 μl of the Tris buffer (pH 7.0) with protease inhibitor cocktail and sonicated for 80 cycles/min. The lysed cells were centrifuged at 10,000 rpm at 4°C for 5 min and protein was estimated by Lowry method.

3.3.2 : Protein Estimation

Protein estimation was done by conventional Lowry method. The assay was run along with a set of standard using BSA, ranging from 50 to 200 µg. The absorbance was read at 660 nm in DU 800 spectrophotometry.

3.3.3 : PAGE electrophoresis

SDS-PAGE electrophoresis was performed using Biorad. 10% separating gel was prepared and poured in the assembled glass plates sealed with 2% agarose gel and left it without disturbing. The separating gel was covered with saturated butanol while left for polymerization. After the separating gel was polymerized, 4% stacking gel was prepared and poured above the separating gel by removing the butanol layer and the comb was placed carefully with out any air bubble and left it aside for solidification. After the gel has been solidified the comb was removed care fully.

The protein samples are mixed with 3X sample loading buffer to attain a final volume of 50 µl and then incubated at 95° C for 2 mins. Then the sample was loaded on the gel and run till the dye reaches the bottom of the gel. The gel was taken carefully from the glass-assembled plates. The loading order was noted by giving a small cut at one end.

3.3.4 : Immunodetection

The proteins were transferred to nitrocellulose at 100V for one hour in cold transfer buffer. Once transferred, the membrane was stained with 0.5% Ponceau to check whether the transfer was done properly. Then the blots were washed with Milli Q water and blocked with 5% blocking solution (Skimmed milk powder). Primary antibodies were added according the standardized dilutions and incubated for 2h to overnight at 4°C. The blots were washed 3 times with TBST buffer 30 minutes each. The presence of the specific proteins was then shown by chemilumniscence of horse raddish peroxidase with chemilumniscence detection using plus Pierce western blot detection reagents. The blot was exposed to X-ray (Kodak X ray film) film for about 5 to 10 mins and then the film was developed.

3.4 : CYTOTOXICITY ASSAY

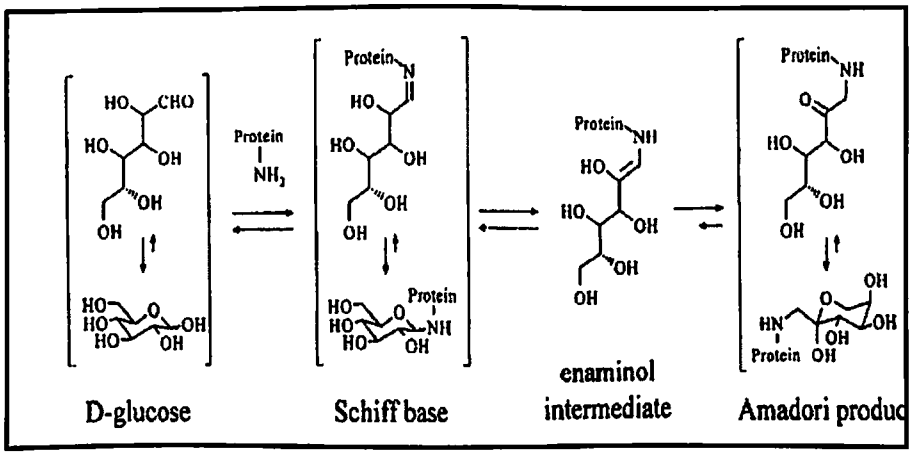
Principle: MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann in 1983, is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals which are solubilized. The number of surviving cells is directly proportional to the level of the formazan product created.

Protocol : The cells after exposure to experimental conditions were assayed by the following method. The medium was carefully removed and replaced with fresh serum free media. To this MTT from (5mg/ml Stock) was added corresponding to $1/6^{th}$ volume of the medium. This was incubated for 4h at 37°C. After the incubation, the medium was discarded and the formed crystals were solubilised in DMSO. After the crystals have dissolved, (5 minutes) the plate was read at 540 nm using multiwell scanning spectrophotometer (ELISA reader). The color can then be quantified using a simple colorimetric assay. Results are expressed as percent viable taking the absorbance of the control cells as 100 % (Mosmann 1983).

3.5 : PREPARATION OF ADVANCED GLYCATION END PRODUCT

Principle: The chemical, non-enzymatic glycation reaction between glucose (and reducing sugars in general) and the free amino group of lysine residues on protein is an inevitable consequence of the reactivity of aldehydes with primary amino groups. The reaction begins with the formation of a Schiff base between glucose and the ε-amino group of lysine that slowly rearranges to the relatively stable Amadori adduct (Fig. 3.1). The Amadori adduct is the first stable product formed during glycation of protein (Thorpe and Baynes 2003) and it has a half-life of several months under physiological conditions in humans (Lyons, Silvestri et al. 1991). The concentration of the Amadori adducts on protein reaches a steady state, reflecting the ambient glucose concentration and the rate of protein turnover. Concentration of Amadori adducts on protein will largely reflect the mean blood glucose concentration and generally only increases 2-3 fold, in proportion to the typical increase in blood sugar, in diabetic individuals.

Figure 3.1: Amodori product formation



Protocol AGE was prepared by slightly modifying the method of Yamagishi et al, by incubating 0.5 M glucose with 50 mg/ml bovine serum albumin (BSA) in phosphate buffered saline for 6 weeks at 37° C. For the control, BSA without glucose was incubated under similar conditions. The preparation was eluted through a PD-10 column (Amersham, Buckinghamshire, UK) with PBS to remove the excess salts and unreacted glucose. The glycated adduct formed was then confirmed by 7.5% SDS PAGE to compare the BSA alone (control) and glucose treated BSA for AGE formation and by Western Blot for Carboxy Methyl Lysine (CML) formation. (Yamagishi, Yonekura et al. 1997)

3.6: $U^{14}C$ GLUCOSE UPTAKE :

Principle: GLUT-1 and GLUT-3 are located in the plasmalemma of cells throughout the body, as they are responsible for maintaining a basal rate of glucose uptake. Basal blood glucose level is approximately 5 mM. The Km value an indicator of the affinity of the transporter protein for glucose molecules; a lower the Km value higher the affinity. GLUT-1 and GLUT-3 proteins have 1 mM; therefore GLUT-1 and GLUT-3 have a high affinity for glucose uptake from the bloodstream. The ECs and pericytes express GLUT-1 and GLUT-3 glucose transporters. GLUT-2 in contrast has a high Km value (15-20mM) and therefore low affinity for glucose. They are located in the plasma membranes of hepatocytes and pancreatic beta cells, and their high Km allows for glucose sensing, GLUT- 4 transporter are insulin sensitive, and are found in skeletal muscle and adipose tissue. As muscle is a principle storage site for glucose and adipose tissue for triglyceride (into which glucose can be converted for storage), GLUT-4 is important in post-prandial uptake of excess glucose from the bloodstream.

Experimental condition: The cells were plated in 6 well plates (15,000 cells/ well) and allowed to grow till 50% confluence in DMEM-F12 with 10% FBS. The cells were then exposed to the experimental condition for 6 days with an addition of fresh media every 48h. Cells without any treatment were used as controls. At the end of the 6^{th} day, the cells were serum depleted (with 1% FBS + DMEM – F12) for an hour followed by glucose depletion for another one hour (with 1% BSA + PBS). To this, 0.25μCi of U ^{14}C glucose was then added and incubated for respective time points like 5 seconds for BREC and 10 seconds for BRP cells as the kinetics of glucose uptake was 5-10s in EC and 10-15 sec for pericytes as reported earlier (Mandarino, Finlayson et al. 1994; Sone, Deo et al. 2000) The glucose uptake was stopped by adding ice cold PBS followed by washing (3 times) in ice cold PBS to remove residual radioactivity. The cells were solubilized using 0.1% SDS with 0.1% NaOH and 200 μl of the solubilised cell lysate was added to 2 ml of the scintillation fluid and the disinteragration per minute was counted in the Liquid scintillation system (Beckman-6500, Fullerton, CA)

3.7: FACS ANALYSIS OF GLUT-1 TRANSLOCATION

Principle: Flow cytometry is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and/or chemical characteristics of upto thousands of particles per second. A beam of light (usually laser light) of a single wavelength is directed onto a hydrodynamically-focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter or FSC) and several perpendicular to it (Side Scatter (SSC) and one or more fluorescent detectors). Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the light in some way, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a longer wavelength than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and, by analysing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive various types of information about the physical and chemical structure of each individual particle. FSC correlates with the cell volume and SSC depends on the inner complexity of the particle (i.e., shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness).

Protocol: For each experiment 30,000 cells were plated in 25 cm^2 flask and allowed to reach 50-70 % confluence depending upon the need. The cells were then exposed to experimental conditions for 6 days depending upon the experimental condition. The controls were the untreated cells and cells treated with BSA alone.

GLUT 1 staining: The cells were detached using 0.1 % trypsin EDTA and were centrifuged. 1 % BSA and 1 % sodium azide was then added to the pellet and incubated for half an hour, after which 100 µl of 1: 50 diluted polyclonal anti-human GLUT-1 raised in goat, Santa cruz was added and incubated for two hours. The cells were again washed in PBS and incubated with 1: 100 dilution of anti-goat tagged with FITC (Santa Cruz) in 3% BSA + 1% sodium azide for 45 minutes. The cells were again washed in PBS, three times and fixed in 0.1% paraformaldehyde for flow cytometric analysis. Control cells which were not treated with primary antibody were

used to set the voltage and those cells which were treated with secondary antibody alone were used to set the background.The flow cytometric analysis was done in BD FACS caliber four-color flow cytometer (BD Biosciences Model no: E 97600177). Data acquisition and analysis were performed using the BD CELL Quest Pro software. The data are expressed as Mean fluorescence intensity (MFI) of the total 10,000 cells which is over and above the fluorescence seen in the control cells (Aller, Ehmann et al. 1997).

3.8 : CALCIUM IMAGING

1. The cells were grown in cover slips in 6 well plates.
2. Before experiments, the cells were serum starved for 1 hr in Hanks balanced salt solution (HBSS) solution and washed with HBSS before addition of fura dye.
3. To 1 ml of the solution, 3 μM Fura AAM dye was added and incubated for 20-30 min.
4. Again the cells were washed with HBSS solution.
5. After which the coverslips were transferred into the Ca chambers in the microscope and the basal levels of Ca fluorescence was recorded till 100 cycles at 340 nm excitation and 380 nm emission in the Axiovision open work space zeiss fluorescent microscope.
6. Then the area of interest was selected and the background basal fluorescence was recorded.
7. Then thrombin was added for positive control and VEGF for the experimental condition and reading were taken till 250 cycles.
8. Then calcium was added and the readings were taken till 600 cycles.

Quality control for the experiment: Ionophore (0.1 μM): Open ups the cell pores so that when calcium is added there will be a rush of calcium and they will fluoresce more. This test will help to decide the cells integrity.

3.9 : TRANSENDOTHELIAL RESISTANCE

1. The cells were grown in electrode containing Petri dish. The cells were plated 2 days before the experiment and were allowed to grow to confluence.

2. The cells were given serum free media and the basal resistance was measured initially. The resistance must be 7000 ohms.
3. The basal reading must be constant and not be wavering.
4. After which the VEGF (50ng/ml) was added and incubated for 30 min and the readings were recorded (Jho, Mehta et al. 2005).

3.10 : mRNA EXPRESSION STUDIES

3.10.1: Total RNA extraction:

Total RNA was extracted (Sigma Genelute mammalian total RNA mini prep kit) according to the manufacturer's instructions as given below from the cell samples.

Table 3.1: Total RNA extraction

Release RNA from Cells or Tissues	➢ Add β-mercaptoethanol to lysis solution (10 µl 2-ME/1 ml of lysis solution) ➢ Lyse cells/homogenize tissue in 250 or 500 µl lysis solution/2-ME mixture ➢ Transfer lysate to blue filtration column. Spin for 2 min. (all spins ≥14000 *g)
Bind RNA to column	➢ Add equal volume of 70% ethanol to filtrate (200 or 500 µl). Mix thoroughly. ➢ Transfer up to 700 µl lysate/ethanol mixture to clear binding column ➢ Spin 15 sec ➢ Discard flow-through and repeat if necessary
Wash to remove contaminants	➢ Add 500 µl wash solution1 to the column ➢ Transfer column to new collection tube ➢ Add 500 µl wash solution 2 to column Note: Ethanol must be added to wash solution 2 concentrate before first use ➢ Spin 15 sec. Discard wash solution ➢ Add second 500 µl wash solution 2 to column ➢ Spin 2 min to remove ethanol
Elute purified RNA	➢ Transfer column to new collection tube ➢ Add 50 µl elution solution to column. Spin 1 min.

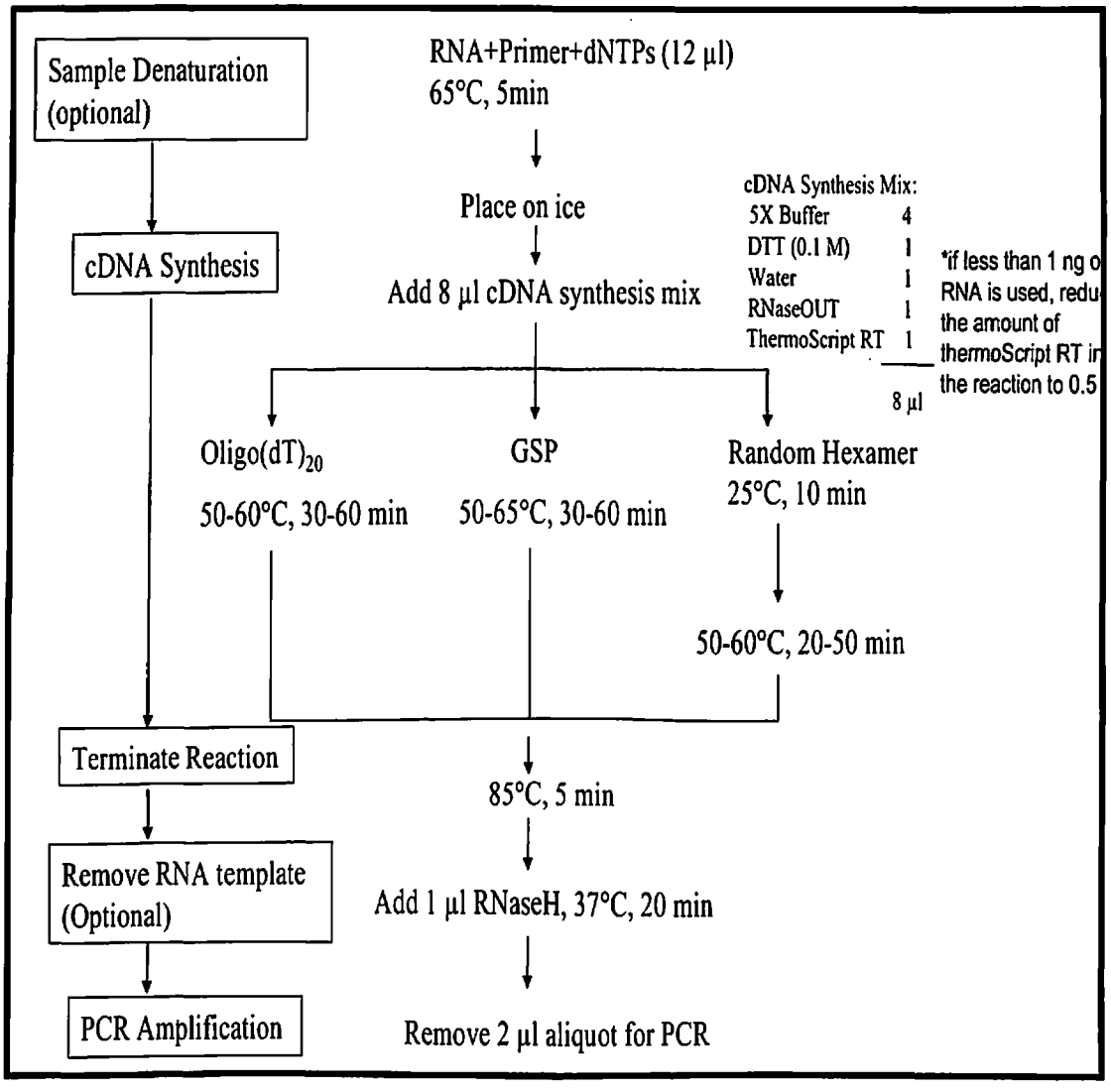
3.10.2: RNA purification

- Isolated RNA was treated by TURBO DNase (Ambion, Genetix Biotech Asia Pvt. Ltd, Chennai, India) to remove the DNA in the sample.
- 50µl reaction containing 7.5-10.0 µg of RNA was mixed with 1µl of TURBO DNase 1µl (2 units) and 0.1 volume 10X TURBO DNase buffer and was incubated at 37 °C for 30 minutes.
- To the reaction mixture, 0.1 µl of resuspended DNase inactivation reagent was added (5 µl) and mixed repeatedly during the incubation period of 2 minutes.
- The mixture was centrifuged for 1.5 minutes at 10,000 rpm and supernatant transferred to a fresh tube.
- The TURBO DNase treated RNA was quantified before reverse transcriptase PCR.

3.10.3: Reverse transcription

- For all samples 1µg of total RNA was used to synthesize first-strand cDNA using Thermoscript RT (Invitrogen, Joyvel, Chennai, India).
- Each component was mixed and centrifuged before use. ThermoScript. RT, an avian reverse transcriptase with reduced RNase H activity, is engineered to have higher thermal stability, produce higher yields of cDNA, and produce more full-length cDNA transcripts. The cDNA synthesis protocol is given below. (figure 3.2)

Figure 3.2: cDNA synthesis



3.10.4: Semiquantitative RT-PCR analysis

Using a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), as an internal control, the mRNA expression of GLUT – 1 VEGF and PON was analyzed by semiquantitative RT-PCR. Briefly, first strand cDNA synthesis was carried and 1.0 μg of cDNA was used for PCR in the thermal cycler. PCR amplifications were performed with the specific primers designed from published bovine gene sequences. Semiquantitation was established by terminating reactions at appropriate intervals for each primer pair to ensure that the PCR products formed were within the linear portion of the amplification curve. PCR products were fractionated by electrophoresis using 2% agarose gel containing 0.5% ethidium bromide with molecular marker *Hinf I* ϕ digest/100 bp ladder to confirm the size of the resultant product. The bands were analyzed using Image J software.

GAPDH primer

Forward primer 5-TGTTCCAGTATGATTCCACCC-3

Reverse primer 5-GTCTTCTGGGTGGCAGTGAT-3

Amplicon size: ~ 424 bp

GLUT -1 primer

Forward primer: 5'-TCC TGC TGC CCT TCT GCC CC -3'

Reverse primer: 5'-AGG TGG CTG CGG AGC -3'

Amplicon size: ~174 bp

VEGF primer:

Forward primer: 5'-CGAAACCATGAACTTTCTGC-3'

Reverse primer: 5'-CCTCAGTGGGCACACACTCC-3'

Amplicon size : ~299 bp

PON 2 primer:

Forward primer: 5' - CCT TCC TAA TTG CCA CCT GA – 3'

Reverse primer: 5' – TGG AGG CCT GGA CAT TTT AG – 3'

Amplicon size: ~150 bp

RT- PCR reaction cocktail:

10 X buffer	:	2.5 µl
dNTPs	:	2.5 µl (1in 10 dil)
Forward Primer	:	1 µl
Reverse Primer	:	1 µl
Taq poly	:	0.3 µl
MQ water	:	µl
cDNA	:	1.0 µg cDNA

The final reaction protocol for RT-PCR for GAPDH

95°C : 2 min

95°C : 30s
60°C : 1 min } 45 cycles
72°C : 1 min

72°C : 10 min

The final reaction protocol for RT-PCR for GLUT-1

95°C : 15 min

95°C : 1 min
60°C : 1 min } 40 cycles
72°C : 1 min 30 s

72°C : 10 min

The final reaction protocol for RT-PCR for VEGF

95°C : 2 min

95°C : 30s
60°C : 30s } 45 cycles
72°C : 30s

72°C : 5 min

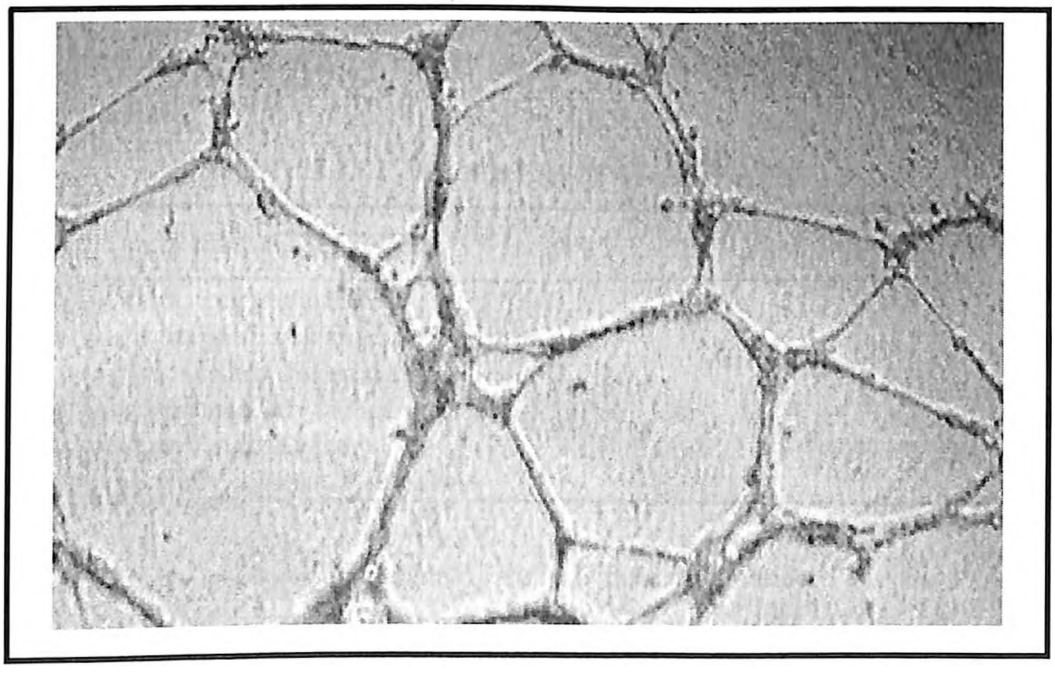
The final reaction protocol for RT-PCR for PON2

95°C	:	2 min	
95°C	:	30s	40 cycles
55°C	:	30s	
72°C	:	30s	
72°C	:	10 min	

3.11 Matrigel assay (tube formation assay)

Principle: When cultured on ECMatrixTM, a solid gel of basement proteins prepared from the Engelbreth Holm-Swarm (EHS) mouse tumor, ECs rapidly aligns and form hollow tube like structures (Sulochana, Fan et al. 2005) Tube formation is a multi-step process involving cell adhesion, migration, differentiation and growth *In Vitro* Angiogenesis Assay Kit represents a simple model of angiogenesis in which the induction or inhibition of tube formation by exogenous signals can be easily monitored. The assay can be used to monitor the extent of tube assembly in various ECs, e.g. HUVEC or bovine capillary endothelial (BCE) cells. (Figure 3.3).

Figure 3.3: Tube formation by endothelial cells



Protocol:

1. The ECM matrix diluent thawed at 4° C overnight.
2. Add 100 μl of the thawed 10X Diluent Buffer to 900 μl of ECMatrixTM solution in a sterile microfuge tube. Mix well slowly, do not pipette air into the solution. Keep solution on ice to avoid solidification.
3. Transfer 50 μl to each well of a pre-cooled 96-well tissue culture plate.
4. Pipette tips and ECMatrixTM solution should be kept cold all the time to avoid solidification.
5. Incubate at 37 °C for at least one hour to allow the matrix solution to solidify.
6. Harvest ECs and resuspend in media. Use EC growth media, or a standard cell growth media supplemented with EC growth supplements.
7. Seed (5×10^3 - 1×10^4)cells per well onto the surface of the polymerized ECMatrixTM.
8. Incubate at 37 °C overnight (4-12 h) in a tissue-culture incubator.
9. Cellular network structures are fully developed by 12-18h, with the first signs apparent after 5-6 h. After 24 h the cells will begin to undergo apoptosis.
10. To study the effect of pro-angiogenic factors, the incubation time should be decreased to 4-8 h. Optimal times may vary depending on the cell type, cell age and media growth conditions.
11. Inspect tube formation under an inverted light microscope using the following guidelines.

Table 3.2: Matrigel assay quantification

Pattern OBSERVED	Value ASSIGNED
Individual cells, well separated	0
Cells begin to migrate and align themselves	1
Capillary tubes visible. No sprouting	2
Sprouting of new capillary tubes visible	3
Closed polygons begin to form	4
Complex mesh like structures develop	5

The experiment was done in duplicates and the mean and standard deviation obtained was plotted as a graph.

3.12 : MIGRATION ASSAY (Sulochana, Fan et al. 2005)

Principle: Cell migration is a highly integrated, multi-step process that plays an important role in the progression of various diseases including cancer, atherosclerosis and arthritis. There are various classifications of cell migration.

- **Chemotaxis** Describes cell migration based on chemicals in the cell surrounding environment. Chemotaxis can indicate cell migration either toward or away from a particular chemical signal. These assays use the traditional Boyden chamber in 24-well or 96-well configurations, and 3 different pore sizes are available to accommodate a wide variety of cell types.
- **Haptotaxis** describes the cell migration up a gradient of extracellular matrix-bound chemoattractants. This is similar to the chemotaxis assays, but the underside of the membrane inserts is coated with a thin layer of either Collagen I or Fibronectin.
- **Transmigration** describes the migration of cells (usually leukocytes or tumor cells) through the vascular endothelium toward a chemo attractant.
- **Wound healing** describes the migration of cells toward a point of injury in order to heal the wound and close the gap.

Protocol: For the migration assay (wound healing) basic fibroblast growth factor (bFGF) was used as the positive control. The BREC cells were grown to confluence in gelatin coated 6 well plates in DMEM/F12 media. An wound was created with a sterile pipette tip in the middle of the confluent layer of the cells and then the cells were treated with bFGF positive control which enhances the migration of the cells and the inhibition of migration was tested with the amino acids glycine and glutamic acid at 5mM concentration and at lower concentrations (0.5mM-2.5mM) for 48 hrs and the effect was recorded.

3.13: Adhesion assay (Bevilacqua, Pober et al. 1987)

Principle: Cell adhesion is a complex mechanism involved in a variety of processes including migration and invasion, embryogenesis, wound healing and tissue remodeling. The recruitment of leukocytes into inflammatory tissues is regulated by

the interaction between blood cells and ECs that is preceded by integrin-mediated cell adhesion. Endothelial-leukocyte cell adhesion plays a major role in cellular communication and regulation, and is of fundamental importance in the development and maintenance of tissues. Expression of surface molecules on the vascular endothelium is altered at sites of pathological inflammation. EC Adhesion Molecules (ECAMs) are important mediators of leukocyte recruitment and adherence to the endothelium. ECAMs such as E-selectin, VCAM-1 and ICAM-1 are upregulated during inflammation, which initiates leukocyte adhesion to the endothelium, and ultimately contributes to disease progression or tissue damage.

Protocol: For the adhesion assay, the monocytes were isolated from the human blood using the histopaque gradient. The retinal ECs were plated in 6 well plates coated with gelatin and allowed to grow to confluence. The cells were exposed to 100 µg/ml of AGE for 24 hrs with and without amino acids. To the treated cells 1500 cells /well monocytes were added and incubated for 4 hrs, the excess cells which were not adhered was removed by PBS wash. The number of cells adhered was counted in four different fields.

3.14: ESTIMATION OF HOMOCYSTEINE THIOLACTONASE USING MASS SPECTROMETRY: LC-MS/MS (Velpandian.T, Angayarkanni.N et al.)

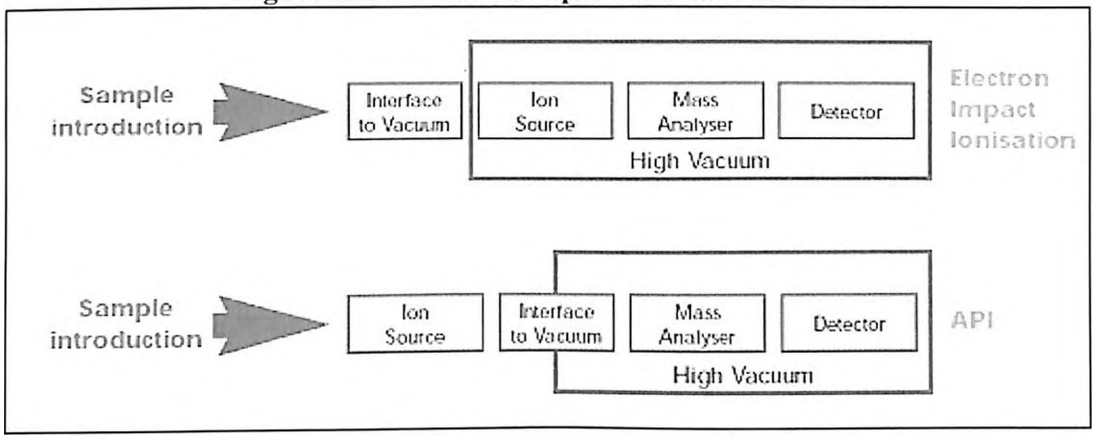
Principle: LC/MS is a hyphenated technique, combining the separation power of HPLC, with the detection power of mass spectrometry. Even with a very sophisticated MS instrument, HPLC is still useful to remove the interferences from the sample that would impact the ionization. The mass spectrometer is an instrument designed to separate gas phase ions according to their m/z (mass to charge ratio) value. The "heart" of the mass spectrometer is the analyzer. This element separates the gas phase ions.

The analyzer uses electrical or magnetic fields, or combination of both, to move the ions from the region where they are produced, to a detector, where they produce a signal which is amplified. Since the motion and separation of ions is based on electrical or magnetic fields, it is the mass to charge ratio, and not only the mass,

which is of importance. The analyzer is operated under high vacuum, so that the ions can travel to the detector with a sufficient yield.

1. Atmospheric pressure ionization (API) technique: The solvent elimination and ionization steps are combined in the source and take place at atmospheric pressure.
2. Electron impact ionization (EI): The solvent elimination and ionization steps are separate.

Figure 3.4: Schematic representation of LC/MS



Protocol: The liquid chromatography was done using the ZIC HILIC column (50 x 4.6 mm, 5μ , 200A, Merck, Darmstadt, Germany), with acetonitrile containing 0.1% formic acid as buffer A and 0.1% Formic acid as buffer B in HPLC system at the flow rate of 0.5ml/min (Thermo Fisher Corp, USA). For tandem mass spectroscopy analysis the mobile phase was passed through Online photodiode array detector Q-Trap API 4000 (Applied Biosystems, USA). For tuning 100 ng of HCYS, HCTL and Homatropine (internal standard) were pumped using Harward pump (Harward Instruments,) using a Hamilton syringe. Table below shows the ms/ms conditions and the product ions formed.

Table 3.3: LC/MS conditions and the product ions formed

Analyte	Q1	Q3	DP	EP	CE	CXP
Homocysteine	136	90	75	10	35	10
Homocysteinethiolactone	118.2	56	75	10	35	10
Homatropine (IS)	276.1	142	75	10	35	10

Ionizing conditions

Gas1 pressure – 20 psi

Gas 2 pressure – 20 psi

Curtain gas pressure- 20 psi

Spray temperature- 300

Ion spray voltage – 5.5 KV

Preparation of the standard: Accurately weighed HCTL were dissolved using 0.82% w/v of formic acid. It was suitably diluted to reach the concentration of 100-3.125 ng/ml and Homatropine (HBr) was used as the internal standard.

Preparation of sample: For the analysis of HCTL, 20 µl of the undiluted vitreous was extracted with 200µl of the extraction solvent (70:30 acetonitrile:water + 10% zinc sulphate). The mixture was centrifuged and 20 µl of the supernatant was used for the analysis

3.15: PARAOXONASE ENZYME ASSAYS

The enzyme Paraoxonase has three enzyme activities depending upon the substrate, it can act as paraoxonase, arylesterase and lactonase. It is associated with the HDL molecule in the serum.

3.15.1 Determination of Aryl Esterase activity: (Cabana, Reardon et al. 2003)

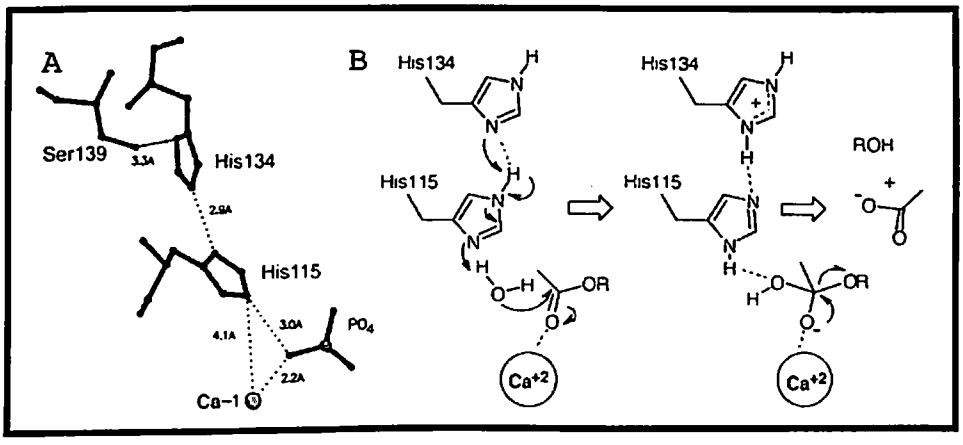
Principle: The postulated catalytic site and mechanism of PON1 for PON-ARE activity

A) The catalytic site: the upper calcium atom (Ca-1), the phosphate ion at the bottom of the active site, and the postulated His-dyad;

B) Schematic representation of the proposed mechanism of action of PON1 on ester substrates such as phenyl and 2-naphthylacetate.

The first step involves deprotonation of a water molecule by the His dyad to generate a hydroxide anion which attacks the ester carbonyl, producing an oxyanionic tetrahedral intermediate. This intermediate breaks down (second step) to an acetate ion and either phenol or 2-naphthol.

Figure 3.5: The mechanism of arylesterase reaction



Reagents required:

1. Phenol (1 mM)
2. Phenylacetate (1 mM / 0.5 mM)
3. Tris Hcl, pH8.0 (10 m M)
4. Calcium chloride (1 mM)
5. Tris Hcl and calcium chloride both are mixed and prepared for 100 ml in the above-mentioned concentration.

Protocol: In the presence of phenylacetate as substrate and calcium (for maintaining the enzyme activity) it acts as an aryl esterase (PON-AREase) and liberates phenol and acetate as the end product. The liberated phenol was measured in the kinetic mode at 270 nm using UV spectrophotometer.

Phenol from 10-50 µg concentration was read at 270nm and it was used as the phenol standard graph for the assay.

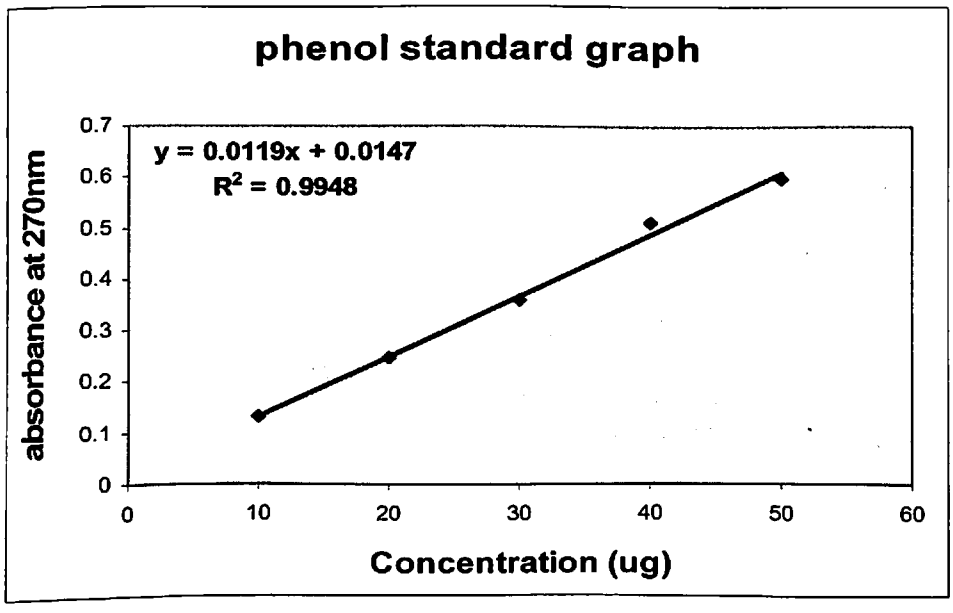
Table 3.4: Determination of Aryl Esterase activity Protocol

REAGENTS	BLANK	TEST
Buffer (µl)	950	940
Substrate (µl)	50	50
Test (µl)	-	10

Calculation

1. Test OD – Substrate OD = x OD value/2 for calculating the phenol liberated per min.
2. OD will be plotted in the phenol standard graph. Enzyme activity was expressed as µM phenylacetate hydrolysed /ml/min.
3. Normal Value : 65-100IU/ml/min

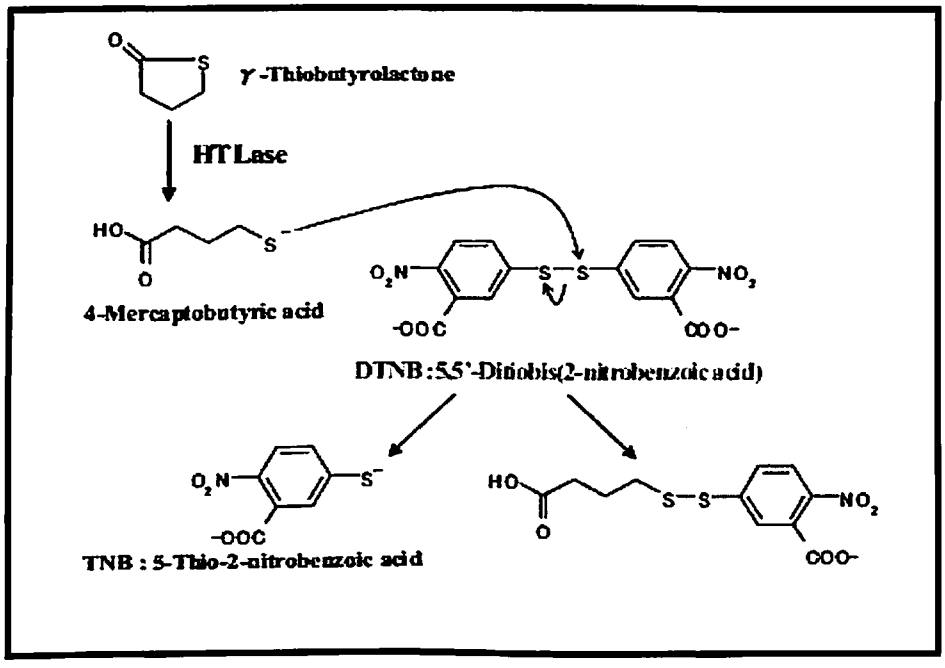
Figure 3.6: Phenol standard graph



3.15.2 : Determination of Homocysteine Thiolactonase activity *(Koubaa, Hammami et al. 2008)*

HTLase hydrolyzes the lactone ring of the substrate (γ-thiobutyrolactone), then free thiols which is released are detected using Ellman's reagent, DTNB; 5, 5'-dithiobis (2-nitrobenzoic acid).

Figure 3.7: The HCTLase rection



Reagents required:

1) Substrate – γ-thiobutyrolactone:

   Intermediate Stock - 0.85µl from stock made up to 2ml with distilled water. [Concentration-5mM]

   Working-4µl from intermediate stock made up to 1ml with distilled water. [Concentration-20 µM]

2) Phosphate buffer pH 7.2-100mM:

   Solution A-Disodium hydrogen phosphate – 1.41g/100ml

   Solution B-Sodium dihydrogen phosphate – 1.56g/100ml

   20ml of solution A and 2.5ml of solution B were mixed, adjusted the pH to 7.2 and made up to 50ml with distilled water.

3) Chromogen-DTNB [5,5'-dithiobis(2-nitrobenzoic acid)]:

   5 mg / 1ml of phosphate buffer. [Concentration-5µg/ µL]

Protocol: Homocysteine Thiolactonase activity was estimated using γ-thiobutyrolactone as the substrate and the rate of hydrolysis was measured spectrophotometrically in kinetic mode at 450 nm (main wavelength) and 546 nm (subwavelength)

- Assay was done in a 96 well ELISA plate. Total reaction volume is 200µl.
- Assay mixture containing 145µl of phosphate buffer, 5µl of 20µM substrate (final concentration is 0.5µM) and 50µl of chromogen (final concentration is 250µg) was used as the reagent blank.
- Test reaction contains 140µl of phosphate buffer, 5µl of 20µM substrate (final concentration is 0.5µM) and 50µl of chromogen (final concentration is 250µg) and 5µl of cell lysate.
- The reading was taken in the kinetic mode for every 1min for five minutes.
- Changes in the absorbance per minute were used for calculation of the activity, using the difference between the change in the absorbance of the sample (ΔEA), the change in the absorbance of the blank (ΔEB) and the extinction coefficient of 5-thio-2-nitrobenzoic acid (TNB).

Calculation of activity:

Activity (U/L) = [(ΔEA-ΔEB) xVx106] / [(ϵ1 – ϵ2) x sv x d]
Where, V – final volume of reaction mixture (200µl)

10^6 – transformation from moles to micromoles

ϵ1 – the extinction coefficient of 5-thio-2-nitrobenzoic acid for the main wavelength. (7667)

ϵ2 – the extinction coefficient of 5-thio-2-nitrobenzoic acid for the sub wavelength. (78)

Sv – sample volume (5µl)

d – Light path (0.5cm)

Substituting these values,

Activity (U/L) = [(ΔEA-ΔEB) x 200 x 10^6] / [(7667-78) x 5 x 0.5]

= (ΔEA-ΔEB) x 10541.57

3.16 : ACTIVITY STAINING FOR PARAOXONASE

Principle: Esterases are enzymes that are capable of hydrolyzing aliphatic and aromatic ester bonds. Depending on their preference for substrate, these esterases have been classified as specific or non-specific esterase. The hydrolyzed alcoholic residue will couple with the hexazotized pararosaniline solution to give an insoluble brightly colored azo dye as the reaction product at the enzyme site(Thiersch, Raffelsberger et al. 2008).

Pararosaniline + sodium nitrite → hexazotized pararosaniline
(Basic fuchsin)

Phenylaceate + H_2O → Phenol + acetic acid

Phenol + hexazotized pararosaniline → azodye

Reagents

1. 30 % Acrylamide
   29.2 g acrylamide
   0.8g bis-acrylamide dissolved in 60ml distilled water and made upto 100ml.
2. Tris-Hcl (pH 8.8)
   18g Tris dissolved in 50ml water. pH was adjusted to 8.8 with 1N Hcl. The solution was then made upto 100ml with distilled water.
3. Tris Hcl (pH 6.8)
   6g of Tris dissolved in 50ml-distilled water. pH was adjusted to 6.8 with 1N Hcl and then made upto 100ml with distilled water.
4. Ammonium per sulphate (10%)
5. TEMED
6. Electrophoresis buffer (pH 8.6)
   0.6g of Tris and 2.88g of glycine dissolved in 1000ml distilled water.
7. Tracking dye: 0.1% bromophenol blue in Tris glycine buffer.
8. Saturated 2 butanol solution

9. Separating gel solution (10%)

30% acrylamide – 3.3ml

Tris Hcl (pH 8.8) – 2.5ml

Distilled water - 4.2ml

10% APS- 50µl

TEMED - 5µl

10. Stacking gel (4%)

30% acrylamide –1.33ml

Tris Hcl (pH6.8) – 2.5ml
Distilled water - 6.1ml
10% APS- 50µl
TEMED - 10µl

11. Pararosaniline: To 1 g of pararosaniline added 20ml distilled water and 5ml of conc Hcl, warm the solution gently, cool to room temperature and store in a refrigerator.

12. Sodium nitrite (4%)

13. Phenylactate (1mM)

14. 0.2N phosphate buffer, pH 7.4

Na_2HPo_4 = 11.36g / 500ml

KH_2Po_4 = 2.7g / 500ml

Staining solution: Pararosaniline = 0.8ml, Sodium nitrite =0.8ml, allow to stand for 2-3min and then added phosphate buffer followed by phenylacetate (5ml) and adjust the pH between 6.8 - 7.2.

50µg of protein was run on a 12% native page and after the electrophoresis the gel was incubated in a staining solution for 1hr at 37° C. Once the bands were visualized the gel was washed throughly in phosphate buffer to remove the precipitate. It was then placed in 0.33% sodium metabisulphite in phosphate buffer to destain the gel for better clarity.

3.17: THIOBARBITURIC ACID REACTING SUBSTANCES (TBARS)

(Devasagayam and Tarachand 1987)

Principle: Malondialdehyde (MDA) produced during peroxidation of lipids, served as an index of lipid peroxidation. MDA reacts with Thiobarbituric acid (TBA) to generate a colored product, which absorbs light at 532 nm.

Reagents

1. 10% TCA
2. Thiobarbituric acid: 500mg of TBA dissolved in 6ml of 1M NaoH and to the solution 69ml of distilled water was added.
3. Stock standard Malondialdehyde:

   0.05ml stock solution of 1,1,3,3 tetraethoxy propane bis (diethyl acetate) was made upto 1ml with 0.9ml and 0.03ml of 6N HCl was added and made upto 100 ml in distilled water.
4. Working standard: 1ml of stock diluted to 50ml with distilled water.
5. Normal saline: 0.9% NaCl

Table 3.5: Thiobarbituric acid reacting substances Protocol

	Blank	Standard	Sample
Working standard MDA (µl)	-	10	-
Standard Concentration (nM)	-	5	-
Test (µl)	-	-	150
Distilled water (µl)	100	90	-
TCA (µl)	300	300	300
Incubate at room temperature for 15 min. 'Test' tubes were centrifuged at 2000 rpm for 10 min. Supernatant used for analysis.			
Supernatant (µl)	-	-	400
Thiobarbituric acid (µl)	350	350	350
Tubes contents were mixed well and heated in a boiling water bath for 15 min, cooled and absorbance was read at 532 nm.			

3.18: TOTAL ANTIOXIDANT CAPACITY : (Koracevic, Koracevic et al. 2001)

Principle: A standardized solution of Fe-EDTA complex reacts with hydrogen peroxide by a Fenton type reaction, leading to the formation of hydroxyl radicals (•OH). These reactive oxygen species degrade benzoate, resulting in the release of TBARS. Antioxidants from the added sample of human fluid cause suppression of the production of TBARS. This reaction can be measured spectrophotometrically and the inhibition of colour development defined as the antioxidant activity.

Reagents:

1. Sodium phosphate buffer: 100 mmol/litre, pH 7.4
2. Sodium benzoate: 10 mmol/litre
3. NaOH: 50 mmol/litre
4. EDTA: 2 mmol/litre in phosphate buffer
5. Fe $(NH4)_2SO4$: 2 mmol/litre.
6. Fe EDTA complex (prepared freshly by mixing equal volumes of solutions 4 and 5, left to stand 60 minutes at room temperature)
7. H_2O_2: 10 mmol/litre
8. 20% acetic acid
9. Thiobarbituric acid (TBA): 0.8% (wt/vol) in 50 mmol NaOH.
10. Uric acid 1 mmol/litre in 5 mmol/litre NaOH

Solution 4-9 should be prepared immediately before use. The sodium phosphate buffer and sodium benzoate should be kept in a refrigerator (0-4° C) and the uric acid solution in a deep freeze (-20° to -30° C)

Table 3.6: Total Antioxidant Capacity Protocol

	TEST		CONTROL		STANDARDS	
	T1	T0	K1	K0	S1	S0
Serum (ml)	0.01	0.01	-	-	-	-
Uric acid (ml)	-	-	-	-	0.01	0.01
Buffer (ml)	0.49	0.49	0.50	0.50	0.49	0.49
Sodium Benzoate (ml)	0.50	0.50	0.50	0.50	0.50	0.50
Acetic Acid (ml)	-	1.0	-	1.0	-	1.0
Iron-EDTA (ml)	0.2	0.2	0.2	0.2	0.2	0.2
Hydrogen Peroxide (ml)	0.2	0.2	0.2	0.2	0.2	0.2
Incubate for 60 min at 37° C and add						
Acetic Acid (ml)	1.0	-	1.0	-	1.0	-
Thiobarbituric Acid (ml)	1.0	1.0	1.0	1.0	1.0	1.0

Incubated for 10 min at 100 °C (in boiling water bath) then cooled in an ice bath. Measured absorbance at 532 nm against deionised water as blank.

Calculation: Antioxidant activity should be calculated as follows:

AOA (mmol/litre) = (C_{UA}) (K – T) / (K – S)

Where, K = absorbance of control $(K_1 - K_0)$

T = absorbance sample $(T_1 - T_0)$

S = absorbance of uric acid solution $(S_1 - S_0)$

C_{UA} = concentration of uric acid (in mmol/litre)

3.19 : TOTAL THIOL *(Coral, Raman et al. 2006)*

Principle: Most of the non-protein sulfhydryl groups are in the form of glutathione.5,5'-dithiobis(2-nitrobenzoic acid) [DTNB] is a disulphide compound that is readily reduced by sulfhydryl compounds, forming a highly colored anion. The absorbance of this yellow substance is measured at 412nm.

Reagents:

1. DTNB (10mM) 4mg/ml in methanol. The reagent can be stored for 2 weeks at 4 °C.
2. Tris base (0.25M) – EDTA (20mM) buffer,pH 8.2.

Table 3.7: Total Thiol Protocol

Reagents	blank	test
sample	-	50 µl
Tris-EDTA buffer	1.0ml	1.0ml
10mM DTNB	20 µl	20µl

Incubate at room temperature for 15 min and the absorbance was measures at 412nm

Calculation: (T-B)*1.57*1000, the value is expressed as µmol

3.20: HUMAN SOLUBLE ICAM 1 (sICAM-1) ASSAY BY ELISA

(R & D syatem)

Principle: This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for sICAM-1 has been pre-coated onto a microplate. Standards, samples, controls and conjugate are pipetted into the wells and any sICAM-1 present is sandwiched by the immobilized antibody and the enzyme-linked monoclonal antibody specific for sICAM-1. Following a wash to remove any unbound substances and/or antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of sICAM-1 bound. The color development is stopped and the intensity of the color is measured.

Protocol: Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards and the sICAM-1 Control be assayed in duplicate.

1. Add 100 μl diluted conjugate to each well.
2. Add 100 μl Standard, sICAM-1 control, or sample to each well with sufficient force to ensure mixing.
3. Cover the plate with a plate sealer provided and incubate at room temperature for 1.5 h.
4. Aspirate or decant each well and wash, repeating the process five times for a total of six washes. Wash by filling each well with Wash Buffer (300 μl) using a multi-channel pipette, complete removal of liquid after each wash is essential to good performance. After the last wash, aspirate or decant the contents and remove any remaining Wash Buffer by tapping the inverted plate firmly on clean paper towels.
5. Immediately add 100 μl Substrate to each well. Cover the plate with a new plate sealer and incubate at room temperature for 30 min.
6. Add 100 μl of Stop Solution to each well. The Stop Solution should be added to the wells in the same order as the Substrate.
7. Determine the optical density of each well within 30 min, using a microplate reader set to 450 nm.
8. The minimum detectable dose of sICAM-1 is less than 0.35 ng/mL

3.21 : HUMAN SOLUBLE VCAM 1 (SVCAM-1) ASSAY BY ELISA:

(R & D systems)

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for sVCAM-1has been pre-coated onto a microplate. Standards, samples, controls, and conjugate are pipetted into the wells and any sVCAM-1 present is sandwiched by the immobilized antibody and the enzyme-linked monoclonal antibody specific for sVCAM-1. Following a wash to remove any unbound substances, a substrate solution is added to the wells and color develops in proportion to the amount of sVCAM-1 bound. The color development is stopped and the intensity of the color is measured.

Protocol:

1. Add 100 µl of sVCAM-1 Conjugate to each well.
2. Add 100 µl of Standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 1.5 h at room temperature. A plate layout is provided to record standards and samples assayed.
3. Aspirate each well and wash, repeating the process three times for a total of four washes.
4. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Immediately add 100µl of Substrate Solution to each well. Cover with a new adhesive strip. Incubate for 20 min at room temperature. Protect from light.
6. Add 50 µl of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
7. Determine the optical density of each well within 30 min, using a microplate reader set to 450 nm.
8. The minimum detectable dose of sVCAM-1 ranged from 0.17 - 1.26 ng/ml

CHAPTER 4: ESTABLISHMENT OF PRIMARY CULTURES OF BOVINE RETINAL CAPILLARY ENDOTHELIAL CELLS AND PERICYTES FOR *IN-VITRO* STUDIES.

4.1: INTRODUCTION:

The retinal capillaries consist of the EC and pericytes in addition to the smooth muscle vascular cells. The EC line the lumen and the pericyte surround the vessel circumferentially. A basement membrane completely envelops the pericyte and separates them from EC except in occasional points of intercellular junctions. The endothelium plays a pivotal role in regulating blood flow and provides an active antithrombotic surface that facilitates transit of plasma and cellular constituents throughout the vasculature. The pericytes are regulators of vascular development, stabilization, maturation and remodeling.

EC are normally quiescent *in vivo*. Most of the EC in the adult have a cell cycle variable from months to years. Only endothelium from endometrium and corpus luteum has a doubling time in weeks. Nevertheless following an injury cells change their phenotype, migrate and proliferate to heal the lesion in a few days. The interaction between the EC and the pericytes has been studied. The pericytes are regulators of EC and known to secrete factors like TGF beta which inhibit the proliferation of EC and also plays a role in cellular signaling.(Antonelli-Orlidge, Saunders et al. 1989). It was reported that EC themselves recruit pericytes or smooth muscle cell during vessel formation (Ekblom, Sariola et al. 1982) and thereby influence each others behavior. The number of pericytes in each tissue varies and there is heterogeneity in the relationship between pericyte and EC in different tissues. Tissues with slowest EC turnover have the greatest coverage by pericytes and a pathological decrease in coverage is associated with EC proliferation. EC and pericytes are in the ratio of 1:1 in the retina. Recent studies intimate that impairments of one cell type will inevitably affect the other. There are huge number of studies which report that the retinal EC and pericyte play an important role in the

development of Diabetic Retinopathy (Mandarino 1992; Fujita 1999; Hammes 2005).The primary morphological change in the diabetic retina is the loss of pericytes that proceeds over the formation of acellular capillaries. Pericytes play an important role in the development and maturation of the vascular system (Lindahl, Johansson et al. 1997) Under normal conditions, pericytes have a survival and growth inhibiting effect on EC (Betsholtz 2004) But in hyperglycemic conditions, the protective function of pericytes is lost. There fore both the cells has been postulated as good models to study the pathophysiological changes associated with DR. The culture of these cells has been established in various tissues and widely used world wide for similar studies. Some of the methods followed as well as the growth factor and matrix required for its growth is listed below

a) Enzymatic digestion: Preferred techniques with large vessel, eg; the human umbilical vein (Capetandes and Gerritsen 1990) and sephanous vein. Collagenase is useful to remove the EC from the basal lamina

Advantage: Good yield and high purity (little contamination with non EC)

Disadvantage: This treatment may exert detrimental effects on certain surface protein that may be relevant in some studies.

b) Mincing and enzymatic digestion: Preferred technique with small vessels eg: Foreskin micro vessel or bovine adrenal cortex capillaries (Folkman, Haudenschild et al. 1979).

The selection of EC is done by

a) Filtering EC clusters on nylon mesh using specific media

b) Paramagnetic beads bearing endothelium specific antibodies.

c) Cell sorting.

c) By mechanical scraping and detachment: Suitable for both micro and macro vascular vessels. EC can be gently scraped by means of a rubber from the walls of large blood vessels. Eg: bovine aorta (Schelling, Meininger et al. 1988)

Advantage: Low damage to EC and high purity, **Disadvantage:** Low yield

These methods have been followed for various sources of tissue in different species like Human, Pig, Bovine, Rat and Rabbit and Sources like Pulmonary artery and vein (Del Vecchio, Siflinger-Birnboim et al. 1992) , saphenous vein (Gosling, Harley et al.

1998), umbilical artery and vein (Jaffe, Nachman et al. 1973), heart microvascualr coronary EC (Gospodarowicz, Moran et al. 1976), sinusoidal and liver EC(Gerlach, Zeilinger et al. 2001) , lungs microvascular EC (Hewett and Murray 1996), kidney (McGinn, Poronnik et al. 2004), brain (Williams, Gillis et al. 1980) and dermis microvascular EC (Folkman, Haudenschild et al. 1979; Kraling, Jimenez et al. 1994)

4.1.1 : Media requirements for endothelial and pericyte culture

The isolated EC require various factors for its growth like FGF fibroblast growth factor (a & b), (Gospodarowicz and Cheng 1986) ECGF EC growth factor (ECGF), Platelet derived EC growth factor (PD ECGF)(Miyazono, Okabe et al. 1987), and vascular endothelial growth factor(VEGF)(Neufeld, Cohen et al. 1999). The pericytes they are mostly cultured in DMEM or DMEM/F12 media.

4.1.2 : Matrix for growth of Endothelial cells and pericytes

It was reported that the optimal matrix for selective growth of retinal EC is fibronectin/ hyaluronic acid matrix or gelatin (Capetandes and Gerritsen 1990). The pericytes don't need any matrix for growth. The role of advanced glycation end products (AGE) in DR is being studied, however the basic metabolic changes induced by AGE in these retinal cells is yet to be understood completely which may be a causative agent for the loss of pericytes and formation of neovascular vessel. The commercial cell line currently available for Human retinal EC is from Cell Systems (Kirkland, WA). ACBRI 181 which can be used only till 10 passages. Since the availability of the human tissue also becomes a concern for continuous culture of cells. We wanted to culture the retinal ECs from the bovine source. Isolation of EC and pericytes from the bovine retinal microvasculature has been reported (Capetandes and Gerritsen 1990; Laver, Robison et al. 1993; Canfield and Schor 1995). Recently a simple and non-enzymatic method for the isolation and large-scale in vitro culture of bovine retinal EC (BRECs) has been described by Banumathi et al (Banumathi, Haribalaganesh et al. 2009) in which they showed a good yield of cells by brisk stirring of the retina with Minimum Essential Medium for 3 min, washing the pellet five times by repeated centrifugation at 300 × *g* and sieving in a single step. Pericyte contamination was eliminated by increasing the incubation period in an enzyme

mixture and passaging the cells at a low concentration of trypsin for 1–2 min. The cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) with 10% fetal bovine serum. Eric F. Grabowski et al has reported species variation in the growth characteristics of EC. The human retinal EC needs fibronectin coated surface for better growth but not the bovine cells. Therefore the first objective was to isolate and establish the culture of bovine retinal capillary EC (BREC) and pericytes (BRP) and characterizes them as EC and pericytes by using specific markers and then use them for *in vitro* experiments.

4.2. MATERIALS AND METHODS

4.2.1: Procuring eye ball

Bovine eyeballs were procured from the slaughter house in a steel container with sterile PBS and antibiotics (amphotericin 25 μg/ml, penicillin 10,000 units/ml and streptomycin10μg/ml). The eyeballs were washed three times in 40 μg/ml of gentamycin followed by sterile PBS wash and kept at 4°C till processing.

4.2.2 : Processing of eye ball (Cogan, Toussaint et al. 1961; Buzney, Massicotte et al. 1983; Bachetti and Morbidelli 2000).

The anterior segment was removed by a circumferential incision about 5 mm posterior to the limbus under sterile conditions. Vitreous was aspirated out using a sterile syringe. To the eyecup, added sterile water with antibiotics and the retina was removed from the eyecup. The removed retina was spread out in a sterile glass petri plate and the capillary was teased out using a sterile blade. The capillaries were then homogenized using a glass homogenizer with 5ml endothelial specific media. The homogenate was then filtered through a 41μ nylon filter for EC and through a 60μ filter for pericytes. The 41μ nylon filter containing the cells was then suspended in a petriplate containing the endothelial specific growth media with gelatin matrix and 60μ filter for pericytes was suspended in DMEM/F12 media with 10% FBS for pericytes and incubated at 37° C in 5% CO_2. Change of media was given every 48hrs till the cells reached confluence. Since the EC and pericytes are in the ratio of 1:1 in the retina and the EC enhance the growth of pericytes by releasing certain growth factors like PDGF and bFGF, which function in a paracrine fashion to promote cell proliferation, there is always a chance of co-culture of pericyte in EC cultures.To

isolate the EC in a pure form, CD31 Ab coated magnetic beads (dynabeads) were used which will specifically bind to the EC. The beads were initially equilibrated in 0.5% BSA in PBS. The initial mixed population of cells grown in the petriplate was trypsinised using 0.1% trpysin and EDTA and resuspended in 0.5% BSA. To this cell suspension 25µl of the equilibrated beads was added and incubated at 4°C for 1 hour for the interaction of the beads with the cells.The tubes were then placed in a magnetic rack and allowed to stand for 10 min. The cells which have adhered to the beads will pellet down due to the magnetic force. The pelleted EC were then plated in gelatin coated 25 cm^2 flask containing endopan media. The supernatant which contains the pericytes was centrifuged and the pellet was resuspended in DMEM/F12 media with 10% FBS. Figure 4.1

Figure 4.1: Steps involved in the processing of eye ball to isolate the retinal capillary cells

4.2.3 : Marker studies

For marker studies the EC were grown in gelatin-coated cover slips. The cover slips were fixed with absolute alcohol for 15 minutes at –20° C for both IHC and IF. Further steps were carried out with Novolink™ Mini polymer detection system (Novacastra laboratories ltd, NE12 8EW, UK) for immunohistochemistry. First the slides were incubated with protein block for 30 min. After this blocking step, slides were incubated for 2 hrs with either a rabbit polyclonal antibody directed against factor VIII (DAKO) or a mouse monoclonal VE-cadherin (R and D systems) antibody for identifying the EC. For identification of pericytes rabbit polyclonal Actin (Gift from Dr. Tombaran Tink) or antibody raised against NG2 (neural /glial antigen 2) (Chemicon) were used. The antibodies were used at a dilution of 1:50 (factor VIII) 10μg/ml (VE-cadherin) and 1:50 and 1:100 for NG2 and Actin, respectively. In negative controls, the primary antibodies were skipped and the same protocol was followed. The same concentration of antibody was used for the IF staining of these cells.

4.3 :RESULTS

4.3.1 : Endothelial cell morphology

Morphological criteria have been used to identify EC and pericytes in culture. The EC were small curved and spindle shaped with a centrally placed nucleus and one to three nucleoli and attained a cobblestone appearance at confluence observed with a phase contrast microscope. Initially the EC form foci in the primary culture and then the cells start spreading and finally become confluent in 20-30 days.Then the cells were trypsinized and separated using dynabeads which grew to confluence in 7 days. The isolated cells were used for experiments till 4 passages and the cells grew better in vented flask. Figure 4.2

4.3.2: Markers for endothelial cells

a) Factor VIII: Vitally important molecule synthesized in EC is Factor VIII, (Von Will brand's Factor). This is essential for the blood clotting reaction, seen as Weibel pallade bodies in human EC. The presence of Factor VIII in our EC culture was

confirmed by doing immunohistochemistry (IHC) as well as IF, using a rabbit polyclonal antibody directed against factor VIII (DAKO). Figure 4.3 (A1 & A2)

b)**VE-cadherin:** It is a strictly endothelial specific adhesion molecule located at junctions between EC. In analogy of the role of E-cadherin as major determinant for epithelial cell contact integrity, VE-cadherin is of vital importance for the maintenance and control of EC contacts. Mechanisms that regulate VE-cadherin–mediated adhesion are important for the control of vascular permeability and leukocyte extravasation. In addition to its adhesive functions, VE-cadherin regulates various cellular processes such as cell proliferation and apoptosis and modulates VEGF receptor functions. Consequently, VE-cadherin is essential during embryonic angiogenesis (Vestweber 2008). The presence of VE-cadherin was confirmed in the cell culture by both IF and IHC using a mouse monoclonal VE-cadherin (R and D systems). Figure 4.3 (B1 &B2) When maintained for long periods the EC disorganize. To test this function of the cell the cells were grown for 3 weeks and photographed under the phase contrast microscope.Figure 4.3 C Another characteristic feature of EC is their capacity to organize into tubules when plated on a three dimensional matrix. Figure 4.3 D

Figure 4.2: Primary culture of endothelial cells

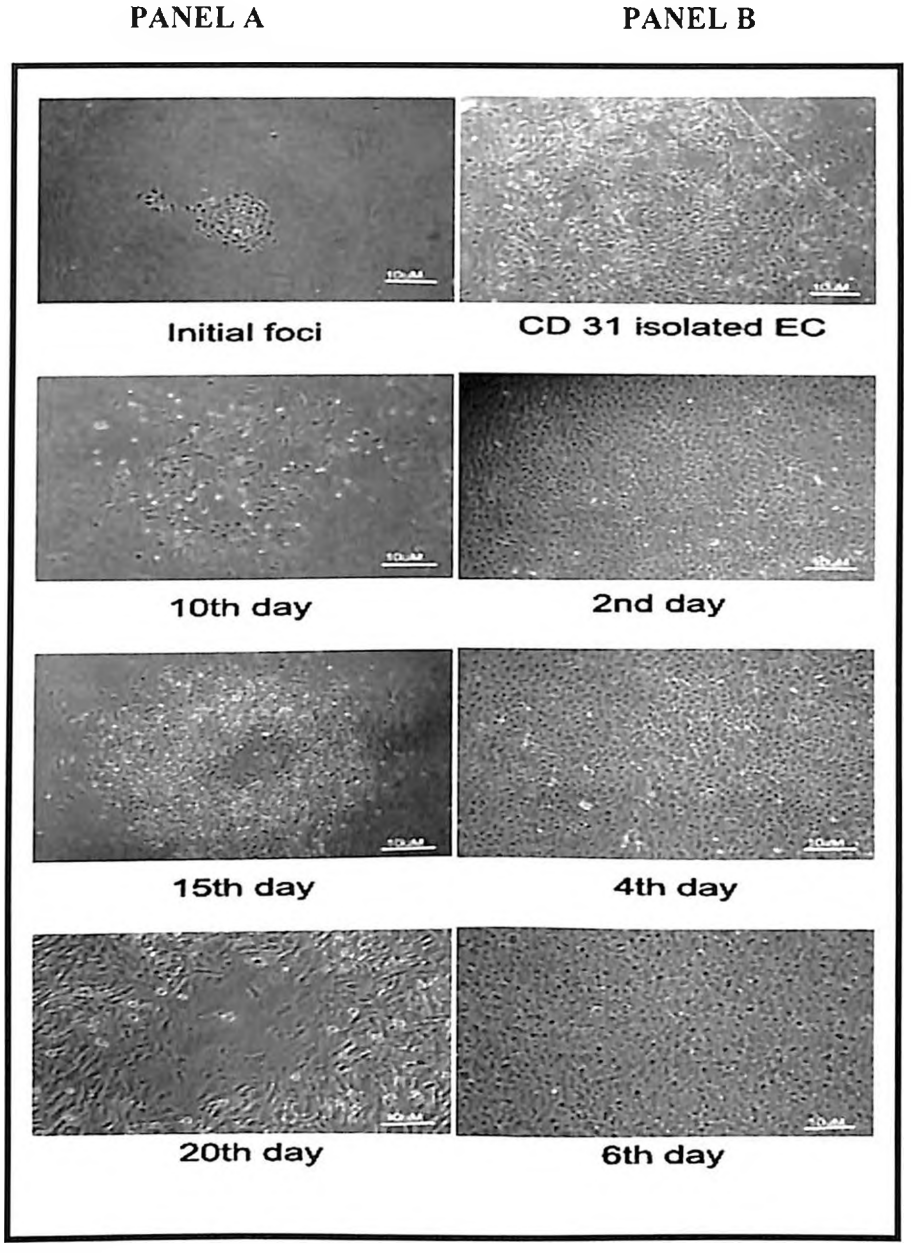
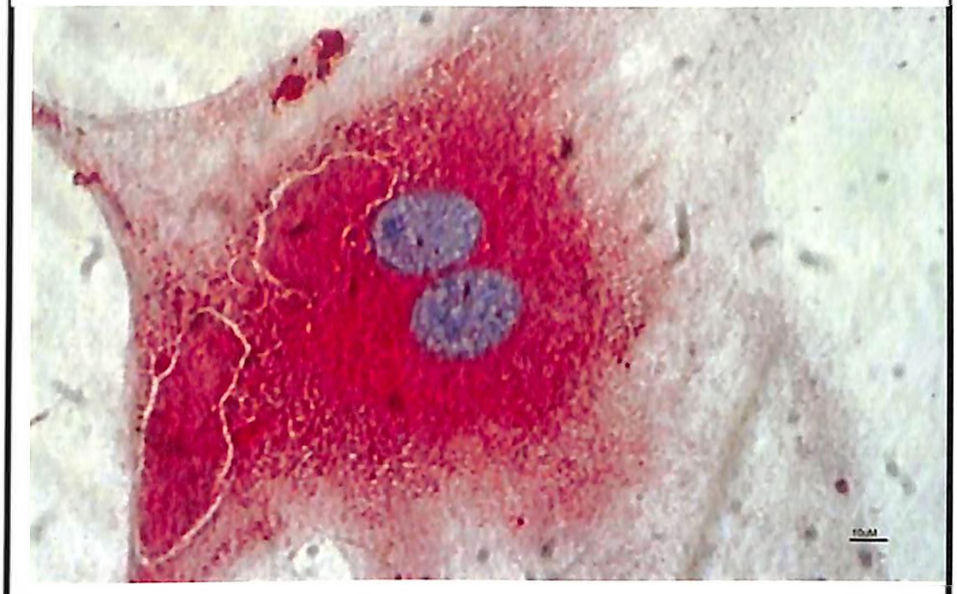


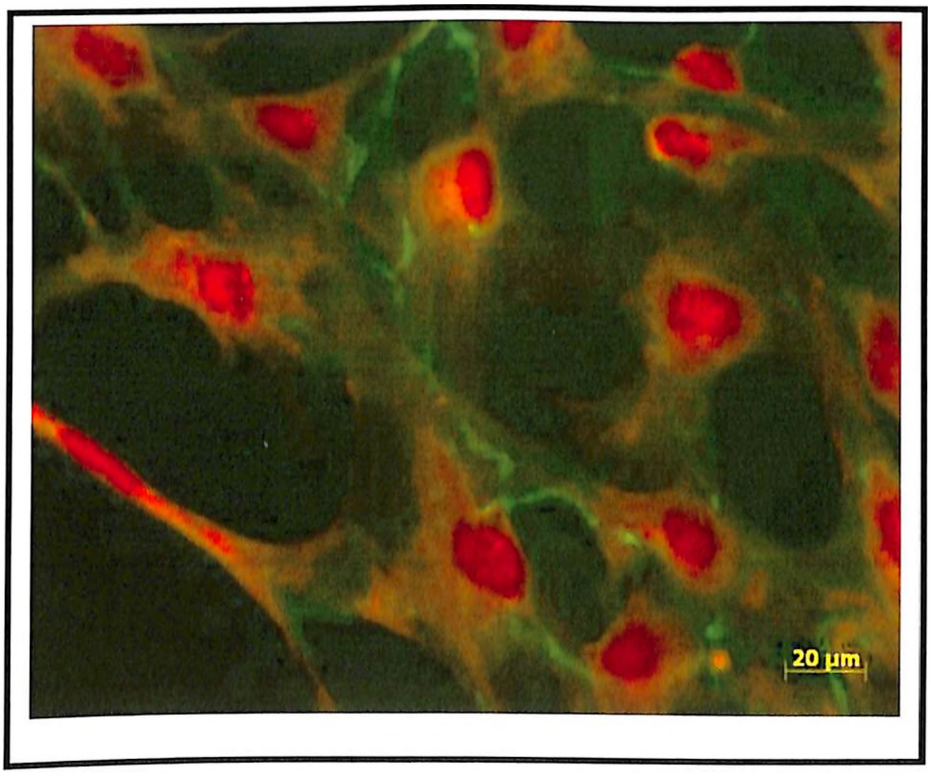
Figure 4.2:
Panel A: Initial growth of primary ECs from bovine retina from day 7 -20.
Panel B: Isolation of the EC to purity using CD 31 coated dynabeads and the cell growth to confluence

Figure 4.3: Endothelial cell markers:

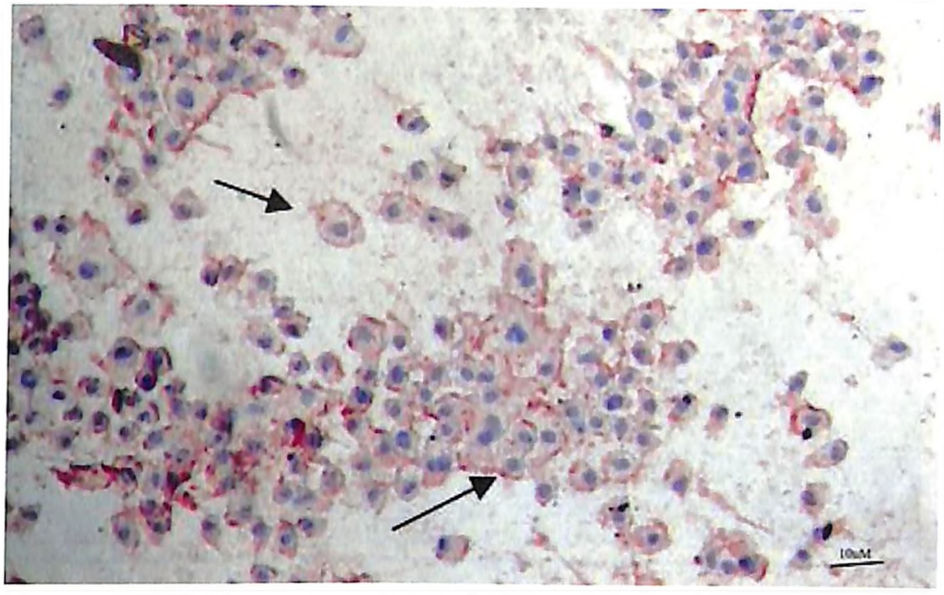
A1: EC showing Factor VIII positivity by IHC



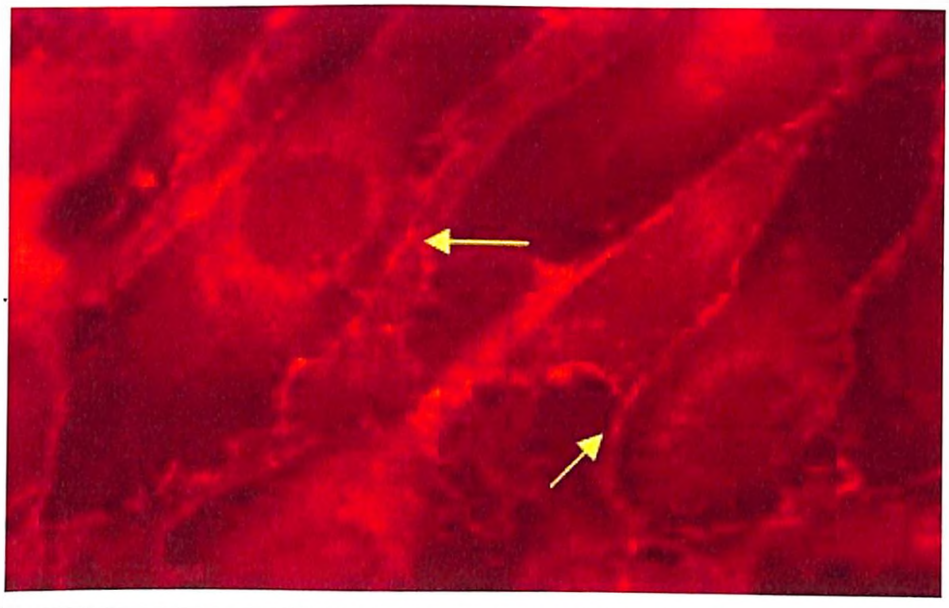
A2: EC showing Factor VIII positivity by IF

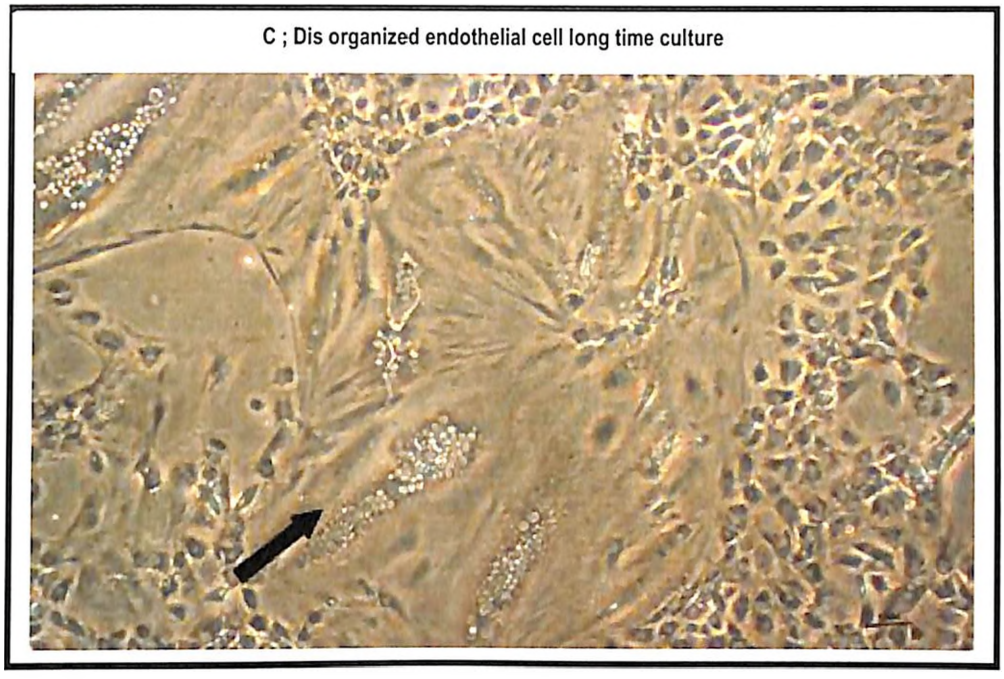


B1: EC showing membrane positivity for VE Cadherin (IHC)



B2: EC showing membrane positivity for VE Cadherin (IF)





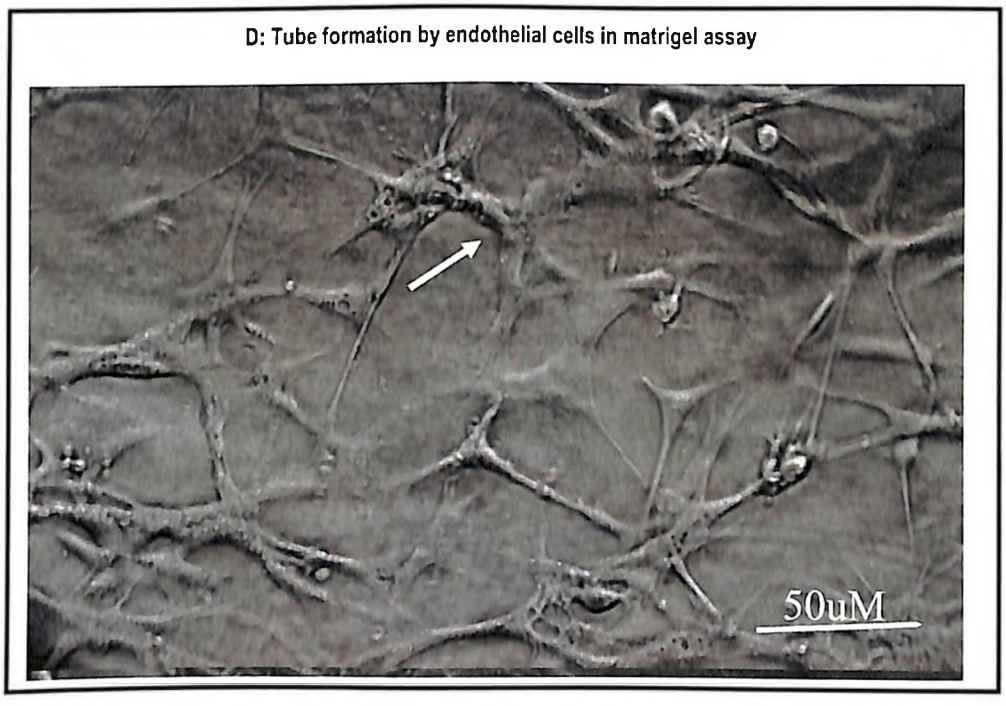


Figure 4.3: A1 & A2) Factor VIII staining of BREC cells showing reddish brown by Amino ethyle carbozone (AEC) in IHC and green fluorescence in IHC with hemotoxylin and eosin as counter stain. B1 & B2) VE cadherin showing reddish

brown membrane positivity in IHC and red fluorescence for rhodamine in IF. C) The disorganized EC indicated in black arrow D) Tube formation by EC in matrigel assay induced by AGE indicated by white arrow.

4.3.3 : Metabolic markers

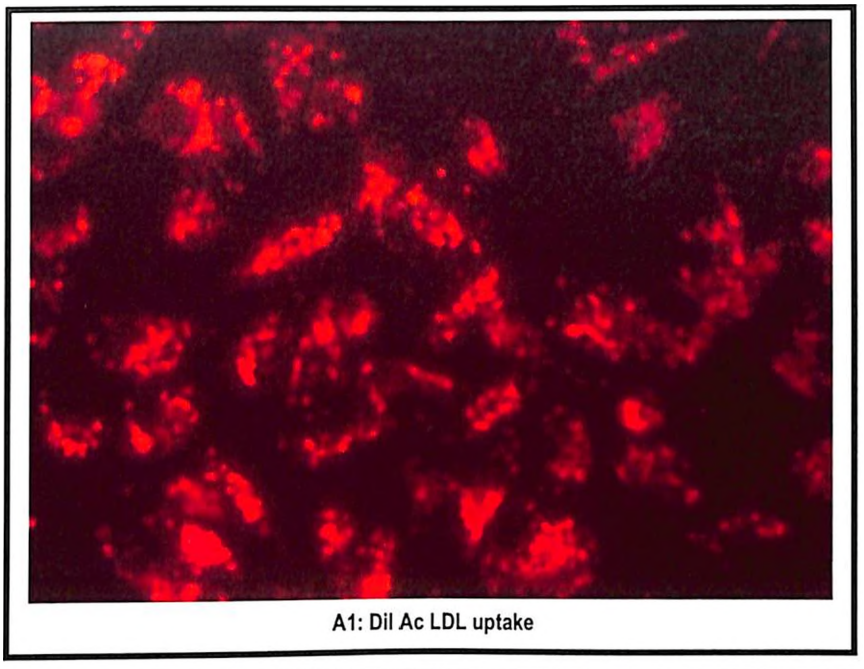
The metabolic markers for EC include Angiotensin converting enzyme and uptake of acetylated LDL by EC. For our isolated BREC cells the ACE activity was measured using a kinetic method and the uptake of acetylated LDL by fluorescence method.

a) Angiotensin I-converting enzyme: It is a circulating exopeptidase enzyme that participates in the body's renin-angiotensin system (RAS), and arterial vasoconstriction process. It is secreted by pulmonary and renal EC and catalyzes the conversion of decapeptide angiotensin I to octapeptide angiotensin II. It is used as a marker for the EC culture.(Auerbach, Alby et al. 1982) (Proc. NatL Acad. Sci. 79:7891-5, 1982).The presence of ACE in the cultured BREC cells was detected by a kinetic method using FAPGG (Furyl acroyl phenylglyclglycine) as substrate. The ACE cleaves the substrate as FAP and GG and the decrease in absorbance is measured at 340 nm for 15 min. The level of ACE is expressed as U/L. The BREC cells showed a basal value of 41 ± 1 U/L of ACE activity.

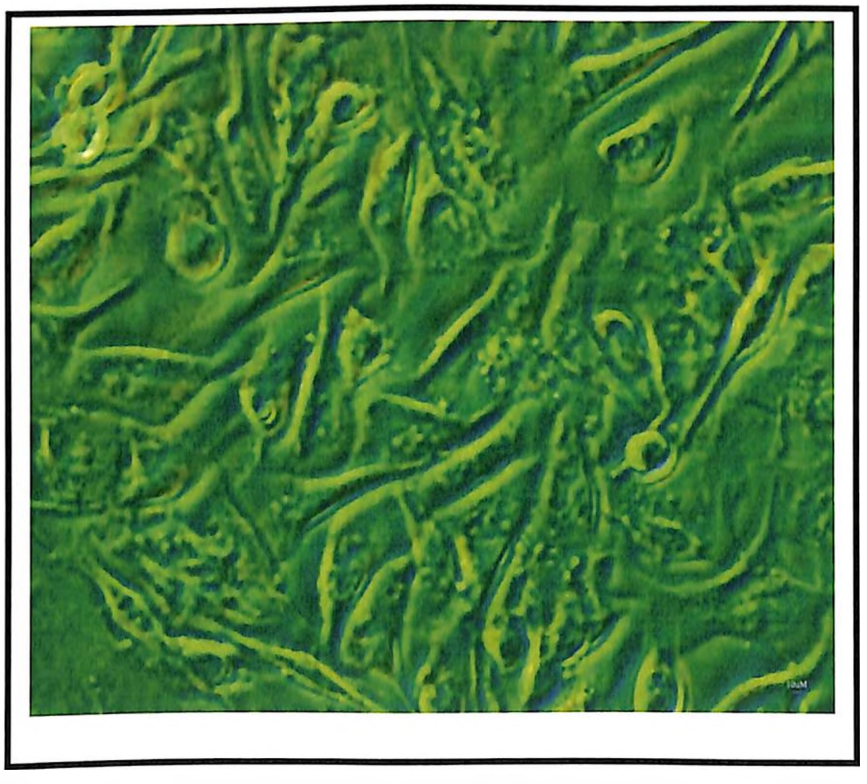
b) Incorporation of acetylated LDL:

The presence of scavenger receptors for acetylated low density lipoprotein (ac-LDL) on micro vascular endothelial cell (MEC) have been reported and detected using 1,1'-dioctadecyl-1,3,3,3',3'-tetramethylindocarbocyanine perchlorate acetylated LDL (DiI-Ac-LDL). The uptake of acetylated LDL is used as a marker to characterize the EC after isolation (Voyta, Via et al. 1984). For the fluorescent labeling, the cells were grown on cover slips in DMEM/F12 media and 10µg/ml of DiI-Ac-LDL was added to it and incubated at 37° C for 4 hrs. Then the cells were washed with PBS and fixed for 10 min with 0.1% Para formaldehyde and viewed under the fluorescent microscope. Figure 4.4

Figure 4.4: Acetylated LDL uptake by bovine retinal endothelial cells



A1: Dil Ac LDL uptake



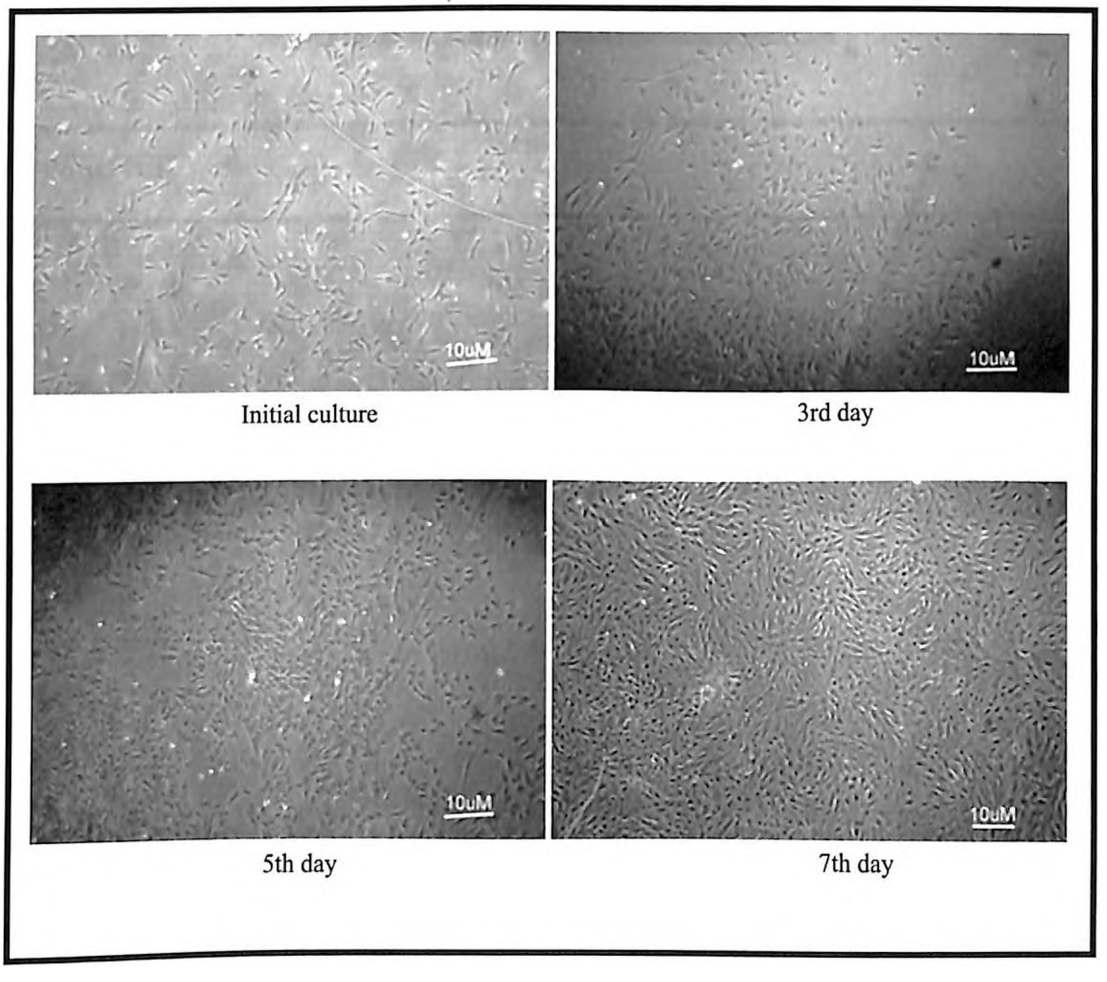
A) The acetylated LDL uptake in BREC cells showed in red with Rhoda mine B) the phase contrast image of the same cells in green filter.

4.3.4 : Morphology and Markers of Pericytes

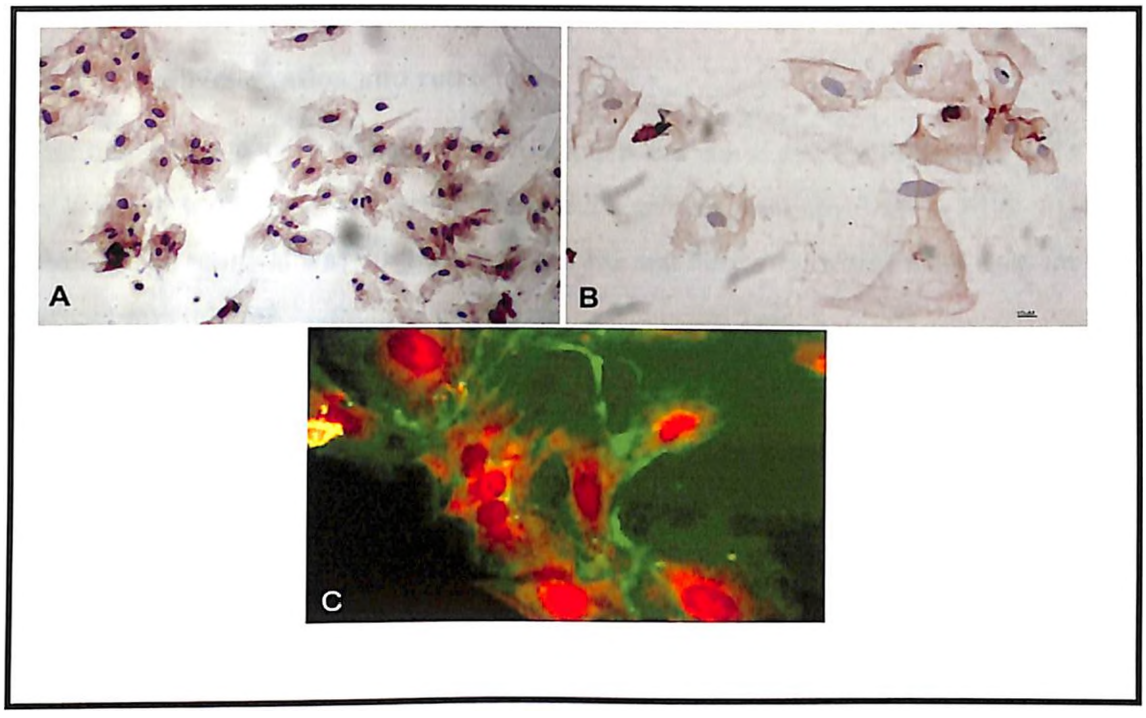
Cultured pericytes grow as large spreading cells with highly irregular edges and prominent intracellular actin bundles or stress fibers. Pericytes were identified by two specific markers which are routinely used to characterize them the alpha smooth muscle actin and the ganglioside antigen (NG2) (Nayak, Berman et al. 1988). For this the cells were grown on cover slips and fixed with 0.1% paraformaldehyde for immunohistochemistry and methanol for immunofluorescence. The pericytes were stained with rabbit polyclonal Actin (Gift from Dr. Tombaran Tink) and NG2 (neural glial antigen 2) (Chemicon) at a dilution of 1: 500 for both the antibody. The isolated cells were used up to 4 passages. Figure 4.5

Figure 4.5: The primary culture of Bovine retinal pericytes

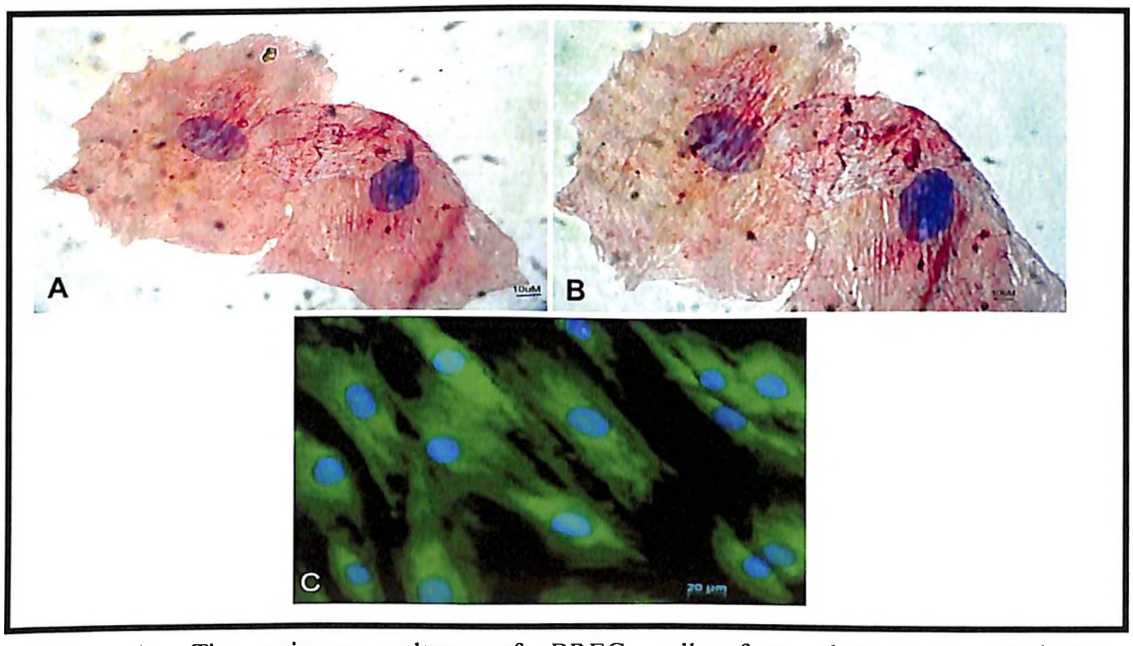
Panel A



Panel B I



Panel B II



Panel A: The primary culture of BREC cells from day 1 to confluence. **Panel B (I):** Reddish brown membrane positive NG2 in IHC by AEC and green fluorescence by IF counterstained by hematoxylin and eosin for nuclear stain.

Panel B (II): Alpha smooth muscle actin filaments reddish brown in IHC and green by IF counterstained with hematoxylin and dapi for nucleus as markers of pericytes.

4.3.5 : Cryopreservation and retrieval

The first passage cells which were confluent and have good morphology ($1x10^6$ cells) were trysinised and cryopreserved in growth medium + 20% FBS + 10% DMSO. The retrieval was 70- 80% for both EC and Pericytes, which were used for the various experiments.

CHAPTER 5: EFFECT OF ADVANCED GLYCATION END PRODUCTS ON GLUCOSE UPTAKE IN RETINAL ENDOTHELIAL CELLS AND PERICYTES

5.1: INTRODUCTION

Glucose uptake in endothelial cells

Glucose enters eukaryotic cells via 2 different types of membrane associated carrier proteins,

(i) Na^{2+} coupled glucose transporters (SGLT) and

(ii) Glucose transporter facilitators (GLUT)

5.1.1 : The SGLT Family

The SGLT family (sodium dependent glucose transporter; gene name SLC5A) comprises Na^{2+}dependent glucose co-transporters (SGLT1 and SGLT2), the glucose sensor SGLT3, the widely distributed inositol and multivitamin transporters SGLT4 and SGLT6 (Balamurugan, Ortiz et al. 2003), and the thyroid iodide transporter SGLT5 (Smanik, Liu et al. 1996) Intestinal glucose absorption and renal reabsorption in proximal tubules via SGLT1 and SGLT2 can be blocked by phlorizin (phloretin-2'-[beta]-glucoside), a plant product from the bark of the apple tree (Gerardi-Laffin, Delque-Bayer et al. 1993) .

5.1.2: The GLUT family

The human genome contains 14 members of the GLUT family which can be divided into 3 subfamilies according to sequence similarities and characteristic elements. The members of the GLUT family (gene name SLC2A) exhibit a striking tissue-specific expression (Gould and Holman 1993) (Kahn 1992). Moreover, they differ in their functional characteristics (eg, their substrate specificity, their Km values, and their binding-affinities to the inhibitory ligands cytochalasin B and forskolin). Furthermore, the function of some transporters (eg, GLUT4) is modified by a

regulated redistribution of the protein between the cell membrane and an intracellular compartment. These different characteristics allow a complex and specific regulation of glucose uptake according to the cellular requirements and the physiologic conditions of substrate supply (Joost and Thorens 2001). *There are four classes.*

a. Class I sugar transport facilitators: Consist of facilitative glucose transporters GLUT 1 – 4. GLUT1 exhibits highest expression levels in erythrocytes and EC of the brain. GLUT2 is a low-affinity glucose transporter with predominant expression in pancreatic [beta]-cells, liver, kidney, and small intestine (basolateral membrane). In all these tissues, the uptake of glucose is not dependent on the number and activity of the glucose transporters but on the blood glucose concentration. GLUT3 (Kayano, Fukumoto et al. 1988) is a high-affinity glucose transporter with predominant expression in tissues with a high glucose requirement (eg, brain). GLUT4 (Fukumoto, Kayano et al. 1989) is a high-affinity glucose transporter expressed in insulin-sensitive tissues (heart, skeletal muscle, adipose tissue).

b. Class II sugar transport facilitators: The class II facultative glucose transporters include the fructose-specific transporter GLUT5 and 3 related proteins, GLUT7, GLUT9, and GLUT11. GLUT5 mRNA (Kayano, Burant et al. 1990) is predominantly expressed in small intestine, testis, and kidney. GLUT5 exhibits no glucose transport activity and is responsible for the uptake of fructose in the mentioned tissues. GLUT7 is a high-affinity transporter for glucose and fructose (Li, Manolescu et al. 2004). GLUT7 mRNA can be detected in small intestine, colon, testis, and prostate; within the small intestine, GLUT9 exhibits highest expression levels in kidney and liver; lower mRNA levels were detected in small intestine, placenta, lung, and leukocytes. GLUT11 (Doege, Bocianski et al. 2001) exhibits a low affinity for glucose and a low-affinity cytochalasin B binding. GLUT11 is expressed predominantly in pancreas, kidney, and placenta and exhibits also moderate expression in heart and skeletal muscle.

c. Class III sugar transport facilitators: Class III comprises the transporter isoforms GLUT6, GLUT8, GLUT10, GLUT12. Because class III sugar transporters show highest homologies with different transporters in yeast, bacteria, and Drosophila

melanogaster, they were speculated to have evolved earlier than class I and class II transporters. The low-affinity glucose transporter GLUT6 (Doege, Bocianski et al. 2000) is predominantly expressed in brain, spleen, and peripheral leukocytes. Like its closest relative GLUT8, the N-terminus of GLUT6 harbors a dileucine motif that directs the protein to intracellular storage compartments when expressed in isolated fat cells and COS-7 cells. (Lisinski, Schurmann et al. 2001). GLUT10 is predominantly expressed in the liver and pancreas. (McVie-Wylie, Lamson et al. 2001) GLUT12 is predominantly expressed in heart and prostate and exhibits glucose transport activity when expressed in X. laevis oocytes. GLUT12 seems to sustain the increased glucose consumption in prostate carcinoma (Chandler, Williams et al. 2003) and breast cancer. (Rogers, Docherty et al. 2003).

5.1.3 : Glucose Metabolism in Endothelial Cells

Glucose is actively metabolized in EC (Gerritsen and Burke 1985) and sustains anaerobic and aerobic metabolism (Mertens, Noll et al. 1990). At physiological concentrations of glucose, the contribution of the hexose monophosphate pathway accounts for 1.2 % of glucose metabolism and the Krebs cycle for only 0.04 %, suggesting that in microvascular EC almost all of the energy obtained from catabolism of glucose is generated glycolytically. At lower glucose concentrations (1mM), oxidation of glucose via the Krebs cycle is higher. Thus oxidative metabolism in EC is inhibited at physiological concentrations of glucose, demonstrating that EC express the Crabtree effect i.e., an inhibitory effect of glucose on mitochondrial respiration (Krutzfeldt, Spahr et al. 1990) .

EC are able to withstand prolonged periods of substrate deprivation and can adapt to hypoxia due to their low energy demand and high glycolytic activity (Culic, Decking et al. 1999), (Mertens, Noll et al. 1990). Recent evidence in HUVEC suggests that fatty acids can also serve as an energy fuel (Dagher, Ruderman et al. 2001) However, oxidation of fatty acids in umbilical vein EC only accounts for 25% of the calculated ATP production in cells incubated with 5 mM glucose.

The effects of elevated glucose on EC function are often tissue specific (Kaiser, Sasson et al. 1993) (Kumagai 1999) (Kumagai, Glasgow et al. 1994) (Mandarino,

Finlayson et al. 1994). The cytosol of EC is reduced by accumulation of NADH and transformation of pyruvic acid to lactate, as described in microvascular EC from bovine corpus cavernosum (Dobrina and Rossi 1983) and brain microvessels (Hingorani and Brecher 1987). In some, but not all, EC types, the polyol pathway can reduce glucose to sorbitol via aldose reductase (AR), which has an extremely low affinity for glucose (*Km* 100 mM) but is activated by glucose. Conversion of glucose to sorbitol by aldose reductase forms NADP+ and may compete with other NADPH-requiring reactions such as conversion of oxidized glutathione (GSSG) to reduced (GSH) glutathione (Asahina, Kashiwagi et al. 1995). Kashiwagi et al. emphasized that glucose induced activation of the polyol pathway in EC may not be directly responsible for the associated decrease in NADPH content, but rather activation of the pentose phosphate pathway and NADP supply to the GSH redox cycle is impaired by H_2O_2 generated in cells exposed to high glucose (Kashiwagi, Asahina et al. 1996). Elevated glucose also increases the generation of superoxide anions known to react with NO to form peroxynitrite, which upon decomposition generates a strong oxidant with reactivity similar to hydroxyl radicals (Beckman 1996). Human EC exposed to hyperglycemia in established diabetes mellitus are more sensitive to reactive oxygen species, since intracellular levels of glutathione, vitamin E, superoxide dismutase, catalase, and ascorbic acid are reduced significantly (Droge 2002) (Wohaieb and Godin 1987; Halliwell 1993) .

The retinal microvasculature is composed of EC and pericytes which line the vessel and rest on a single basement membrane forming the inner blood retinal barrier. The cellular and biochemical abnormalities which are responsible for the pericyte loss and endothelial dysfunction in DR are still under investigation. Studies emphasize on the role of tight control of glucose in order to reduce the complication of diabetes (Busik, Olson et al. 2002; Artwohl, Brunmair et al. 2007). In the retina, AGEs are known to accumulate during diabetes. Advanced glycation end products a heterogeneous group of compounds and have been implicated in micro vascular complications of DR by promoting endothelial cell barrier permeability, proliferation, migration and elaboration of growth factors (Mandarino, Finlayson et al. 1994) (Goh and Cooper 2008). In addition, AGE also promotes apoptotic signaling in pericytes, the loss of

which is associated with DR. (Chen, Jiang et al. 2007) (Lecomte, Denis et al. 2004; Chen, Jiang et al. 2007; Goh and Cooper 2008). However, the effect of this AGE on the biochemical process of glucose uptake and the GLUT-1 translocation in these cells has not been clearly looked into. Therefore, the objective of this study is to see the response of the bovine retinal EC (BREC) and bovine retinal pericytes (BRP) to AGE treatment at the level of labeled glucose uptake and GLUT – 1 expression.

5.2 : MATERIALS AND METHODS

Fetal bovine serum (Gibco), Factor VIII antibody (Dako), Actin antibody (santa cruz), NG2, VE–cadherin and carboxyl methyl lysine antibody (chemicon), GLUT-1 primary antibody raised in goat, FITC conjugated anti goat secondary antibody from Santa cruz, ^{14}C- labeled glucose (BARC, India), Primers for GAPDH and GLUT-1 were designed using primer 3 software and synthesized from Bangalore Genei, India and Endopan media for culturing retinal EC was from Genex. DMEM/F12 media was obtained from GIBCO.

5.2.1 : Cell culture

Primary cultures of BREC and BRP were isolated from the bovine retina by homogenization of the bovine retinal capillaries under sterile conditions followed by filtration through a 41 μ nylon filter for BREC cells, and through a 60 μ filter for BRP. To further purify the isolated BREC cells, CD31 antibody coated Dynabeads (Invitrogen) were used. Cells were cultured in 5 % CO_2 at 37°C, and the media was changed every 3 days. Primary culture of BREC was grown on 0.1 % gelatin coated petridish using endopan media (commercial media for growing EC) containing 20 % FBS. The BRP cells were grown in DMEM/F12 (containing 17.5 mM glucose) + 10 % FBS. BREC and BRP cell population was confirmed by monitoring expression of cell surface markers specific to each cell type. Thus, purity of BREC cells was confirmed by immunoreactivity with factor VIII and VE-cadherin, while the purity of BRP cells was confirmed by immunoreactivity with NG2 and Actin. The cells from passage 2–5 were used for the experiments.

5.2.2 : Preparation of advanced glycation end products

AGE was prepared by slightly modifying the method of Yamagishi et al (Yamagishi, Yonekura et al. 1997), by incubating 0.5 M glucose with 50 mg/ml bovine serum albumin (BSA) in phosphate buffered saline for 6 weeks at 37° C. For the control, BSA without glucose was incubated under similar conditions. The preparation was eluted through a PD-10 column (Amersham, Buckinghamshire,UK) with PBS to remove the excess salts and unreacted glucose. The glycated adduct formed was then run in 7.5 % SDS PAGE to compare the BSA alone (control) and glucose treated BSA for AGE formation and confirmed by Western Blot for Carboxy Methyl Lysine (CML) .

5.2.3 : MTT assay

To test for cytotoxic effect of AGE on BREC and BRP cells. The BREC and BRP cells were grown independently in 96 well plate (1000 cells/well) and exposed to various concentrations of AGE (50,100 and 200 μg/ml) for 6 days in DMEM / F12 + 1 % FBS with change of media every 48 hours. The formazan, formed after treatment with MTT, were dissolved in dimethyl sulphoxide (DMSO) and read at 570 nm to assess the cell viability.

5.2.4 : Immufluorescence staining of GLUT-1 in BREC and BRP cells:

The BREC cells and the BRP cells were grown in cover slips in 24 well plate and exposed to AGE (100 μg/ml) for 6 days with change of media every 48 hours. The BSA treated and unexposed cells to AGE were used as controls. After 6 days the cells were serum starved and glucose starved as mentioned in the uptake experiment and exposed to 5 mM glucose for 5 seconds and 10 seconds for BREC and BRP respectively. The cells were fixed in 100 % methanol and stained for GLUT-1 protein (Santa Cruz). The flourescent cells were observed under the Carl Zeiss Axiovision fluorescent microscope.

5.2.5 : U^{14}C-labelled Glucose uptake in BREC and BRP

BREC and BRP were plated in 6 well plates (15,000 cells/ well) and allowed to grow till 50 % confluence in DMEM-F12 with 10 % FBS. The cells were then exposed to 100 μg/ml AGE-BSA for 6 days with an addition of fresh media every 48 hrs. Cells with BSA and without any treatment were used as controls. 0.25 μCi of ^{14}C glucose was added and incubated for 5 seconds for BREC and 10 seconds for BRP. The uptake was done as described in materials and methods.

5.2.6 : Fluorescence-activated cell sorting (FACS) for GLUT-1 in BREC and BRP

For each experiment 30,000 cells were plated in 25cm^2 flask and allowed to reach 50 % confluence. The cells were then exposed to 100 μg/ml AGE for 6 days with change of media every 48 hrs. The controls were the untreated cells unexposed to AGE and cells treated with 100 μg/ml BSA alone. At the end of the 6th day the same procedure of cell treatment was followed and 5mM glucose was added and incubated for 5seconds for BREC and 10seconds for BRP cells respectively. The uptake was stopped by adding ice cold PBS followed by profuse washing (3times) of cells in ice cold PBS. Then the cells were detached using 0.1 % trypsin EDTA and were centrifuged. 1 % BSA and 1 % sodium azide was added to the pellet and incubated for half an hour, after which 100 μl of 1: 50 diluted polyclonal GLUT-1 raised in goat, (Santa cruz) was added. The cells were again washed in PBS and incubated with 1: 100 dilution of anti-goat tagged with FITC (Santa cruz) in 3 % BSA + 1 % sodium azide. The cells were again washed in PBS, three times and fixed in 0.1 % paraformaldehyde for flow cytometric analysis. Control cells which were not treated with primary antibody were used to set the voltage and those cells which were treated with secondary antibody alone were used to set the background. The flow cytometric analysis was done in BD FACS caliber four-color flow cytometer (BD Biosciences Model no: E 97600177). Data acquisition and analysis were performed using the BD CELL Quest Pro software. The data are expressed as % FITC positive cells of the total 10,000 cells which is over and above the fluorescence seen in the control cells.

5.2.7 : RT-PCR for GLUT-1 in BREC and BRP

Total RNA was extracted (Sigma Genelute mammalian total RNA mini prep kit) according to the manufacturer's instructions. For RT-PCR (reverse transcription polymerase chain reaction), 1μg of total RNA was treated with DNAse I (invitrogen), reverse transcription was carried out using random hexamer (Thermoscript, Invitrogen) using the manufacturers protocol. The PCR was carried out using the following primers for bovine glyceraldehyde 3 phosphate dehydrogenase GAPDH Forward primer 5-TGTTCCAGTATGATTCCACCC-3 and Reverse primer 5-GTCTTCTGGGTGGCAGTGAT-3 corresponding to 424 bp and for GLUT-1 Forward primer: 5'-TCCTGCTGC CCTTCTGCCCC -3' and Reverse primer: 5'-AGGATGGGCTGGCGGTAGGC -3'. Corresponding to 174 bp size, the bands obtained were quantified using NIH image J software after normalization to GAPDH.

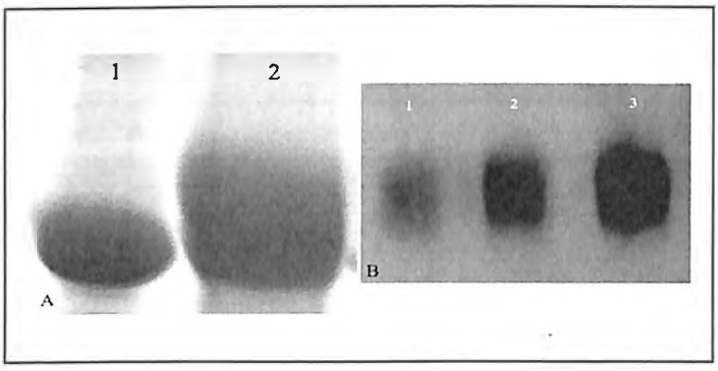
5.2.8: Statistical analysis

Determinations were performed in triplicate, and experiments were repeated at least three times. Results were expressed as means ± SD unless otherwise indicated. Statistical analysis was done using wilcoxins test.

5.3: RESULTS

BREC and BRP cultures were established and cells from passages 2 to 5 were used in all experiments. The formation of glycated BSA was seen by increased molecular weight of the band as seen in 7.5 % SDS PAGE and confirmed by western blot for the glycation moiety for CML with specific antibody (Figure 5.1)

Figure 5.1: The glycated adduct and the confirmation of AGE- BSA formation by western blot.



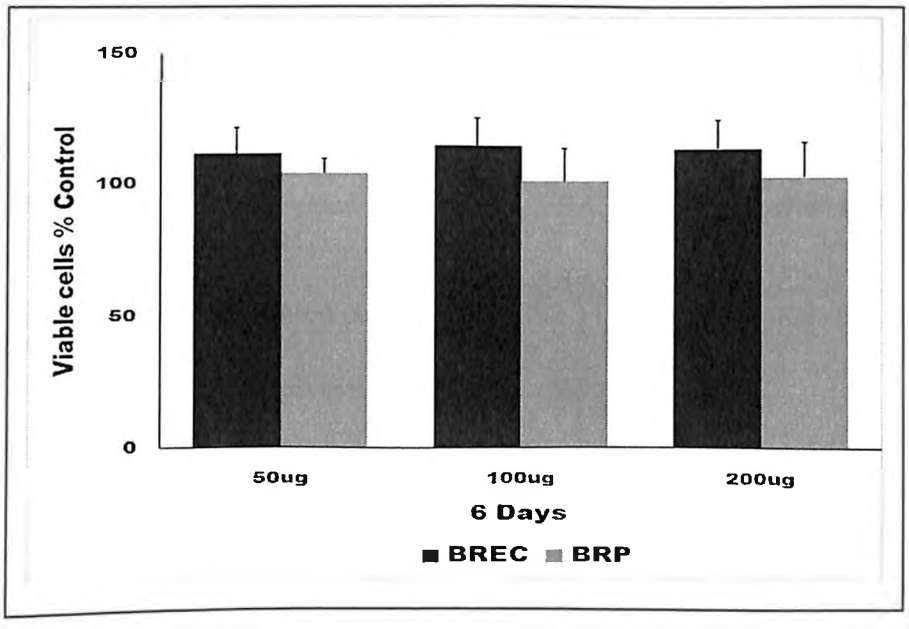
A: Lane 1: BSA alone, Lane 2: Glycated BSA

B: Lane 1: Varying concentration of AGE- BSA (25 , 50 and 100 μg blotted for CML)

5.3.1 : MTT assay

The effect of AGE, in terms of varying concentration (50,100 and 200 µg/ml) on the 6th day of exposure to BREC and BRP cells, showed that both the cells were viable till 6 days of AGE exposure, 100 µg of AGE was then used for the rest of the experiments (Figure 5.2).

Figure 5.2: MTT of BREC and BRP cells with varying concentration of AGE

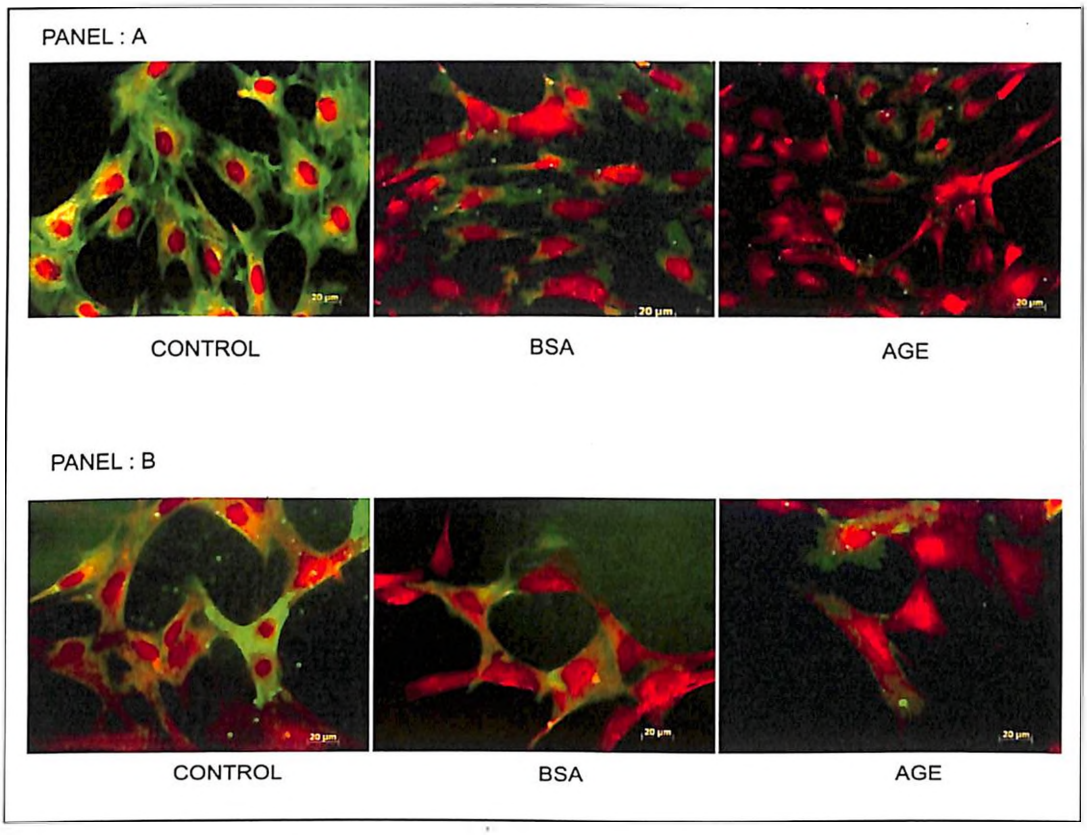


There is no change in the viability of the cells after treatment with AGE (50, 100 and 200 µg) on 6th day.

5.3.2 GLUT-1 protein expression by immunofluorescence

There was a decrease in the expression of GLUT-1 by immunofluorescence in the AGE-BSA treated cells when compared to BSA treated and the untreated controls in both the BREC and BRP cells on the 6th day of AGE-BSA exposure (Figure 5.3).

Figure 5.3: GLUT-1 expression in BREC cells and BRP by Immunoflourescence



Panel A: BREC cells – Untreated control cells, BSA(100µg/ml) treated cells, AGE - BSA (100µg/ml) treated cells,

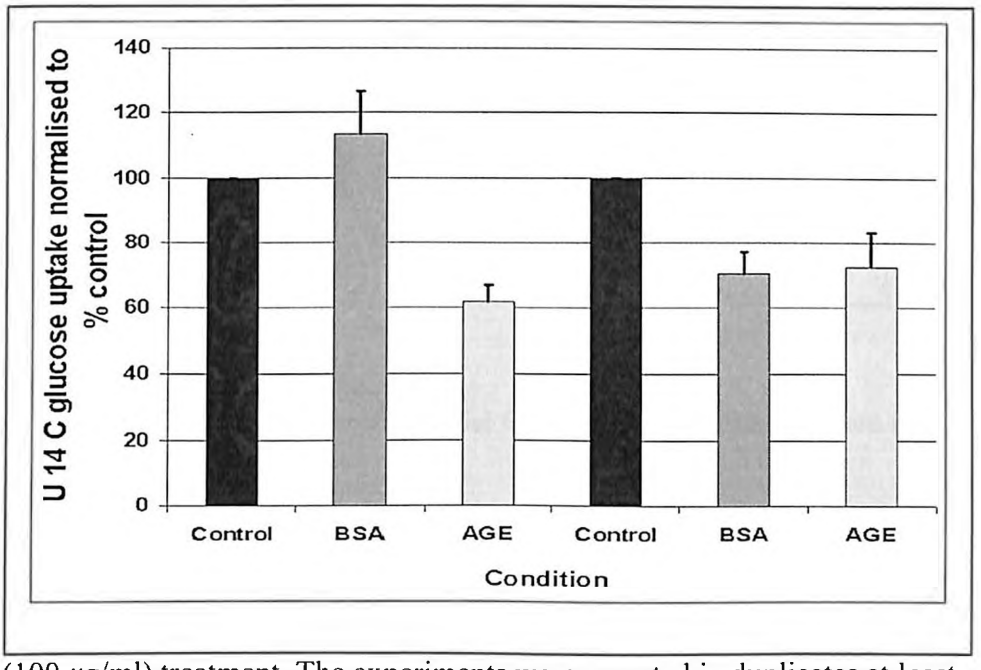
Panel B: BRP cells – Untreated control cells, BSA (100µg/ml) treated cells, AGE – BSA (100µg/ml) treated cells
Green immunoflourescence indicates GLUT-1 expression

5.3.3 : $U^{14}C$ glucose uptake in BREC and BRP

There was a decrease in the glucose uptake in the BREC cells when compared to control and BSA treated but it was not statisticaly significant. There was no change in the glucose uptake in case of pericytes. Figure 5.4

Figure 5.4: $U^{14}C$- labeled glucose uptake in BREC and BRP cells.

Figure 5.4: ^{14}C-labeled glucose uptake in BREC and BRP cells after 6 days of AGE -



BSA (100 μg/ml) treatment. The experiments were repeated in duplicates at least thrice. The result are expressed normalizing to % control

5.3.4: FACS assay of GLUT- 1 protein in BREC and BRP

The GLUT-1 protein expression in these cells was analyzed by FACS .There was a drop in the GLUT-1 expression in the AGE treated BREC cells when compared to control and BSA treated. But this trend was not observed in pericytes. **(Figure 5.5, 5.6)**

Figure 5.5: The GLUT- 1 protein expression by FACS in BREC cells

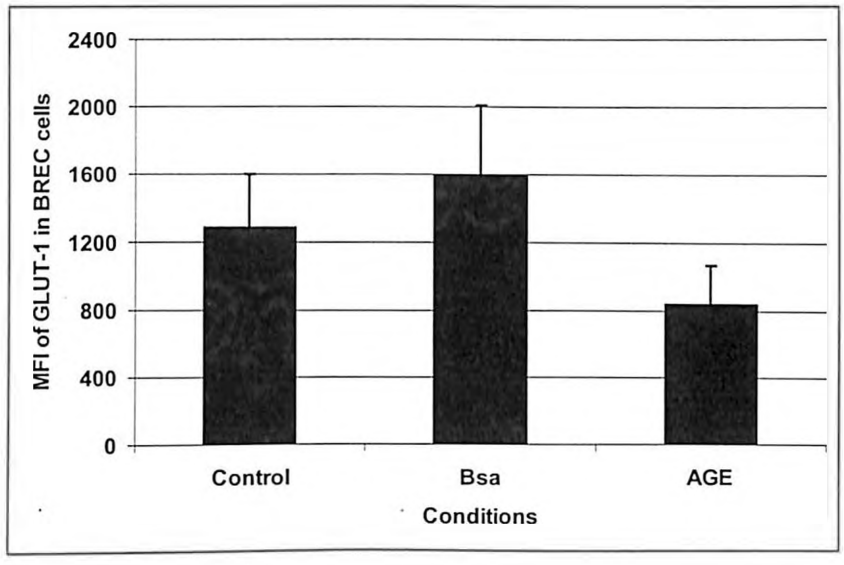


Figure 5.5: There is a slight decrease in the GLLUT-1 mean fluorescence intensity in the AGE treated BREC cells.

Figure 5.6: The GLUT- 1 protein expression by FACS in BRP cells

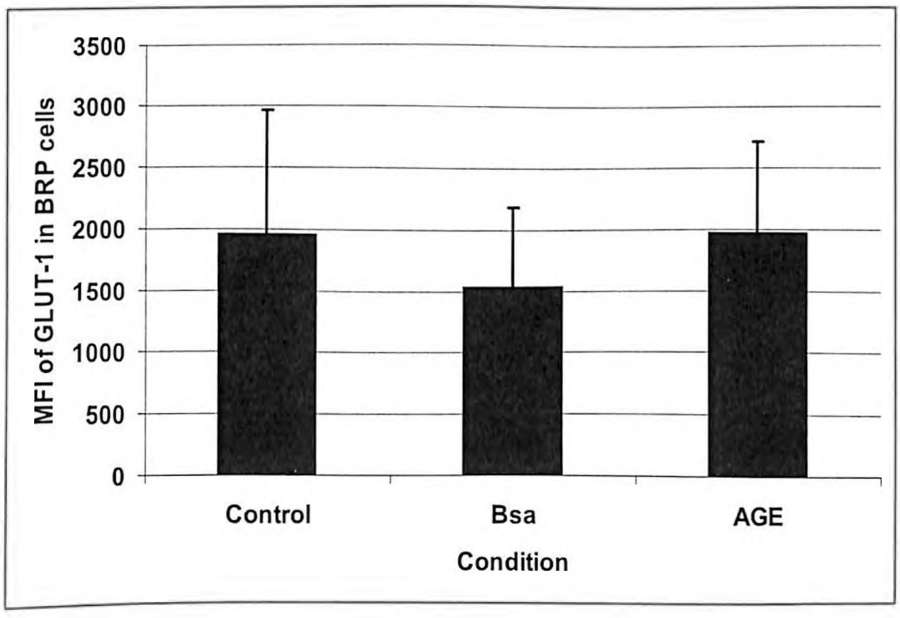
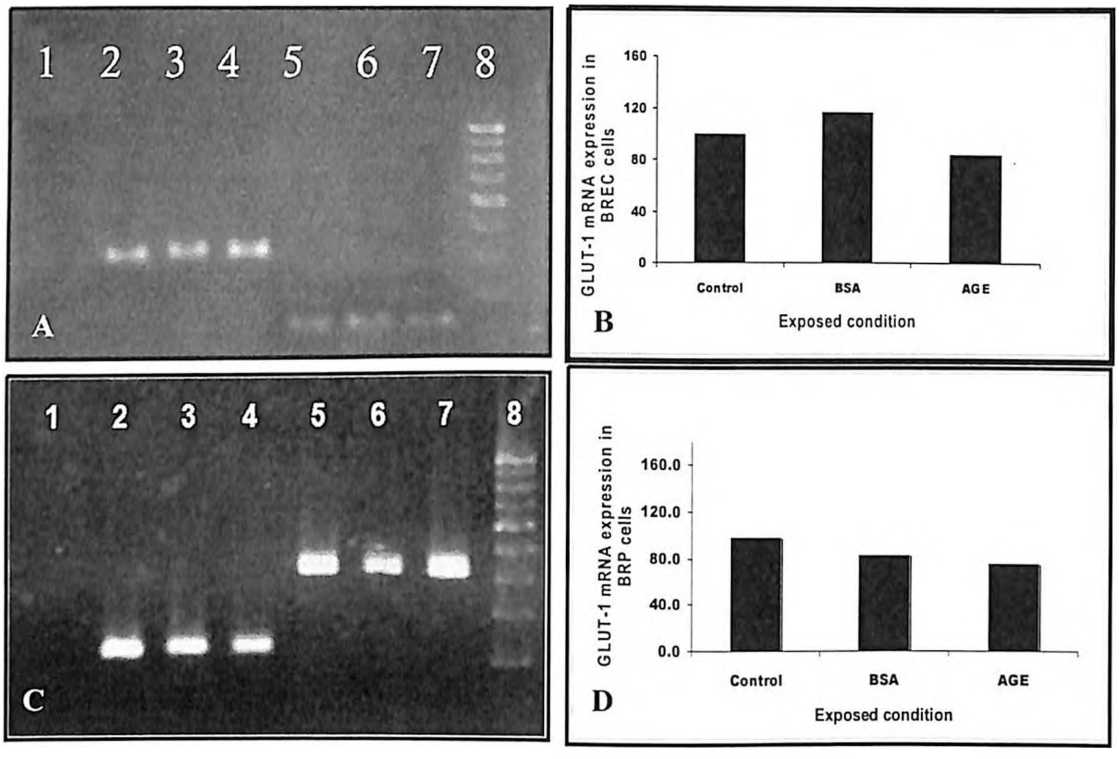


Figure 5.6: There is no change in the GLUT-1 mean fluorescence intensity in the AGE treated cells.

5.3.5: mRNA expression of GLUT-1 in BREC and BRP

The mRNA expression of GLUT-1 by RT-PCR showed a 15% drop in the GLUT-1 expression in AGE treated BREC cells when compared to control and a 23% drop in BRP cells treated similarly when compared to controls after normalizing to GAPDH **(Figure 5.7).**

Figure 5.7: mRNA expression of GLUT-1 by RT-PCR



Panel A: GLUT-1 mRNA expression in BREC cells
A: Lane 1: Negative control, Lane 2: Control GAPDH, Lane 3: BSA GAPDH, Lane 4: AGE GAPDH, Lane 5: Control GLUT-1, Lane 6: BSA GLUT-1, Lane 7: AGE GLUT-1, Lane 8: Mol.wt. GLUT-1 174 bp and GAPDH- 424 bp
B: Histogram based on the band intensity, showing the expression of GLUT-1 in BREC cells normalized to GAPDH
Panel B: GLUT-1 mRNA expression in BRP cells.
C: Lane 1: Negative control, Lane 2: Control GLUT-1, Lane 3: BSA GLUT-1, Lane 4: AGE GLUT-1, Lane 5: Control GAPDH, Lane 6: BSA GAPDH, Lane 7: AGE GAPDH, Lane 8: Mol.wt. GLUT-1 174 bp and GAPDH- 424 bp
D: Histogram based on the band intensity, showing the expression of GLUT-1 in BRP cells normalized to GAPDH

The lacunae in the AGE mediated oxidative stress, apoptosis and angiogenic Signaling in the retinal capillary cells have to be explored to further understand the mechanisms involved.

CHAPTER 6: EFFECT OF ADVANCED GLYCATION END PRODUCTS AND HOMOCYSTEINE ON THE ENZYME PARAOXONASE *IN VITRO* AND THE ROLE OF PARAOXONASE IN DIABETIC RETINOPATHY *IN VIVO*

6.1 : INTRODUCTION

Several studies have observed a relationship between homocysteine levels and chronic complications of diabetes, and it has been reported that hyperhomocysteinaemia is associated with coronary heart disease in diabetes (Becker, Kostense et al. 2003). Recent reports are indicative of increased Homocysteine thiolactone levels associated with the development and progression of diabetic macrovasculopathy (Gu, Lu et al. 2008). Serum PON1 is low in subjects with Type 1 or Type 2 diabetes (Mackness, Durrington et al. 2000; Kordonouri, James et al. 2001; Kao, Donaghue et al. 2002; Ferretti, Bacchetti et al. 2004), leading to dysfunctional HDL with impaired antioxidant capacity. In Type 2 diabetes there is an inverse relationship between PON1activity and circulating oxidized LDL levels (Sharma, Singh et al. 2007), indicative of the major role of PON1 in retarding LDL oxidation. It has previously been reported that *in vitro* glycation inhibits PON1 activity towards paraoxon. In the recent study they found that glycation of PON1 inhibited both paraoxon hydrolysis and membrane lipid hydroperoxide metabolism by approximately 50 %; however, glyoxidation caused 80 % inhibition of these two PON1 activities. It is therefore possible that the increased *in vivo* glycation of PON1 leads to its glyoxidation and is responsible for the derangement of membrane hydroperoxide metabolism found in HDL from people with Type 2 diabetes and those with Coronary heart disease (Mastorikou, Mackness et al. 2008).

6.1.1 : History of paraoxonase

Paraoxonase-1 (PON1) is a calcium dependent esterase that is synthesized in the liver microsomes associated with vesicles derived from endoplasmic reticulum it is exclusively located in HDL. It is highly conserved in mammals but is absent in fish, birds, and invertebrates such as arthropods. PON 1 can bind reversibly to

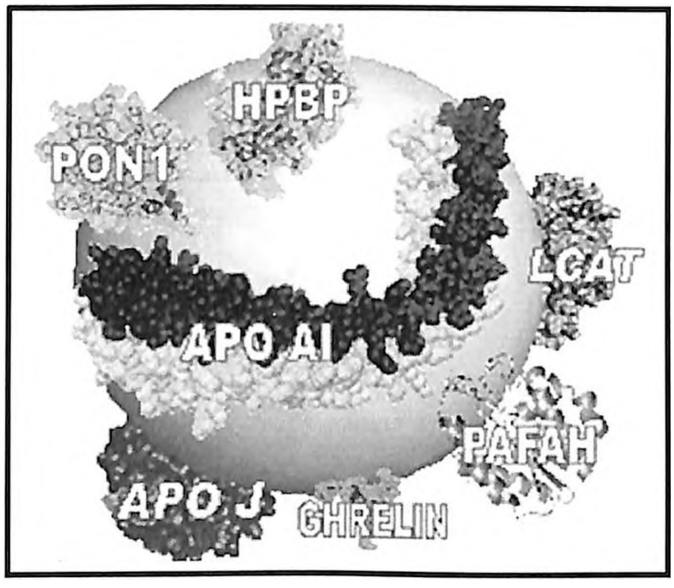
organophosphate substrates, which it hydrolyzes. PON1 is thus the main means of protection of the nervous system against the neurotoxicity of organophosphates entering the circulation. There is wide inter individual variation in the capacity of PON1 to hydrolyze organophosphates and other organic esters(Durrington, Mackness et al. 2001). In contrast, organophosphates are detrimental for other serum organic esterases, such as pseudo cholinesterase, and for the acetyl cholinesterase at synapses and the neuromuscular junctions because they bind irreversibly to them (Georges and Jason 2004). It was in this context that it was first discovered, and its name reflects its ability to hydrolyze paraoxon, a metabolite of the insecticide parathion and named paraoxonase. Mazur is credited as being the first investigator to report that Organophosphorous compounds could be hydrolyzed enzymatically by several animal tissues (Mazur 1946). This finding led to the series of definitive studies in the early 1950's by Aldridge on the hydrolysis of paraoxon (diethyl p-nitrophenyl phosphate) and arylesters such as phenylacetate and p-nitrophenyacetate). He proposed the definition of A-esterases for the enzyme hydrolyzing organophosphate (Aldridge and Davison 1953). For many years the Nomenclature Committee of the International Union of Biochemistry classified both paraoxonase and arylesterase under EC 3.1.1.2. Recently they have found that the enzyme is more a lactonase than an arylesterase or organophosphatase as it was found to cleave thiolactones (Khersonsky and Tawfik 2005).

6.1.2 : Synthesis of paraoxonase

PON1 is synthesized in the liver and secreted into serum. The mechanism of PON1 secretion is important, because factors that modulate its release from the cell will, in turn, affect serum levels of the enzyme. In the absence of lipoproteins, little PON1 was secreted. Addition of phospholipids micelles or HDL stimulated secretion, whereas LDL and lipid free ApoA1 had no effect. The secreted protein retains its hydrophobic leader sequence, which is a structural requirement for PON1 association with HDL. The potential interface with HDL has an aromatic belt rich in tryptophan and tyrosine residues, which has been described in a number of membrane-binding proteins (Figure 6.1). This suggests that PON1 requires an appropriate acceptor for

release into serum. HDL appears to be the predominant physiological acceptor, but the presence of apoA1/apoA2 is not essential. There are three isoforms of PON. PON 1, 2 and 3. Similar to PON 1, PON 3 is widely distributed in liver and also in kidney, intestine and testis. PON2 is found to be ubiquitously present in all tissues.

Figure 6.1: LOCATION OF PON IN HDL



Adapted from: www.biochemsoctrans.org

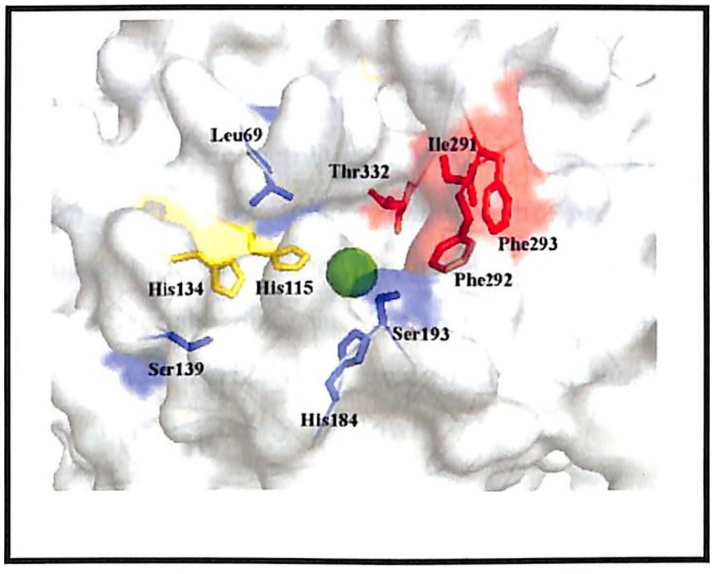
6.1.3 : Isoenzymes of paraoxonase

There are 3 paraoxonase genes (PON1, PON2 and PON3) all of which are clustered in the same region on chromosome 7q21.3-22.1. All the three share 60% sequence homology. All the three isoforms have a MW of 43-45kDa with 355 amino acids for PON1, 354 amino acids for PON2 and PON3. The identity between PON1 and 2 is 64 % and between PON1 and 3 is 60 %. The similarity between PON2 and 3 was found to be 63 %.

6.1.4 : Active site in Paraoxonase 1

The enzyme has two calcium binding sites: the higher affinity calcium is required for the structural integrity, whereas the lower affinity calcium is involved in catalysis Asp269 and Glu53 participate in the ligation of catalytic Calcium. The active site of PON1 (Fig.6.2) contains four histidines (His115, His134, His285, and His184), two aspartates (Asp183and Asp269), and one glutamic acid (Glu53). All these residues are conserved in all mammalian PONs. A histidine dyad composed of His115 and His134 was suggested to be directly involved in the catalytic mechanism of PON1 for both ester and phosphotriester hydrolysis.

Figure 6.2: The active site of PON 1.



Adapted from: Olga Khersonsky et al JBC 2006.

Figure 6.2: Surface view of PON1 active site. Catalytic His115-His134dyad (yellow), catalytic calcium(green)and the residues that govern the substrate selectivity of the Lactonase/arylesterase activities(red) and the phosphodiesterase activity(blue).His115, being only 4 Å from the catalytic calcium, is the most essential amino acid for the general base role. It can form a His-His dyad with His134, in which His115 is activated by His134 via a proton shuttle mechanism.

Table 6.1: Human PON1: structure activity relationships

Contents	AREase/Organphos-phatase	Lactonase	Antioxidative
Calcium binding sites	Essential	Essential	Not essential
N-terminus	Important	Important	Not essential
Essential amino acids			
a. His	115,134,155,243,285	-	-
b. Asp	56,169,183,269,279	-	-
c. Glu	53,195	-	-
d. Trp	281	Essential	Essential
e. Free cys 284	Not essential	-	-
f. Gln/Arg 192 polymorphism	Essential	-	-

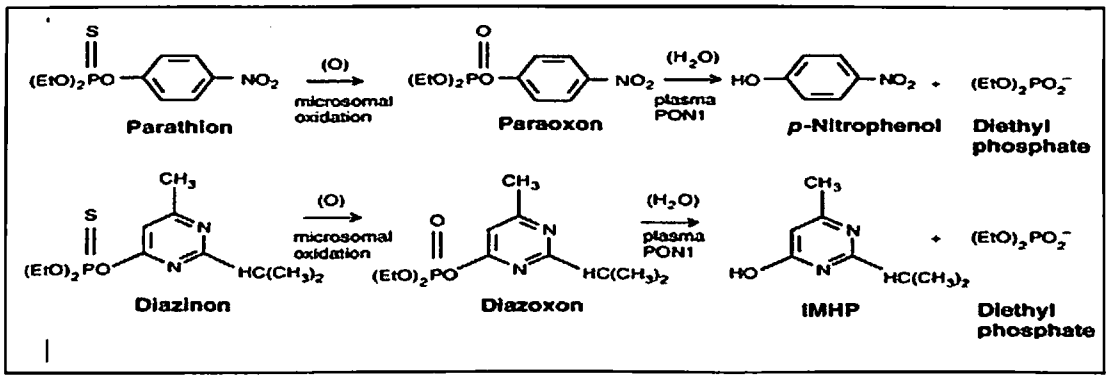
It was found that all hydrolytic activities of PON1 (lactonase, esterase, and phosphotriesterase) are mediated by amino residues with a pKa of 6.3–7.4 that is active in the deprotonated form. Site directed mutagenesis studies have shown that, although all these activities take place in the same active site, the residues that mediate the lactonase and esterase activities are different from those involved in the phosphotriesterase activity (Khersonsky and Tawfik 2006).

6.1.5 : Detoxification of organophosphates

Organophosphates are a group of synthetic chemical compounds, composed of variable mixtures of phosphorous, carbon and hydrogen. The chemicals in this class kill insects by disrupting their brains and nervous systems. Unfortunately, these chemicals also can harm the brains and nervous systems of animals and humans. The toxicity induced by organophosphates results from inhibiting the enzymes acetyl cholinesterases (ChE), in the nervous system of the exposed organisms. These enzymes remove acetyl choline (ACh) which carries electrical signals across the synapse. Diazinon prevents the ChE from removing the ACh and thus synapses get jammed with ACh, this results in rapid twitching of voluntary muscles and finally paralysis. Three compounds, diazinon, chlorpyrifos and paraoxon, chosen to represent the class of organophosphates compound.Sarin and soman are the nerve agents which are hydrolysed by paraoxonase. These agents also inhibit tissue

cholinesterases at synaptic site, and cause the accumulation of excess aetylcholine at nicotinic and muramic receptors. Pesticides are widely used in agriculture as relatively nontoxic sulphur derivative; cytochrome P450 dependent microsomal monoxygenases activate them in vivo to their toxic oxygen analogues. Hydrolysis of the organophosphates by serum and hepatic A-esterses to less toxic metabolites is the most important route for their detoxification. **Figure 6.3** shows how paraoxonase hydrolyse various organophosphate like parathion, chlorpyrifos and diazinon (Navab, Berliner et al. 1996).

Figure 6.3: Proposed paraoxonase mechanism of PON



Adapted from: Bharti Mackness et al

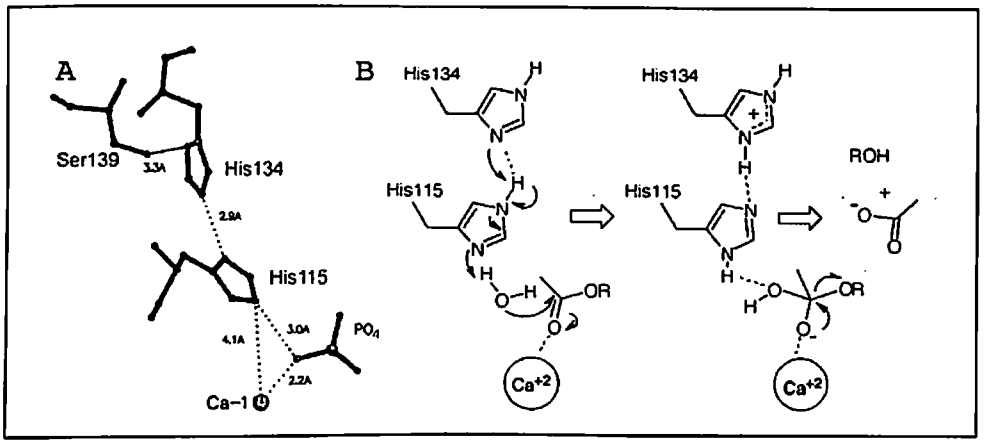
Figure 6.3: the hydrolysis of paraxon and diazoxon the organophosphate compounds by PON is given.

6.1.6 Arylesterase activity of PON:

Oxidation of phospholipids in plasma lipoproteins produces a spectrum of phosphatidylcholine derivatives including hydroperoxides, isoprostanes and core aldehydes which are inactivated through enzymatic hydrolysis. PON1 metabolizes oxidized arachidonic acid derivatives. Serum PON1 plays a role in the protection against oxidative stress, macrophage foam cell formation, and the development of atherosclerosis. Purified PON1 inhibits LDL oxidation in vitro and treatment of mildly oxidized LDL with purified PON1 significantly reduces the ability of this modified LDL to induce monocyte-endothelial interactions. PON1 was found to be an independent risk factor for coronary artery disease(Harel, Aharoni et al. 2004). Serum level of arylesterase activity increases gradually after birth, particularly during the

first six months, and reaches adult level by the age of two years. There is a slight decrease in the serum PON level in the people over 50 yrs old, and there are no significance in adult males and females (Jakubowski 1999). Figure 6.4

Figure 6.4: proposed arylesterase mechanism of PON



Adopted from: Harel et al *Nature Structural & Molecular Biology* (2004)

Figure 6.4 : **A)** The catalytic site: the upper calcium atom (Ca-1), the phosphate ion at the bottom of the active site, and the postulated His-dyad; **B)** Schematic representation of the proposed mechanism of action of PON1 on ester substrates such as phenyl and 2-naphthylacetate. The first step involves deprotonation of a water molecule by the His dyad to generate a hydroxide anion which attacks the ester carbonyl, producing an oxyanionic tetrahedral intermediate. This intermediate breaks down (second step) to an acetate ion and either phenol or 2-naphthol (Harel, Aharoni et al. 2004).

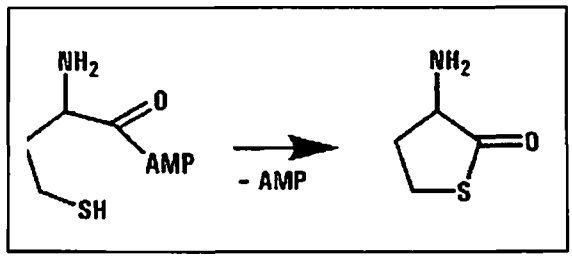
6.1.7 : Homocysteine thiolactonase activity of Paraoxonase

Homocysteine thiolactone (HCTL) is a biologically active thiolactone synthesized by several aminoacyl-tRNA synthetases in an error editing reaction that prevents transitional incorporation of homocysteine into proteins.

1. Carboxyl group of Hcy is activated by ATP, forming a MetRS-bound homocysteinyl adenylate

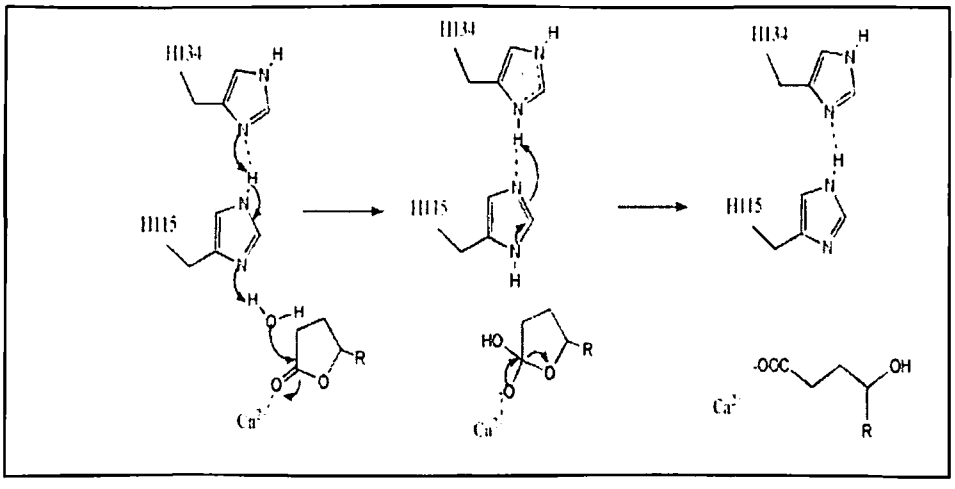
$$\mathbf{MetRS + Hcy + ATP \rightleftharpoons MetRS \bullet Hcy \sim AMP + PPi}$$

2. The side chain thiolate of Hcy displaces the AMP group from the activated carboxyl group of Hcy, forming Hcy thiolactone as a product. The energy of the anhydride bond of Hcy;AMP is conserved in the intermolecular thioester bond of Hcy thiolactone.



HCTL damages protein by modifying their lysine residues (Jakubowski 1999; Jakubowski 2000). HCTL is found to inhibit the insulin signaling through an oxidative stress mediated mechanism (Najib and Sanchez-Margalet 2001; Najib and Sanchez-Margalet 2005). HCTLase, activity of paraoxonase, hydrolyses homocysteine thiolactone back to homocysteine (Jakubowski 2000).

Figure 6.5: Proposed lactonase mechanism of PON



Adapted from: Olga Khersonsky et al JBC 2006.

Figure 6.5: The mechanism in which His115 acts as a general base and deprotonates a water molecule, and generates a hydroxide ion that attacks the carbonyl of the lactone/ester substrate. His134 activates His115 by serving as a proton shuttle. Catalytic Ca^{2+} serves as an oxyanion hole and stabilizes the negative intermediate produced by the attacking hydroxide ion.

Mutation studies have shown that site directed mutation in His-His dyad region causes several fold decrease in the activity of the enzyme proving that this is the active site responsible for its reaction (Khersonsky and Tawfik 2006). PON1 exhibits all three activities with weak paraoxonase activity. PON2 and 3 has higher lactonase activity and exhibits no paraoxonase activity (Draganov and La Du 2004).

6.1.8 : Polymorphisms of Paraoxonase

PON 1 polymorphism reported Paraoxonase exhibits two sequence polymorphisms- Arg(192)→Gln and Met(55)→Leu, the former being responsible for distinct catalytic activity of the two corresponding alloenzymes.. Amino acids (Q or R) occupying position 192 in human PON1 would exert large influence on the catalytic properties of the enzyme. The Paraoxon is hydrolysed six times faster by PON1192R than by PON1192Q, whereas the Q form is more active towards sarin, soman and diazoxon (Mohamed Ali and Chia 2008) The Q192R polymorphism also alters the enzyme's ability to protect LDL from oxidation *in vitro* with the Q form being the most protective (Aviram, Billecke et al. 1998). The next common polymorphism site at position 55 has very little effect on the catalytic properties of the enzyme, but is associated with lower serum activity and concentration of the enzyme (Leviev and James 2000; Draganov and La Du 2004). Leviev *et al.* (Leviev, Deakin et al. 2001) found lower PON1 mRNA levels in individuals carrying the M allele. One of the recent study reports show that low Homocysteine thiolactonase activity is associated with M55 and Q192 alleles associated with acute coronary syndrome (Koubaa, Nakbi et al. 2009).

PON 2 polymorphism reported

The PON2/C311S (SS) genotype was found to be lower in coronary artery disease patients than that in controls The T allele of PON1/T(-107)C (promoter polymorphism) and S alleles of PON2/C311S polymorphism were associated with lower plasma homocysteine and homocysteine thiolactone complex. The genotype PON2 - allele C were higher in caronary artery disease patients with type 2 diabetes than that in CAD patients without type 2 diabetes and controls ($P < 0.005$). The elevation of serum Hcy and the complex of HTL were associated with increased risk

of coronary heart disease. The allele PON1/(-107)T and PON2/311S might be protective for the development of atherosclerosis (Qin, Li et al. 2006).

To consolidate, two particular polymorphisms, namely PON1-192 and PON2-311, in the genes encoding the antioxidant enzymes paraoxonase-1 (PON1) and paraoxonase-2 (PON2) have been associated with an increased risk of acute myocardial infarction (AMI) (Guxens, Tomas et al. 2008).

PON 3 polymorphism The PON 3 polymorphism at A10340C,and A2115T along with PON1/L55M and PON1/Q192R showed a 2%, 1%, 8% and 19% variation in PON1 activity. Reports say that PON3 gene influences serum PON1 activity independently of the effect of PON1 genetic variation (Sanghera, Manzi et al. 2008).

6.1.9 : Paraoxonase in Pathology other than diabetes

Uremic predialysis and hemodialysis: In uremic patients, there is a reduction in HDL cholesterol whereas triglycerides were significantly increased. Reduced PON activity could be related to reduced HDL cholesterol and apoA1 (Dirican, Akca et al. 2004).

Liver cirrhosis: Pioneer studies in the 1970's observed for the first time a significant decrease in serum PON1 activity in small groups of patients with liver cirrhosis (Burlina and Galzigna 1974; Burlina, Zaninotto et al. 1977). This results were confirmed by Ferre et al (Ferre, Marsillach et al. 2005), in a wider series of patients with various degrees of chronic liver damage. Changes in serum PON1 activity has also been studied in relation to outcomes of liver transplantation in patients with severe liver disease (Xu, Lv et al. 2005). Ferre et al proposed the addition of serum PON1 paraoxonase activity measurement as a biomarker of liver impairment.(Ferre, Camps et al. 2002) and (Camps, Marsillach et al. 2009).

Bechet's disease: A chronic relapsing inflammatory process and its predominant histopathology is vasculitis with vessel walls and perivascular tissue infiltration by lymphocytes, monocytes, plasma cells and neutrophils. Neutrophil derived O_2 intermediates may contribute to tissue damage, especially in the endothelial tissue. Oxidative stress is increased in this disease due to over production of ROS and

decreased efficiency of antioxidant defenses was reported along with decreased PON activity (Karakucuk, Baskol et al. 2004).

Chronic obstructive pulmonary disease (COPD): It is characterized by progressive, irreversible airflow limitation associated with airway inflammation. Oxidative stress has an important role to play in this disease where oxidants cause damage to extracellular matrix, to biological membrane of cell and ciliary function. Free radicals cause imbalance between oxidants and antioxidants . PON is localized in clara cell, EC and typel cells of the alveolar epithelium. In lungs it protects airway from oxidative stress .In COPD due to damage in airspace epithelium, PON level is decreased (Isuk B, Isuk SR et al. 2005).

Atherosclerosis and Coronary artery disease: Gln^{192}-Arg polymorphism of the paraoxonase gene (B allele) is associated with coronary heart disease in Japanese patients with NIDDM. The precise mechanism by which the Gln^{192}-Arg polymorphism influences susceptibility to CHD remains unknown.(Odawara, Tachi et al. 1997) .There is increasing evidence from both animal and human studies linking low PON1 activity to an increased likelihood of cardiovascular diseases. PON1 is a potentially antiatherogenic HDL-associated enzyme that protects LDL from oxidative modification. Enhancing PON1 activity could be an important target for future pharmacological agents aimed at decreasing cardiovascular risk (Soran, Younis et al. 2009).

PON1 activity in serum is found to be lowered in diabetic cases and recent reports have shown decreased PON activity associated with diabetic retinopathy (Nowak, Wielkoszynski et al. 2009). There are no reports on the PON activity in ocular fluids therefore the objective was to measure the activity of the same in the vitreous of the PDR cases. The PDR is associated with proliferation of EC. The role of the enzyme paraoxonase in the retinal capillary EC has nto been looked into. Since the enzyme activity is reportedly altered in diabetes the effect of AGE as well as homocysteine and homocyteine thiolactone on this enzyme is tested *in vitro* in BREC cells.and *in vivo* in the vitreous of PDR cases.

6.2 : MATERIALS AND METHODS

Homocysteine (Hcys), Homocysteinethiolactone HCl (HCTL)–L Homatropine HBr (Borringer Inglem, Germany), Mercaptoethanol (MSgrade). Acetonitrile (MS grade), Formic acid (MS grade), Ammmonium acetate (GR grade) obtained from Merck, Germany, Water for HPLC was freshly deionized using MilliQ-Gradient (18.2 MΩ) using double distilled water as a feed. Phenyl acetate, γ thiobutyrolactone,5,5'-Dithiobis(2-nitrobenzoic acid),TBA, Iron(Fe), EDTA, Benzoic acid, TCA, Acetic acid and dimethyl sulphoxide (DMSO) were obtained from Sigma USA. DMEM/F12 media (GIBCO), Endopan media (Genex), Fetal bovine serum (GIBCO), Factor VIII antibody (Dako),VE–cadherin (chemicon), Sigma Total RNA was extracted (Sigma Genelute mammalian total RNA mini prep kit), cDNA conversion (Thermoscript, Invitrogen).

All experiments involving human subjects adhered to the tenets of the Declaration of Helsinki. In patients with PDR, the clinical ocular findings were graded at the time of vitrectomy for the presence of hemorrhage, tractional retinal detachment, and presence or absence of new vessels in the retina or optic disc. Active PDR was graded in patients on the basis of visible patent new vessels in the retina or optic disc, and their absence was deemed inactive PDR. All cases of Type -1 or 2 diabetes mellitus with ocular signs namely (NVE) Neovscularisation elsewhere, (NVD) Neovascularization disc, (FVP) Fibrovascular proliferation, vitreous hemorrhage, tractional retinal detachment or combined retinal detachment were included in the study for the vitreous. In patients with MH, all patients with idiopathic full thickness retinal defect of more than 400μm and with posterior vitreous detachment were included in the study for the vitreous specimen as the disease control. Clinical details of the patients with MH and PDR are given in Tables 6.2 and 6.3 given in Appendix Undiluted vitreous samples from 13 patients (mean age, 52 ± 7 Yrs ; 7 :M and 6 :F) with Proliferative Diabetic Retinopthy (PDR) and 8 Macular Hole (mean age 56 ± 10 Yrs ; 5:M, 3:F) were collected during vitreoretinal surgery. The samples were transported on ice and centrifuged at 3000 rpm for 10 minutes at 4 °C. The centrifuged samples were frozen at - 80 °C until they were assayed with correspondingly stored control

specimens. Vitreous HCTL levels, PON-AREase and HCTLase activity, total protein and oxidative stress parameters like Thiobarbituric acid reacting substances (TBARS), Total antioxidant capacity (TAC) and total thiols were measured for all the cases and controls as per the protocols described in materials and method section.

***In vitro* experiments in BREC** : The primary bovine retinal capillary EC (BREC) were cultured from the retina and characterized as EC using Factor VIII and VE–Cadherin. The cells were exposed to varying concentration (25, 50 ,100 ,500 ,1000 μg AGE) for 24 ,48 ,72 hrs and (25, 50, 100 and 200 µM) of Homocysteine (Hcys) and Homocysteine thiolactone (HCTL) at varying time points (3, 6, 12, 24 & 48 h) in DMEM/F12 media independently and the PON-HCTLase and AREase activity were estimated in all the cell lysates.

6.2.1 : MTT assay to test for cytotoxic effect of HCTL and Hcys in BREC

The BREC cells were grown in 96 well plate (1000 cells/well) and exposed to various concentrations of Hcys (25,50,100 and 200 µM) for 3 ,6 ,12 ,24 and 48 h respectively in DMEM / F12 and as well as with AGE (25,50,100,500 and 1000µg) and exposed until 48 hrs. The formazan, formed after treatment with MTT, were dissolved in DMSO and read at 570 nm to assess the cell viability.

6.2.2 : Homocysteine thiolactone was estimated using LC MS/MS

The liquid chromatography separation of the HCTL in the vitreous was done by gradient elution using acetonitrile with 0.1 % formic acid as (A) and water containing 0.1 % formic acid (B) in the ratio of 70:30 pumped at a flow rate of 0.5 ml/min in Thermo Surveyor quaternary HPLC pump (Thermo Electron Corp, Waltham, MA, USA) coupled with Applied Bio Systems 4000 Q Trap (ABS Biosystems, Foster City CA, USA) was used with Positive Electron Spray Ionization mode (ESI). The analytical separation was achieved by using Chromolith, SpeedROD, RP-18e (50 X 4.6 mm) Merck, Darmstadt, Germany) within the run time of 5 minute where homatropine was used as a internal standard. Analyst software version 1.4.2 was used to control all parameters of mass spectrometry. Quantification was performed using multiple reaction monitoring (MRM) mode based on the parent → product ion

transitions for homocysteine thiolactone (118.2→56) and homatropine (276.1→142). Source dependent parameters optimized were gas 1 (40 psi); gas 2 (40 psi); curtain gas (10 psi); ion spray voltage (5500 V); temperature (300 °C). Compound dependent parameters declustering potential, entrance potential, and collision energy and cell exit potential and dwell time were set at 75, 10, 35, 10 and 200 for both analyte and the internal standard respectively. Standard HCTL (Sigma, USA) was accurately weighed and dissolved in MilliQ water and was suitably diluted to reach the concentration varying from 100- 3.125 ng/ml. 20 µl of either standard or sample were mixed with 200 µl of extraction solvent (70:30 ratio of acetonitrile:water with 10% zinc sulphate) containing homatropine at the concentration of 250 ng as an internal standard (Velpandian.T, Angayarkanni.N et al.).

6.2.3 : Determination of Aryl Esterase activity

PON-AREase activity was measured using the method of Cabana et al (Cabana, Reardon et al. 2003). Phenyl acetate was used as the substrate and the rate of hydrolysis was measured spectrophotometrically in kinetic mode by detecting the increase in phenol concentration at 270 nm. Undiluted vitreous was added to the buffer consisting 10 mM Tris and 1mM $CaCl_2$, pH 8.0. Enzyme activity was expressed as μM phenylacetate hydrolysed/ml/min. A blank containing incubation mixture without vitreous was run simultaneously to correct for spontaneous substrate breakdown for activity determination.

6.2.4 : Determination of Homocysteine Thiolactonase activity

Homocysteine Thiolactonase activity assay was standardized in-house using γ-thiobutyrolactone as the substrate and the rate of hydrolysis was measured spectrophotometrically in kinetic mode at 450 nm (main wavelength) and 546 nm (subwavelength) modifying the method followed by Koubaa et al for HCTLase estimation,(Koubaa, Hammami et al. 2008) suitably for assay in 96 well plate. 5 µl of vitreous sample was used for the assay with DTNB as chromogen at pH 7.2 using 100mM phosphate buffer. Enzyme activity was expressed in U/L. A blank containing incubation mixture without vitreous was run simultaneously to correct for spontaneous substrate breakdown for activity determination.

6.2.5 : Expression of Paraoxonase 2 in BREC by RT-PCR

Total RNA was extracted (Sigma Genelute mammalian total RNA mini prep kit) according to the manufacturer's instructions. For RT-PCR (reverse transcription polymerase chain reaction), 1μg of total RNA was treated with DNAse I (invitrogen), reverse transcription was carried out using random hexamer (Thermoscript, Invitrogen) using the manufacturers protocol. The PCR was carried out using the following primers for bovine glyceraldehyde 3 phosphate dehydrogenase GAPDH Forward primer 5-TGTTCCAGTATGATTCCACCC-3 and Reverse primer 5-GTCTTCTGGGTGGCAGTGAT-3 corresponding to 424 bp and for PON2 Forward primer: 5' - CCT TCC TAA TTG CCA CCT GA – 3'and Reverse primer: 5' – TGG AGG CCT GGA CAT TTT AG – 3' corresponding to ~150bp size. The bands obtained were quantified using NIH image J software after normalization to GAPDH.

6.2.6 : Activity stain for paraoxonase using phenyl acetate as substrate

The AREase activity of PON protein in the vitreous was observed by doing an activity stain using phenyl acetate as substrate. The liberated phenol couples with the hexazotized pararosaniline solution to give an insoluble brightly colored azo dye seen as pink bands in the gel. Briefly 50 μg of the vitreous protein from MH and PDR was run on a native page (12 %). The gel was then immersed in a staining solution (Pararosaniline: 0.125 M, Sodium nitrite:4 %, phenylacetate: 1mM in phosphate buffer 6.8-7.2.), for 1 hr at 37° C. Once the bands were visualized the gel was destained with 0.33% sodium meta bisulphate in phosphate buffer (Thiersch, Raffelsberger et al. 2008). The presence of PON2 protein in the vitreous was also confirmed by western blot.

6.2.7 : Determination of Thiobarbituric Reactive Substance

Estimation of vitreous TBARS was done spectrophotometrically based on absorbance of the chromophore at 530nm. The results were expressed as nmol MDA released /ml/mg protein.(Devasagayam and Tarachand 1987).

6.2.8 : Determination of Total Antioxidant Capacity

Estimation of vitreous TAC was done spectrophotometrically by a Fenton type reaction. A standardized solution of Fe-EDTA complex reacts with hydrogen peroxide leading to the formation of hydroxyl radicals (•OH). These reactive oxygen species degrade benzoate, resulting in the release of TBARS. Antioxidants from the added sample of human fluid cause suppression of the production of TBARS. This reaction can be measured spectrophotometrically at 532 nm and the inhibition of color development defined as the TAC. (Koracevic, Koracevic et al. 2001)

6.2.9: Determination of Total Thiols

Estimation of Total Thiols was measured spectrophotometrically using DTNB as chromogen, a disulphide compound that is readily reduced by sulfhydryl compounds like glutathione, homocysteine and cysteine, forming a highly coloured complex. The absorbance is measured at 412 nm and it is expressed in μmol (Coral, Raman et al. 2006).

6.2.10: Statistical analysis

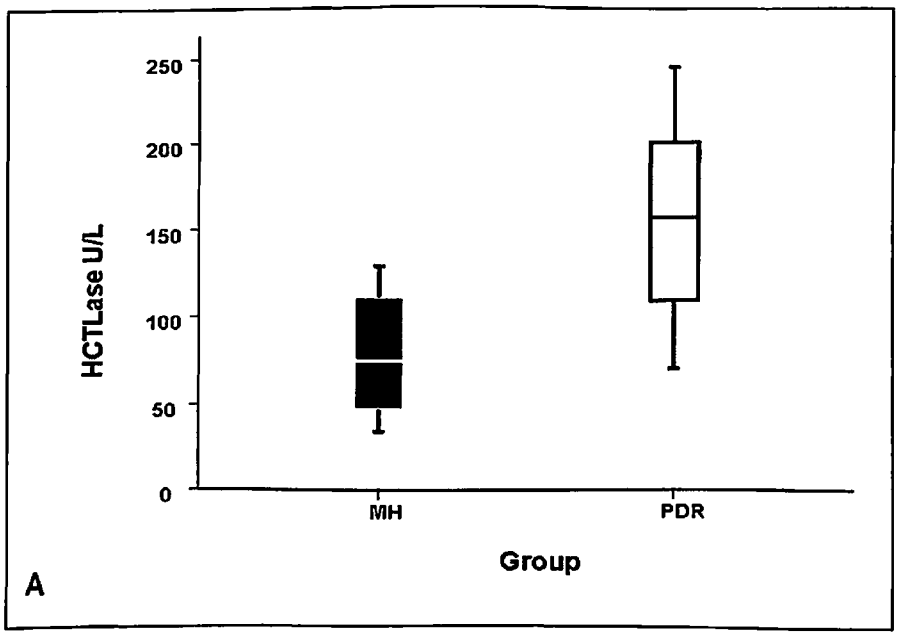
Students *"t"* test was used to compare continuous variables between groups. Pearson's correlation was employed to calculate the 'r' value. Statistical significance was defined as $p < 0.05$. The statistical analysis was done using SPSS version 14.0.

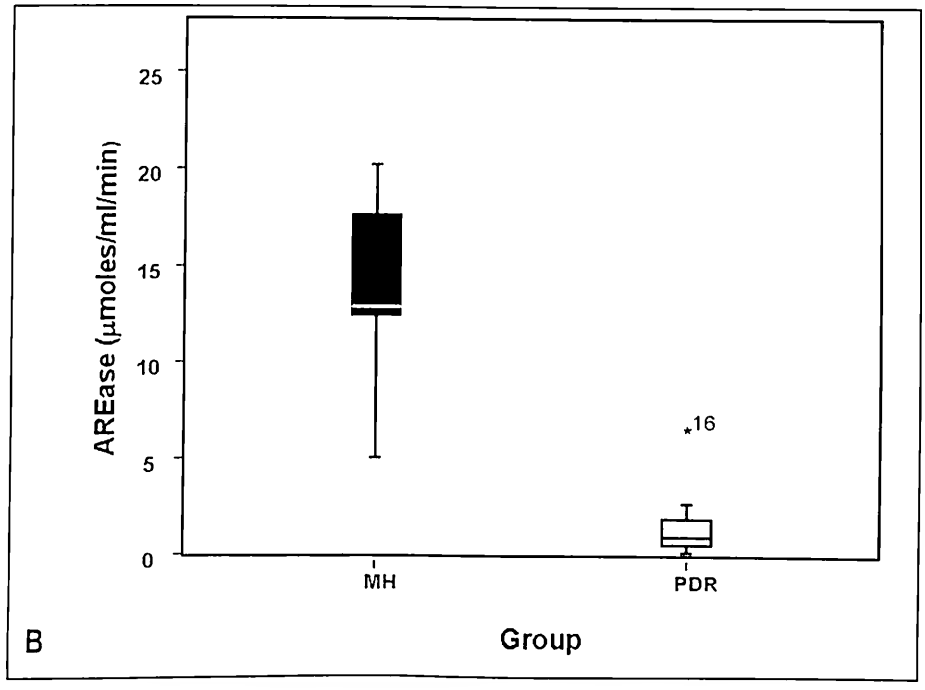
6.3. RESULTS

6.3.1 : PON-HCTLase/AREase activity in vitreous

The PON-HCTLase activity in the vitreous of PDR cases was found to be significantly elevated with a mean of 176.7 ± 16.2 U/L compared to MH which showed an activity of 94.74 ± 13.7 U/L (p=0.00). Correspondingly a significant decrease in PON-AREase activity was also observed in these PDR cases compared to MH (p = 0.00). The mean enzyme activity of PON-AREase was 1.5 ± 1.7 μmoles/ml/min in PDR as against 13.8 ± 1.6 μmoles/ml/min in MH. (p=0.00). Distribution of PON-AREase and PON-HCTLase activity in PDR and MH shows a shift in the median with 9 fold drop in the PON-AREase activity and a 2.2 fold increase in PON-HCTLase in the PDR cases when compared to MH **(Figure 6.6 A, B).**

Figure 6.6: Distribution graph showing reciprocal relationship of HCTLase and AREase in PDR





(A)- HCTLase activity, (B) – AREase activity. PDR (n =13) and MH (n = 8).

6.3.2 : Detection of Paraoxonase

Activity staining was done for the presence of PON protein in the vitreous using phenyl acetate as the substrate. Figure6.7. The PON2 in the vitreous was confirmed by western blot. This showed a band at 45 kDa. Figure 6.7

Figure 6.7: Activity stain for PON using phenyl acetate as substrate

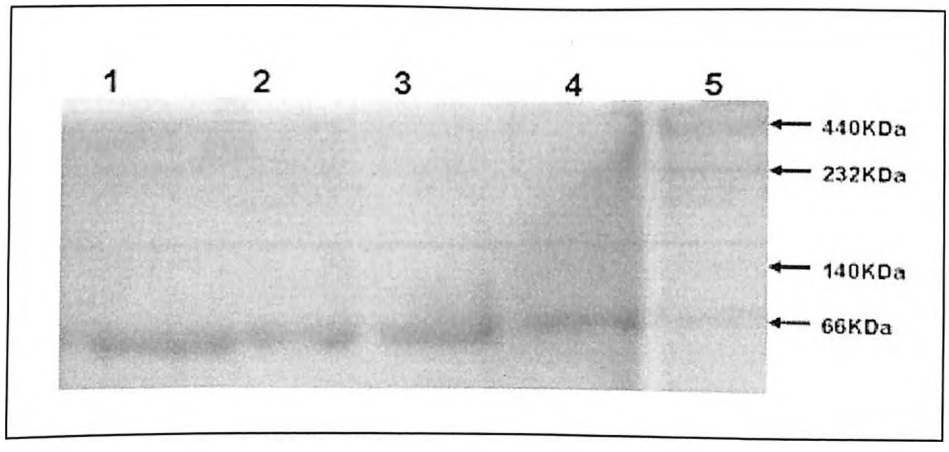


Figure 6.7: Lane 1: MH, Lane 2-4: PDR, Lane 5 : HMW. The hydrolyzed alcoholic residue will couple with the hexazotized pararosaniline solution to give an insoluble brightly colored azo dye observed as pink colour in this figure. The molecular weight is stained with fast blue which is shown in blue colour. The stained bands are having a molecular weight of 66 KDa.

Figure 6.8: PON2 protein in the vitreous of PDR by western blot

45 kDa

6.3.3 : Mass spectrometry for Homocysteine thiolactone in vitreous:

In order to see if this increase in PON-HCTLase is associated with increase in the levels of HCTL, the levels of the same were detected in the vitreous by mass spectrometry in the PDR and MH cases (**Figure 6.9 A,B,C).**

Figure 6.9 A: Mass spectrum of Standard homocysteine thiolactone

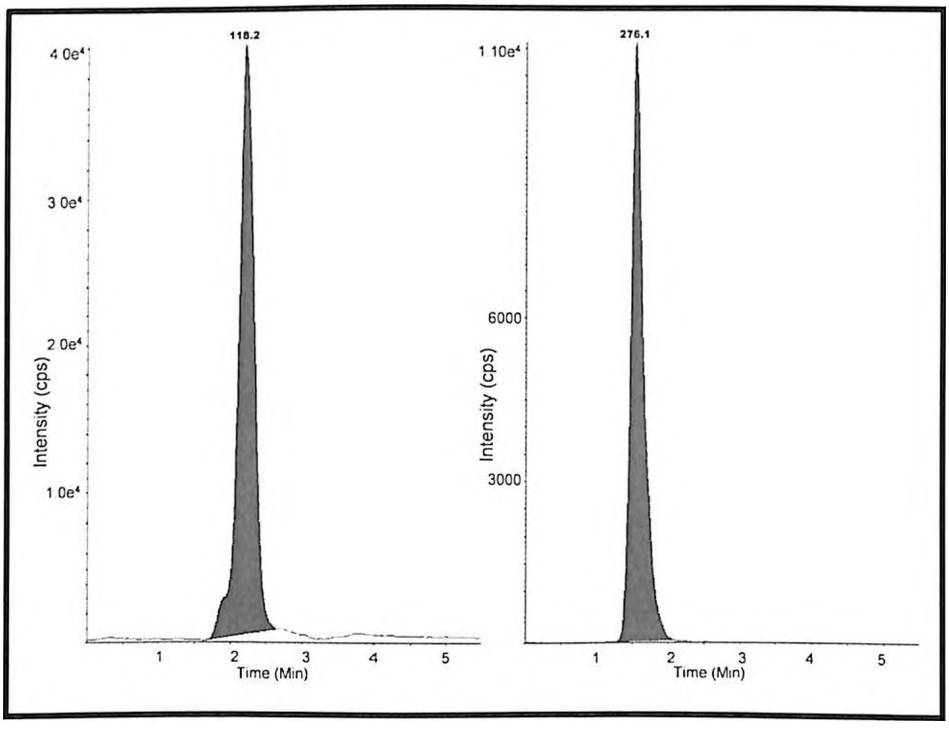


Figure 6.9 B: MH vitreous showing homocysteine thiolactone peak

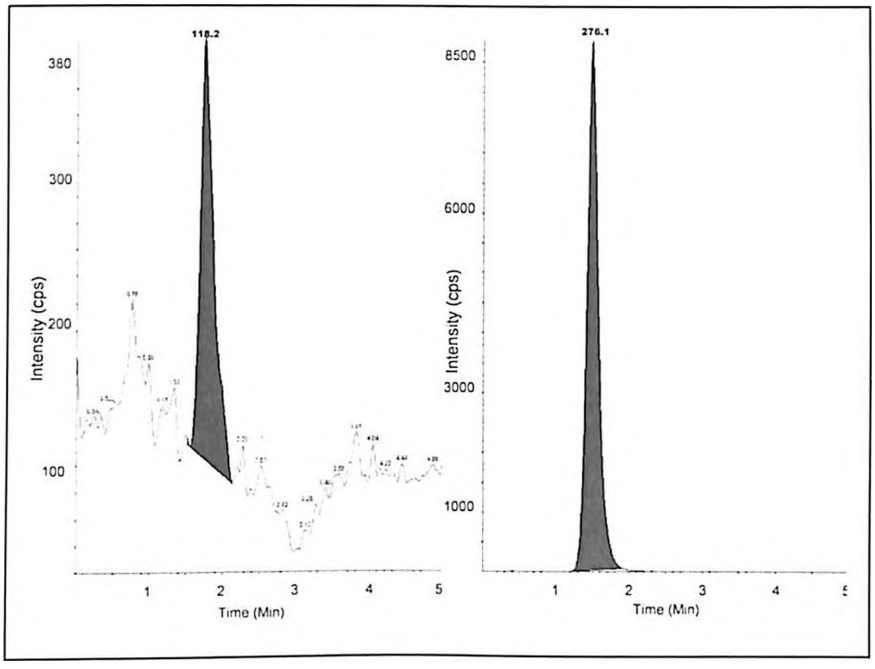


Figure 6.9 C: PDR vitreous showing homocysteine thiolactone peak

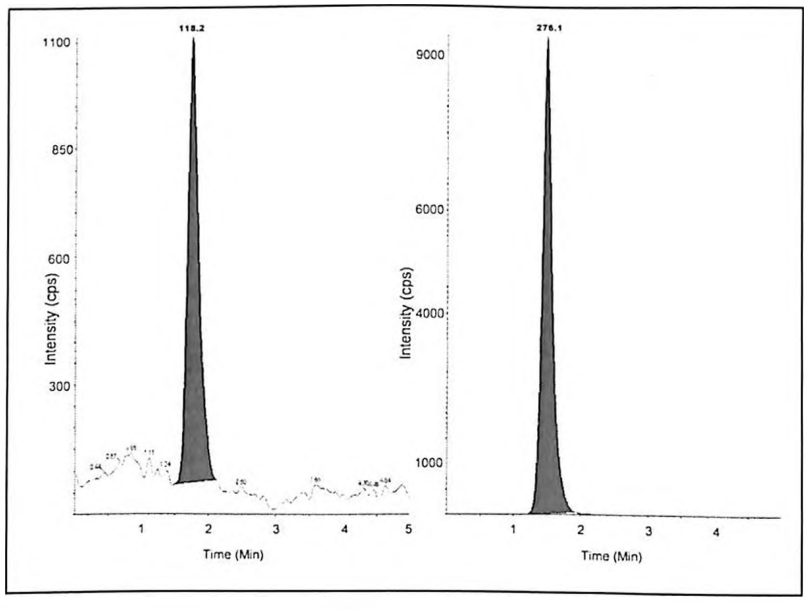


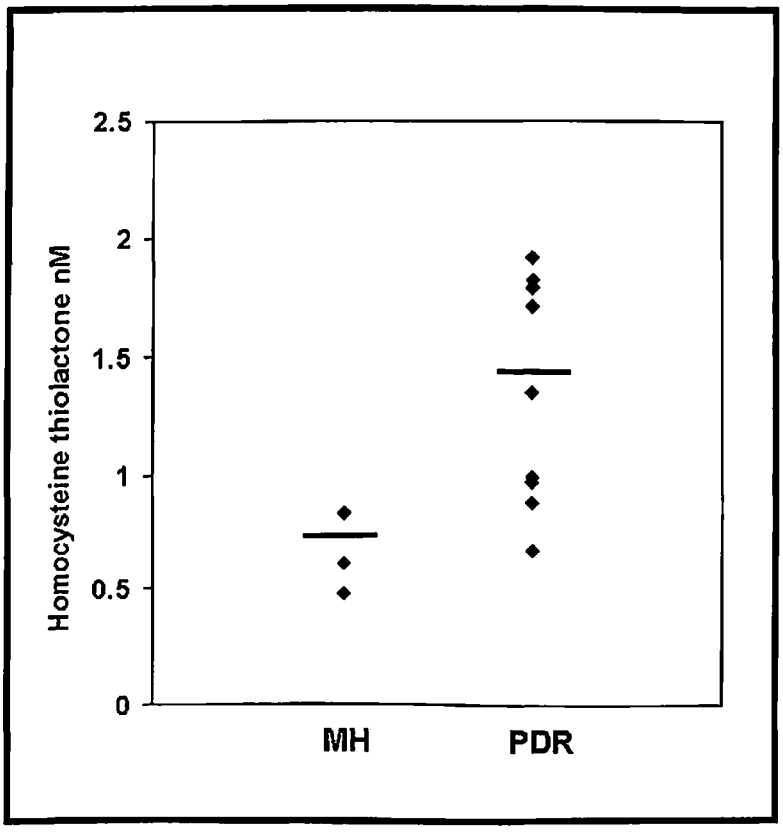
Figure 6.9: The representative LC/-MS/MS chromatogram showing the HCTL (left) and the corresponding internal standard namely Homatropine (right)

(A). Standard (B) MH vitreous (C) PDR vitreous.

The *m/z* of HCTL is 118.2 and Homatroprine is 276.1 (seen as the peak.)

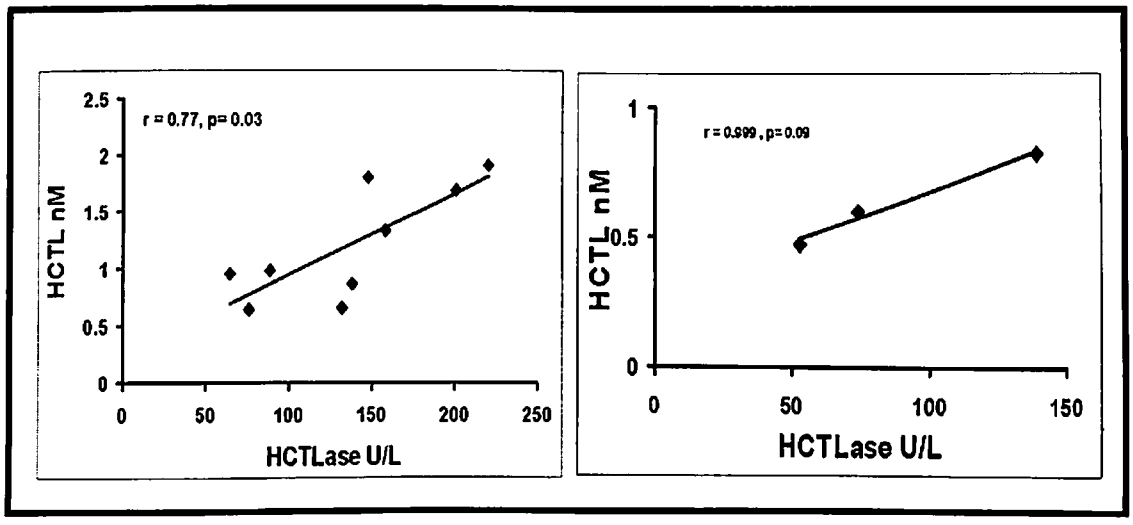
The following figure shows significant increase in the levels of HCTL in the vitreous of PDR cases when compared to MH. The mean level of HCTL was found to be 1.37 nM ± 0.51 nM in PDR compared to 0.65 nM ± 0.18 in MH. **(Figure 6.10).**

Figure 6.10: Distribution of HCTL levels in PDR and MH.



6.3.4: Correlation between HCTLase/AREase with HCTL: A significant positive correlation was observed between vitreous HCTL levels and the corresponding HCTLase activity, in PDR (r =0.77, p= 0.033) **(Figure 6.11 A,B).** However no significant correlation was observed with PON-AREase and HCTL.

Figure 6.11: Correlation between HCTL and HCTLase in PDR and MH:



6.3.5:Oxidative stress parameters in the Vitreous:

There was a significant decrease in the vitreous levels of total thiols in PDR cases compared to MH (p= 0.00), with significant increase in the TAC levels in the PDR cases compared to MH (p=0.0001). This increase in TAC value in spite of reduced thiol status can be attributed to the cumulative effect of other small molecule antioxidants such as Vitamin E and C. Izuta et al suggests that the thioredoxin and Nrf2/ARE pathways can also mediate the redox status in the vitreous body of PDR cases. He also reports on the increased antioxidant potential of the vitreous of PDR (Izuta, Matsunaga et al.). However the alteration in the TBARS levels was not significant with the median showing 11.6 in PDR (IQ range: 22.3) and 26.05 in MH (IQ range 2.7). Total protein level in the vitreous was found to be significantly elevated in PDR (p=0.002) **(Table 6.4).**

Table 6.4: Homocysteine thiolactonase, Aryl esterase activity and oxidative stress parameters in the vitreous of PDR compared to MH cases

S.No	Parameter	Macular Hole (n = 8)	PDR (n = 13)	p value
1.	Homocysteine Thiolactonase (U/L)	78.5 ± 12.7	175.1 ± 16.4	p = 0.000
2.	Arylesterase activity (μmoles/ml/min)	13.8 ± 1.6	1.5 ± 1.7	p = 0.000
3.	TBARS (nmol/ml/mgprotein)	24.42 ± 0.20	17.1 ± 15.2	p = 0.090
4.	TAC (mmol)	0.22 ± 0.03	0.319 ± 0.24	p = 0.0001
5.	Total Thiols (μmol)	43.88 ± 6.3	28.7 ± 12.9	p = 0.000

***6.3.6; In vitro* studies on BREC cells.** The cytototoxic effect of HCTL and Hcys was tested in BREC cells and the MTT assay revealed that the cells were viable at all the concentrations of HCTL and Hcys tested until 48 hours (**Figure 6.12 A & B**). The *in vitro* experiments showed an increase in HCTLase activity, dose and time dependently, when exposed to both HCTL and Hcys (**Figure 6.13 A & B**). HCTLase activity was found to be maximal at 200 μM at the end of 24 hours for both HCTL & Hcys exposure, which was a significant increase compared to the untreated control (p=0.000). Correspondingly, the PON-AREase activity was significantly decreased. (p= 0.000) (**Figure 6.14**). In the same experimental condition, the mRNA expression of the PON enzyme all the three isoforms was tested and only PON2 was found to be expressed by the BREC cells as reported in other ECs.An increase in the expression of PON 2 was seen in cells treated with Hcys and HCTL and the effect was much pronounced in Hcys. (**Figure 6.15**)

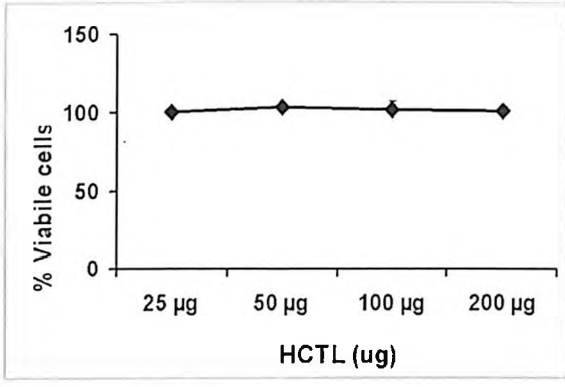


Figure 6.13 (A)

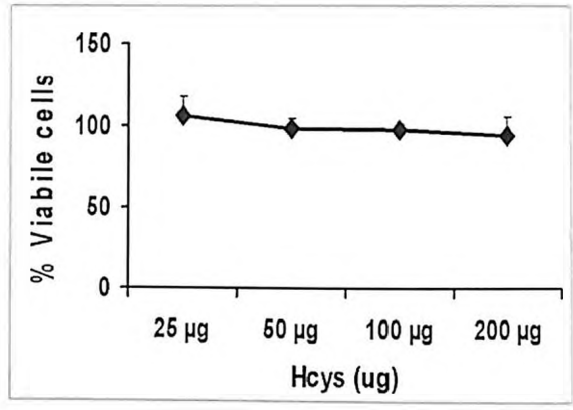


Figure 6.13 (B)

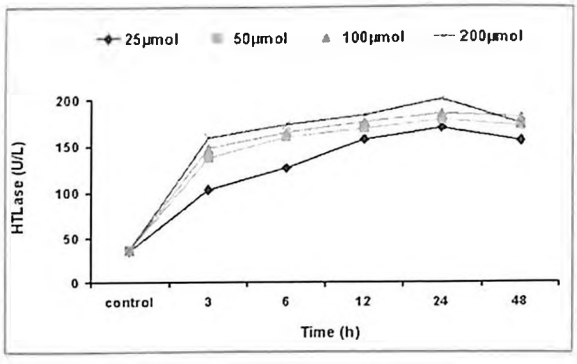


Figure 6.14 (A)

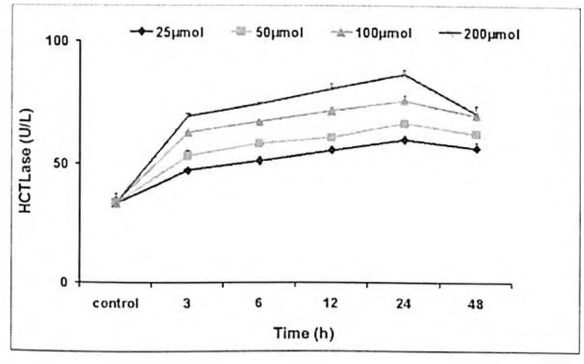


Figure 6.14 (B)

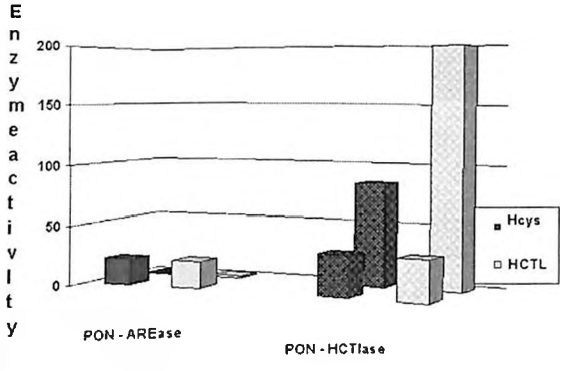


Figure 6.15

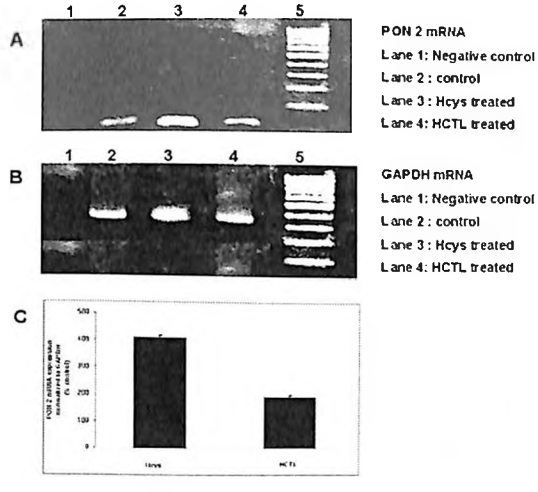


Figure 6.16

Figure 6.12: MTT assay for the cytotoxic effect of HCTL and Hcys: A: HCTL at various concentrations at 48hrs, B: Hcys at various concentrations at 48 hrs

Figure 6.13 : HCTLase levels in BREC cells exposed to HCTL and Hcys
A: Graph showing the dose and time dependent increase in HCTLase activity after treatment with HCTL, B: Graph showing the dose and time dependent increase in HCTLase activity after treatment with Hcys

Figure 6.14: HCTLase and AREase activity comparison in BREC cells exposed to Hcy and HCTL at 200 µM for 24hrs.

Figure 6.15: mRNA expression of PON2 in BREC cells exposed to Hcys and HCTL (200 µM AT 24 h).

6.3.7: Effect of amino acids on Homocysteine thiolactonase activity:

The effect of AGE on PON activity was tested at 24 hrs since we observed the maximal activity with Hcys at 24hrs. The PON HCTlase activity was found to be decreased when exposed to AGE at 24 hrs. There was a dose dependent decrease in the HCTLase and ARease activity of PON with increasing concentration of AGE and it was maximam at 1000µg/ml showing 50% inhibition (Figure 6.16).

Figure 6.16: A.The effect of AGE on HCTLase activity:

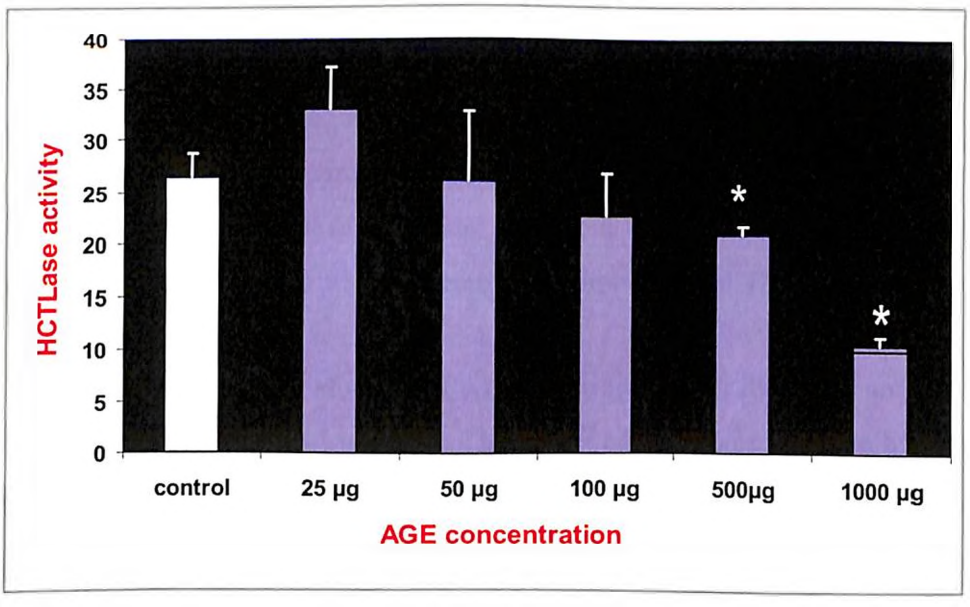


Figure 6.16: B.The effect of AGE on AREase activity

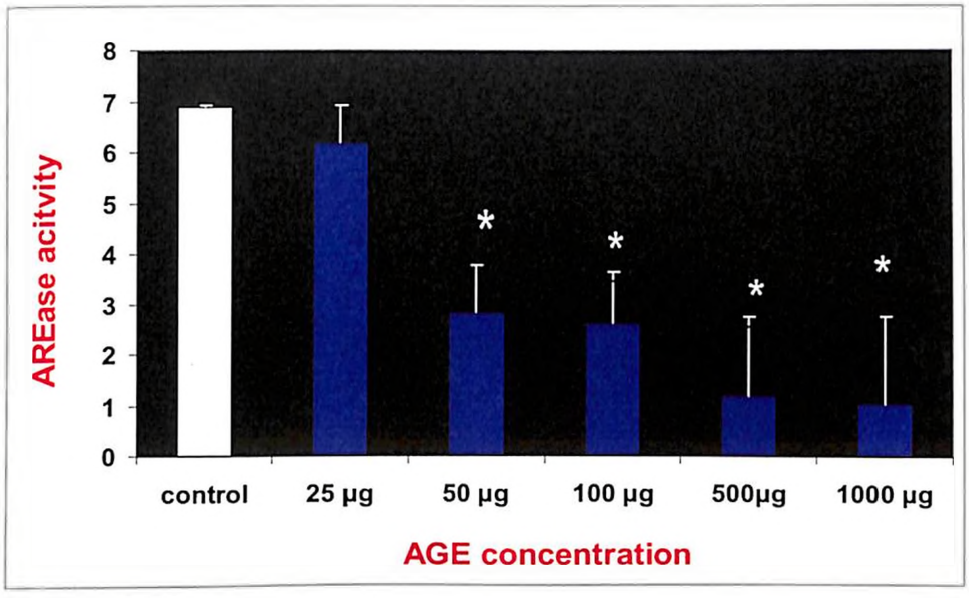


Figure 6.16: The effect of varying concentrations of AGE (50, 100, 500 and 1000 µg AGE) on HCTLase activity of PON .

Since there was a drop in the activity of paraoxonase with advanced glycation end products compounds that block the glycation process would be useful. In our previous studies we have shown that the amino acids have the capability to reduce the glycation process. Free lysine, Alanine, Aspartic acid, or Glutamic acid has been shown to reduce the glycation of human lens proteins, *in vitro* (Ramakrisahnan and Sulochana et al.1996, 1997). There fore out of these and also from the other ongoing studies in our lab a set of 5 amino acids at 5mM concentration comprising of glycine, glutamic acid, leucine, lysine and cysteine was studied to check for its effect on PON activity in BREC cells.The amino acids along with AGE were incubated for 24hrs and the PON activity in the cell lysates was measured. This effect of AGE was found to be retrieved by all the amino acids in the order of leucine to nearly the control levels, lysine and cysteine increasing the activity 2 fold, glutamic acid and glycine increasing the activity 4 fold. Figure 6.17.

Figure 6.17 A: The effect of amino acids on the HCTLase activity of the BREC Cells treated with 500 µg AGE

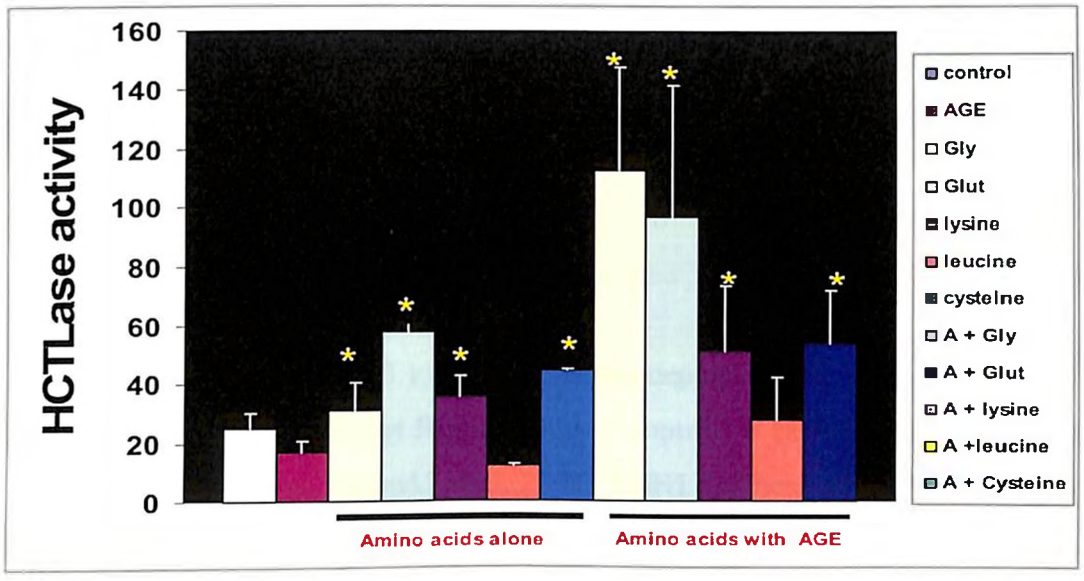
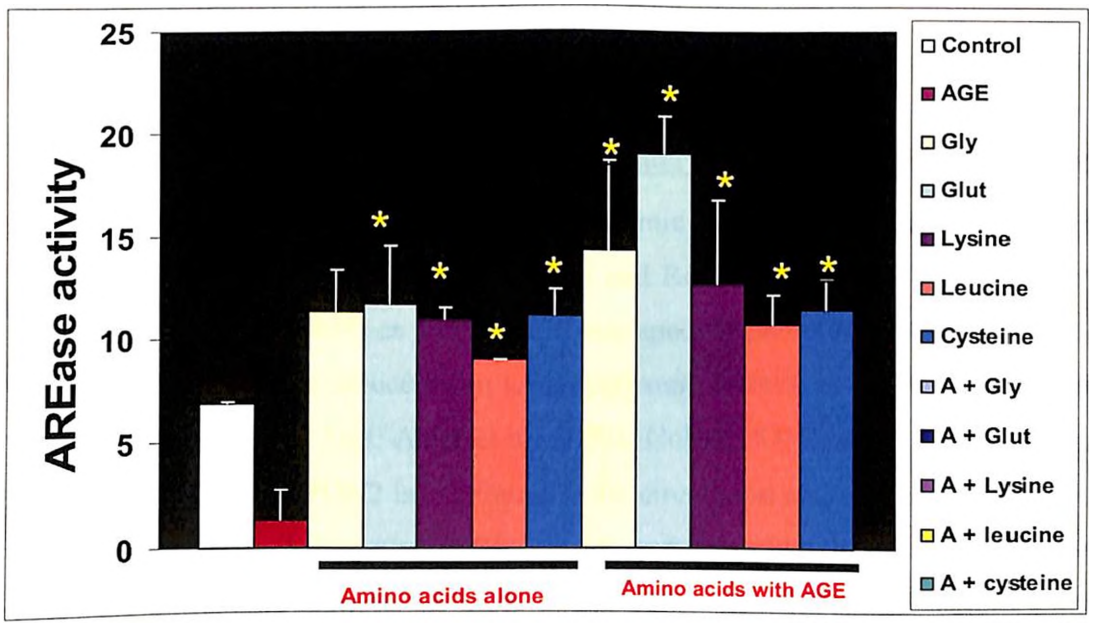


Figure 6.17 B: The effect of amino acids on the AREase activity of the BREC Cells treated with 500 µg AGE



CHAPTER 7: COMPARATIVE MODELING OF PON2 AND ALYSIS OF ITS SUBSTRATE BINDING INTERACTONS USING COMPUTATIONAL METHODS

7.1: INTRODUCTION

Paraoxonase-1 (PON1) (EC 3.1.8.1) is a calcium-dependent esterase synthesized in the liver and contained in plasma High-Density Lipoproteins (HDL) (Aviram, Rosenblat et al. 1998; Domagala, Lacinski et al. 2006). PON1 has been found to have essential functions of Arylesterase (ARE), organophosphatase and lactonase activity depending on the substrate they act upon (Durrington, Mackness et al. 2001). PON1 is an essential antioxidant enzyme, which possess peroxidase like activity and also hydrolyzes platelet-activating factor, bioactive phospholipids which are involved in lipid peroxidation and vascular disease development (Yeung, Josse et al. 2004). PON constitutes a family of mammalian enzymes with three members including PON1, PON2 and PON3. The members of the PON family share ~60% sequence identity. PON1 has been reported to beneficially influence atherogenesis *via* inhibition of Low Density Lipoprotein (LDL) oxidation (Mackness, Arrol et al. 1993). This PON enzyme has been associated with various systemic diseases like atherosclerosis (Seo and Goldschmidt-Clermont 2009), (Aviram and Rosenblat 2008), Diabetes (Nowak, Wielkoszynski et al. 2009) as well as in tissue specific pathologies such as in ocular disease like retinal venous occlusion (Angayarkanni, Barathi et al. 2008), age-related macular degeneration (Ates, Azizi et al. 2009). Unlike PON1 and 3 which is mainly associated with HDL, PON2 is not found in the circulation and acts as an intracellular antioxidant (Zhang, Azhar et al. 2001; Aviram and Rosenblat 2004) and may provide an innate antioxidant activity in most of the cells, independent of secretory PON. PON2 is ubiquitously present in all tissues and also reportedly present intra cellularly in the 3 major vascular cell types namely, cultured HUVEC, smooth muscle vascular cells (SMVC) and aortic adventitial fibroblasts with the major function of reducing the

reactive oxygen species (ROS) mediated EC dysfunction. (Mackness, Hunt et al. 1997). PON 2-deficient mice exhibit elevated tissue levels of lipid hydroperoxides and increased migration of macrophage into the artery wall, leading to formation of atherosclerotic lesion as compared to their wildtype controls (Ng, Bourquard et al. 2006). It is suggested that PON2 protects macrophages from foam cell formation and thus contributes to the prevention of atherogenesis (Ng, Hama et al. 2006). Importantly, a decreased PON2 expression has been observed in hypercholesterolemic patients (Rosenblat, Hayek et al. 2004) and during progression of atherogenesis (Fortunato, Di Taranto et al. 2008). There are several reports which show Hcys to be a risk factor for cardiovascular disease and atherosclerosis (Kazemi, Eshraghian et al. 2006) and recent reports have shown that the concentration of Hcys is increased in the serum and vitreous of diabetic patients.(Aydemir, Turkcuoglu et al. 2008; Coral, Angayarkanni et al. 2009) In all these conditions the level of PON is found to be lowered.(Ikeda, Suehiro et al. 2007; Jayakumari and Thejaseebai 2009). This gives a clue that Hcys may have an influence on the AREase activity. Further to strengthen this hypothesis in our study we observed a drop in AREase activity in the vitreous of PDR cases but the HCTLase activity high. The increase in HCTLase activity correlates with the proportionate increase of HCTL by increasing concentration of Hcys. The presence of PON2 in the vitreous was confirmed by western blot in our study. PON2, the oldest member of the family, has not been crystallized so far and PON1 is the only best- studied member of the family (Tavori, Khatib et al. 2008) .In this context, modeling and docking techniques could add insight towards structure-based analysis, pertaining to molecular interaction studies. Hence, the present study aims at predicting the three dimensional (3D) structure of PON2 by employing *in silico* modeling techniques and also to characterize its interactions with ligands of biological importance.

7.2: METHODS

The 3D structure of Human PON2 (Uniprot Accession No: Q15165) (2009) was modeled by Homology modeling using MODELLER 9v7. (Fiser and Sali 2003) Template for modeling the 3D structure was identified by blastP (Altschul, Gish et al. 1990).search against Protein Data Bank (PDB) with default search parameters PON 1(1V04) (Harel, Aharoni et al. 2004) was selected as template to model PON2. The template structure was downloaded from PDB(Berman, Westbrook et al. 2000).

The mRNA sequence alignment of the PON family is given below:

```
PON1    ATGGCGAAGCTGATTGCGCTCACCCTCTTGGGGATGGGACTGGCACTCTTCAGGAACCAC
PON2    ATGGGGCGGCTGGTGGCTGTGGGCTTGCTGGGGATCGCGCTGGCGCTCCTGGGCGAGAGG
PON3    ATGGGGAAGCTCGTGGCGCTGGTCCTGCTGGGGGTCGGCCTGTCCTTAGTCGGGGAGATG
        **** *  ***  * **  *   * *  ***** * *  *** *  *  *  *  *

PON1    CAGTCTTCTTACCAAACACGACTTAATGCTCTCCGAGAGGTACAACCCGTAGAACTTCCT
PON2    CTTCTGGCACTCAGAAATCGACTTAAAGCCTCCAGAGAAGTAGAATCTGTAGACCTTCCA
PON3    TTCCTGGCGTTTAGAGAAAGGGTGAATGCCTCTCGAGAAGTGGAGCCAGTAGAACCTGAA
              *     *    *  * ** **     **** **  *  * ***** * *

PON1    AACTGTAATTTAGTTAAAGGAATCGAAACTGGCTCTGAAGACTTGGAGATACTGCCTAAT
PON2    CACTGCCACCTGATTAAAGGAATTGAAGCTGGCTCTGAAGATATTGACATACTTCCCAAT
PON3    AACTGCCACCTTATTGAGGAACTTGAAAGTGGCTCTGAAGATATTGATATACTTCCTAGT
         ****  *  *  ** * * * * ***  ************  * ** ***** ** * *

PON1    GGACTGGCTTTCATTAGCTCTGGATTAAAGTATCCTGGAATAAAGAGCTTCAACCCCAAC
PON2    GGTCTGGCTTTTTTTAGTGTGGGTCTAAAATTCCCAGGACTCCACAGCTTTGCACCAGAT
PON3    GGGCTGGCTTTTATCTCCAGTGGATTAAAATATCCAGGCATGCCAAACTTTGCGCCAGAT
        ** ********  *       **  **** *  ** **  *    * ***    **  *

PON1    AGTCCTGGAAAAATACTTCTGATGGACCTGAATGAAGAAGATCCAACAGTGTTGGAATTG
PON2    AAGCCTGGAGGAATACTAATGATGGATCTAAAAGAAGAAAAACCAAGGGCACGGGAATTA
PON3    GAACCAGGAAAAATCTTCTTGATGGATCTGAATGAACAAAACCCAAGGGCACAAGCGCTA
           ** ***  ***  *  ******* ** ** *** ** * ****  *     *   *

PON1    GGGATCACTGGAAGTAAATTTGATGTATCTTCATTTAACCCTCATGGGATTAGCACATTC
PON2    AGAATCAGT---CGTGGGTTTGATTTGGCCTCATTCAATCCACATGGCATCAGCACTTTC
PON3    GAAATCAGT---GGTGGATTTGACAAAGAATTATTTAATCCACATGGGATCAGTATTTTC
           **** *    **   *****       * *** ** ** ***** ** ** *  ***

PON1    ACAGATGAAGATAATGCCATGTACCTCCTGGTGGTGAACCATCCAGATGCCAAGTCCACA
PON2    ATAGACAACGATGACACAGTTTATCTCTTTGTTGTAAACCACCCAGAATTCAAGAATACA
PON3    ATCGACAAAGACAATACTGTGTATCTTTATGTTGTGAATCATCCCCACATGAAGTCCACT
        *  **  * **  *  *  * ** **    ** ** ** ** **  *    ***   **

PON1    GTGGAGTTGTTTAAATTTCAAGAAGAAGAAAAATCGCTTTTGCATCTAAAAACCATCAGA
PON2    GTGGAAATTTTTAAATTTGAAGAAGCAGAAAATTCTCTGTTGCATCTGAAAACAGTCAAA
PON3    GTGGAGATATTTAAATTTGAGGAACAACAACGTTCTCTGGTATACCTGAAAACTATAAAA
        *****  * ********* * ***  * **   ** **  *  * ** *****  * * *

PON1    CATAAACTTCTGCCTAATTTGAATGATATTGTTGCTGTGGGACCTGAGCACTTTTATGGC
PON2    CATGAGCTTCTTCCAAGTGTGAATGACATCACAGCTGTTGGACCGGCACATTTCTATGCC
PON3    CATGAACTTCTCAAAAGTGTGAATGACATTGTGGTTCTTGGACCAGAACAGTTCTATGCC
        *** * *****    * * ******* **    * * * ***** *  ** ** **** *
```

```
PON1        ACAAATGATCACTATTTTCTTGACCCCTACTTACAATCCTGGGAGATGTATTTGGGTTTA
PON2        ACAAATGACCACTACTTCTCTGATCCTTTCTTAAAGTATTTAGAAACATACTTGAACTTA
PON3        ACCAGAGACCACTATTTTACCAACTCCCTCCTGTCATTTTTTGAGATGATCTTGGATCTT
            ** *  ** ***** **     *  *   * *    *  *  ** *     ***    *

PON1        GCGTGGTCGTATGTTGTCTACTATAGTCCAAGTGAAGTTCGAGTGGTGGCAGAAGGATTT
PON2        CACTGGGCAAATGTTGTTTACTACAGTCCAAATGAAGTTAAAGTGGTAGCAGAAGGATTT
PON3        CGCTGGACTTATGTTCTTTTCTACAGCCCAAGGGAGGTTAAAGTGGTGGCCAAAGGATTT
               *** *  ***** * * *** ** **** ** ***  ****** **  ********

PON1        GATTTTGCTAATGGAATCAACATTTCACCCGATGGCAAGTATGTCTATATAGCTGAGTTG
PON2        GATTCAGCAAATGGGATCAATATTTCACCTGATGATAAGTATATCTATGTTGCTGACATA
PON3        TGTAGTGCCAATGGGATCACAGTCTCAGCAGACCAGAAGTATGTCTATGTAGCTGATGTA
              *   ** ***** ****   * *** * **    ****** ***** * *****  *

PON1        CTGGCTCATAAGATTCATGTGTATGAAAAGCATGCTAATTGGACTTTAACTCCATTGAAG
PON2        TTGGCTCATGAAATTCATGTTTTGGAAAAACACACTAATATGAATTTAACTCAGTTGAAG
PON3        GCAGCTAAGAACATTCACATAATGGAAAAACATGATAACTGGGATTTAACTCAACTGAAG
               *** *  * *****  *    ***** **   ***   *  ********   *****

PON1        TCCCTTGACTTTAATACCCTCGTGGATAACATATCTGTGGATCCTGAGACAGGAGACCTT
PON2        GTACTTGAGCTGGATACACTGGTGGATAATTTATCTATTGATCCTTCCTCGGGGGACATC
PON3        GTGATACAGTTGGGCACCTTAGTGGATAACCTGACTGTCGATCCTGCCACAGGAGACATT
                *  *  *    **  * ********  *  ** * ******    * ** *** *

PON1        TGGGTTGGATGCCATCCCAATGGCATGAAAATCTTCTTCTATGACTCAGAGAATCCTCCT
PON2        TGGGTAGGCTGTCATCCTAATGGCCAGAAGCTCTTCGTGTATGACCCGAACAATCCTCCC
PON3        TTGGCAGGATGCCATCCTAATCCTATGAAGCTACTGAACTATAACCCTGAGGACCCTCCA
            * **  ** ** ***** ***     ***  *  *    *** ** *  *  * *****

PON1        GCATCAGAGGTGCTTCGAATCCAGAACATTCTAACAGAAGAACCTAAAGTGACACAGGTT
PON2        TCGTCAGAGGTTCTCCGCATCCAGAACATTCTATCTGAGAAGCCTACAGTGACTACAGTT
PON3        GGATCAGAAGTACTTCGCATCCAGAATGTTTTGTCTGAGAAGCCCAGGGTGAGCACCGTG
               ***** ** ** ** ********  ** *  * **  * ** *  ****     **

PON1        TATGCAGAAAATGGCACAGTGTTGCAAGGCAGTACAGTTGCCTCTGTGTACAAAGGGAAA
PON2        TATGCCAACAATGGGTCTGTTCTCCAAGGAAGTTCTGTAGCCTCAGTGTATGATGGGAAG
PON3        TATGCCAACAATGGCTCTGTGCTTCAGGGCACCTCTGTGGCTTCTGTGTACCATGGGAAA
            *****  * *****  * **  * ** ** * *  * ** ** ** *****  * *****

PON1        CTGCTGATTGGCACAGTGTTTCACAAAGCTCTTTACTGTGAGCTCTAA
PON2        CTGCTCATAGGCACTTTATACCACAGAGCCTTGTATTGTGAACTCTAA
PON3        ATTCTCATAGGCACCGTATTTCACAAAACTCTGTACTGTGAGCTCTAG
             * ** ** *****  * *  **** * *  * ** ***** *****
```

The protein sequence alignment of the PON family is given below

```
PON1        MAKLIALTLLGMGLALFRNHQSSYQTRLNALREVQPVELPNCNLVKGIETGSEDMEILPN
PON2        MGAWVGCGLAGDRAGFLGERLLALRNRLKASREVESVDLPHCHLIKGIEAGSEDIDILPN
PON3        MGKLVALVLLGVGLSLVGEMFLAFRERVNASREVEPVEPENCHLIEELESGSEDIDILPS
            *.  :.  * *   .:. :   : : *::* ***:.*:  :*:*:: :*:****::***.

PON1        GLAFISSGLKYPGIKSFNPNSPGKILLMDLNEEDPTVLELGITGSKFDVSSFNPHGISTF
PON2        GLAFFSVGLKFPGLHSFAPDKPGGILMMDLKEEKPRARELRIS-RGFDLASFNPHGISTF
PON3        GLAFISSGLKYPGMPNFAPDEPGKIFLMDLNEQNPRAQALEIS-GGFDKELFNPHGISIF
            ****:* ***:**: .* *:.** *::***:*:.* .  * *:   **   ******* *

PON1        TDEDNAMYLLVVNHPDAKSTVELFKFQEEEKSLLHLKTIRHKLLPNLNDIVAVGPEHFYG
PON2        IDNDDTVYLFVVNHPEFKNTVEIFKFEEAENSLLHLKTVKHELLPSVNDITAVGPAHFYA
PON3        IDKDNTVYLYVVNHPHMKSTVEIFKFEEQQRSLVYLKTIKHELLKSVNDIVVLGPEQFYA
            *:*:::** *****. *.***:***:* :.**::***::*:** .:***..:** :**.
```

```
PON1        TNDHYFLDPYLQSWEMYLGLAWSYVVYYSPSEVRVVAEGFDFANGINISPDGKYVYIAEL
PON2        TNDHYFSDPFLKYLETYLNLHWANVVYYSPNEVKVVAEGFDSANGINISPDDKYIYVADI
PON3        TRDHYFTNSLLSFFEMILDLRWTYVLFYSPREVKVVAKGFCSANGITVSADQKYVYVADV
            *.**** :. *.  *  *.* *: *::*** **:***:**  ****.:*.* **:*:*::

PON1        LAHKIHVYEKHANWTLTPLKSLDFNTLVDNISVDPETGDLWVGCHPNGMKIFFYDSENPP
PON2        LAHEIHVLEKHTNMNLTQLKVLELDTLVDNLSIDPSSGDIWVGCHPNGQKLFVYDPNNPP
PON3        AAKNIHIMEKHDNWDLTQLKVIQLGTLVDNLTVDPATGDILAGCHPNPMKLLNYNPEDPP
            *::**: *** *  ** ** ::::.*****:::** :**: .*****  *:: *:.::**

PON1        ASEVLRIQNILTEEPKVTQVYAENGTVLQGSTVASVYKGKLLIGTVFHKALYCEL
PON2        SSEVLRIQNILCEKPTVTTVYANNGSVLQGSSVASVYDGKLLIGTLYHRALYCEL
PON3        GSEVLRIQNVLSEKPRVSTVYANNGSVLQGTSVASVYHGKILIGTVFHKTLYCEL
            .*********:* *:* *: ***:**:****::*****.**:****::*::*****
```

ClustalW (Chenna, Sugawara et al. 2003) was used to align the target and template sequences and the resultant alignment was stored as PIR format.The alignment and the template atom files were given as input to MODELLER 9v7, to generate the 3D structure of PON2. Since PON family has 2 Ca^{2+} ions (Horke, Witte et al. 2008) which is the characteristic feature of the family, the modeling process was customized to accommodate 2 Ca^{2+} ions in the predicted structure, as present in template. Finally, 10 CHARMM (Sali and Blundell 1993) optimized decoys were dynamically generated using MODELLER 9v7 and were validated using PROCHECK for proper Phi/Psi conformation (Laskowski, Rullmannn et al. 1996). The results indicate that 2 models out of 10 generated models were found to be perfectly fit with no residues in the disallowed region of Ramachandran plot. Furthermore, the prediction quality was assessed using Protein Quality Predictor (ProQ) based on LGscore (Wallner and Elofsson 2003).

7.2.1 : Two dimentonal structure of ligands :

The set of biologically relevant ligand molecules studied in this study include Homocysteine thiolactone [Pubchem: 107712], γ- thiobutyro lactone[Pubchem: 13852], Δ- valero lactone[Pubchem: 10953], Benzyl acetate[Pubchem: 8785], 2-Naphthyl acetate[Pubchem: 73709], Phenyl acetate [Pubchem: 31229] and paraoxon[Pubchem: 9395] .The structure of these ligand molecules were retrieved from NCBI-Pubchem Compound database (Wang, Xiao et al. 2009). (Figure7.1)

Figure 7.1: Two dimensional structures of ligands:

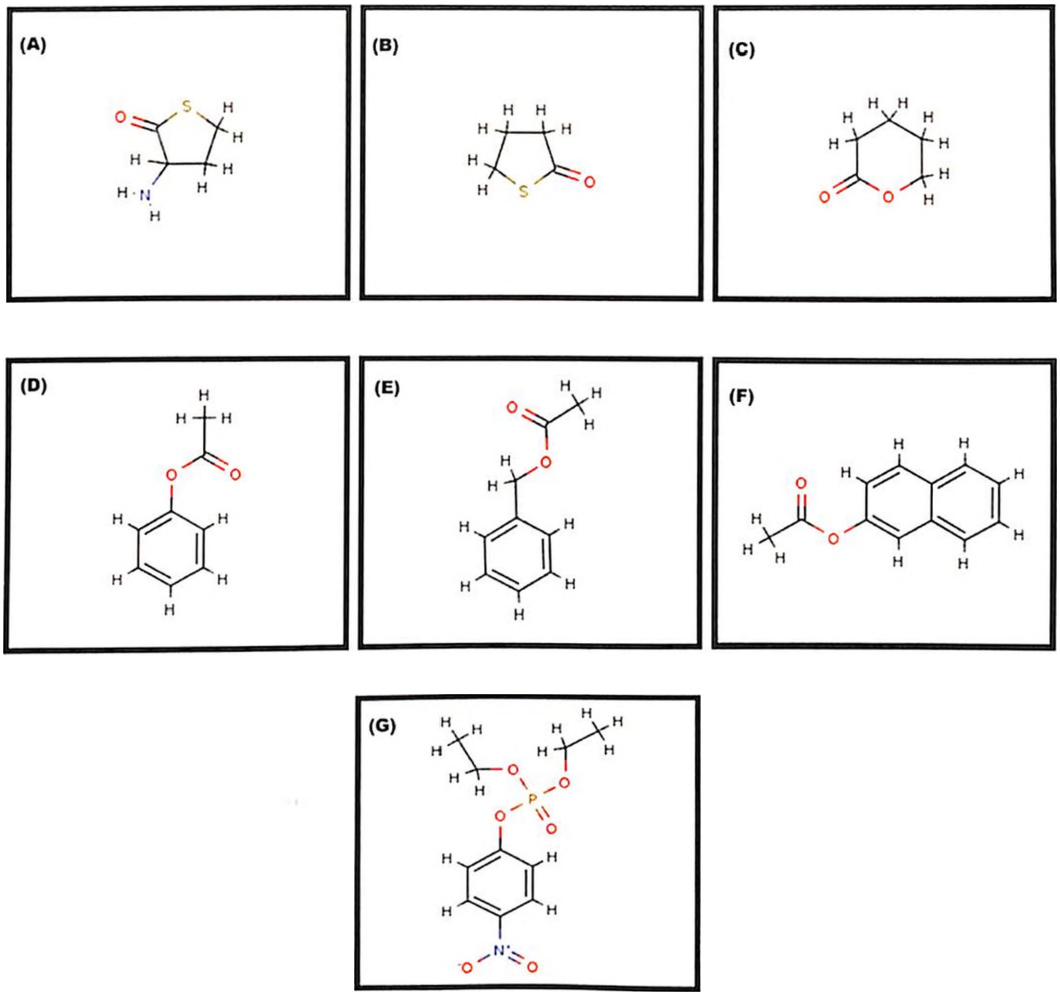


Figure 7.1: Two dimensional structures of ligands: (A) Homocysteine thiolactone (HCTL), (B) Gamma thiobutyrolactone (GTBL) ,(C) Delta Valero lactone (DVL) (D) Phenyl acetate (PA), (E) Benzyl acetate (BA), (F) 2- Naphthyl acetate (2-NA), (G) Paraoxonase (PAR). These images were generated using MarvinSketch.

The geometry of the ligands were optimized via geometry optimization protocol, (Broyden- Fletcher- Golfarb-Shanno line search method set to 1000 steps) using ArgusLab(Thompson) , a popular free suite of molecular modeling and simulation solutions for drug discovery. Each of the minimization was carried out using Universal Force Field (UFF)(Thompson). (Table 7.1)

Table 7.1: Energy minimized values of ligands of PON 2

Molecules	Energy levels with minimization maximum of 1000 steps	
	Initial Potential Energy (Kcal/mol)	Final Potential Energy (Kcal/mol)
Homocysteine thiolactone	26.572	13.957
Δ Valero lactone	35.825	11.541
Γ Thiobutyro lactone	18.379	11.080
2- Naphthyl acetate	47.984	22.663
Benzyl acetate	40.820	30.240
Phenyl acetate	36.688	27.350
Paraoxon	198.835	78.844

7.2.2: Docking of ligands with paraoxonase 2

Docking calculations for the selected compounds with PON2 were carried out using AutoDock 4.0 suite, a comprehensive software for performing automated docking of ligands to their macromolecular receptors (Morris GM 1998). In this docking simulation, we used semi-flexible docking protocols (Gowthaman, Jayakanthan et al. 2008), in which the target protein PON2 was kept rigid and the ligands being docked were kept flexible in order to explore an arbitrary number of torsional degrees of freedom. Graphical User Interface (GUI) program "AutoDock Tools" was used to prepare, run, and analyze the docking simulations. Kollman United atom charges and polar hydrogens were added into the receptor PDB file for the preparation of protein in docking simulation. Gasteiger charges were also assigned prior to docking. The rigid roots for each ligand were defined automatically. The amide bonds were made non-rotatable and the peptide backbone bonds were made rotatable. The modified ligand molecules were saved as PDBQT. The grid parameter files are to be set before running a docking calculation, as AutoDock 4.0 requires pre-calculated grid maps for each atom type present in the ligand being docked. The grid which is set must cover the area of interest in the protein. Since, the active binding site is unknown; the grid box was set to cover the entire protein. The grid box size in x-, y- and z- axes was set to 126 Å x126 Å x126 Å and was kept constant for all the ligands. The spacing between the grid points was 0.375 Å. Autogrid 4.0 program, provided along with AutoDock 4.0

was used to produce grid maps. To search for the best conformers, Lamarckian Genetic Algorithm (LGA/ GALS) was chosen. A maximum of 10 conformers were considered for each compound and a maximum of 2500000 energy evaluations, maximum number of generations set to 27000, maximum number of top individual that automatically survived was set to 1, rate of mutation was set to 0.02 and the rate of crossover was set to 0.8. Finally the Protein- ligand complexes were analyzed using DS Visualizer (Talley, Harel et al. 2008) and PYMOL visualization tool (DeLano 2002).

7.3 : RESULT

Human PON2 shares 81% sequence similarity to recombinant variant PON1 (1V04), this close homology indicates the fold equivalence of the 3D structures. Hence, the homology model predicted in this study is highly plausible (Figure 7.2).

Figure 7.2: The modeled structure of Human PON2

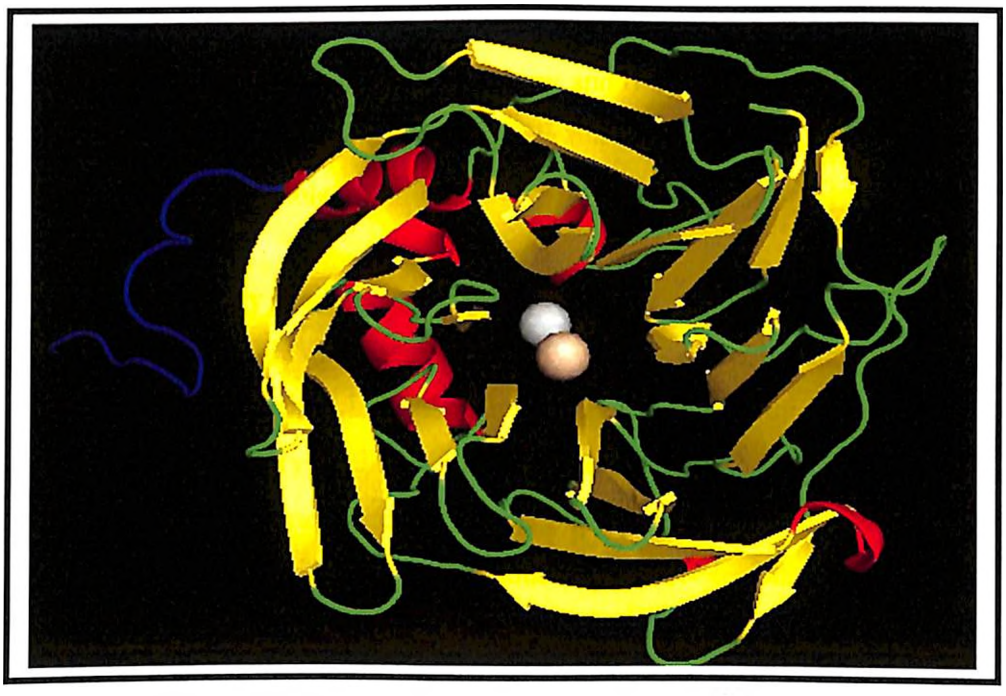


Figure 7.2: The modeled structure of Human PON2: Ca^{2+} involved in interactions with ligand is colored in wheat. Other Ca^{2+} ion is colored white. The N terminal region which is not similar to PON1 is colored blue. This image was generated using PyMol visualization tool.

The best refined model generated had a ground state energy value of -40123.82Kcal/mol. The Structural alignment of Human PON2 model to the template using Combinatorial Extension of Polypeptides(Shindyalov and Bourne 1998) exhibited 0.4 Å of RMSD of the backbone superimposition. This confirms that the folds shared are highly similar. The backbone conformation of the model generated was inspected using Phi/Psi Ramachandran plot obtained in PROCHECK server. Eighty- nine percent of the residues lie in the most favorable regions of the Ramachandran plot (100% in allowed region). Moreover, the ProQ prediction for the model also indicates significant predictive accuracy, wherein, the LGscore (Cristobal, Zemla et al. 2001) obtained was 5.486 (LGscore Range for excellent model >5). Docking simulation of 10 runs of GA- LA was performed for a set of 7 minimized ligands into PON2. The best docked conformation of each ligand with PON2 was determined as the one having the lowest binding free energy among the 10 different poses generated. The binding free energy and binding site residues of PON2 which are involved in hydrogen bond and hydrophobic interactions with the ligands used for the study are given in table 7.2 given in appendix

7.3.1: Homology modeling of PON2

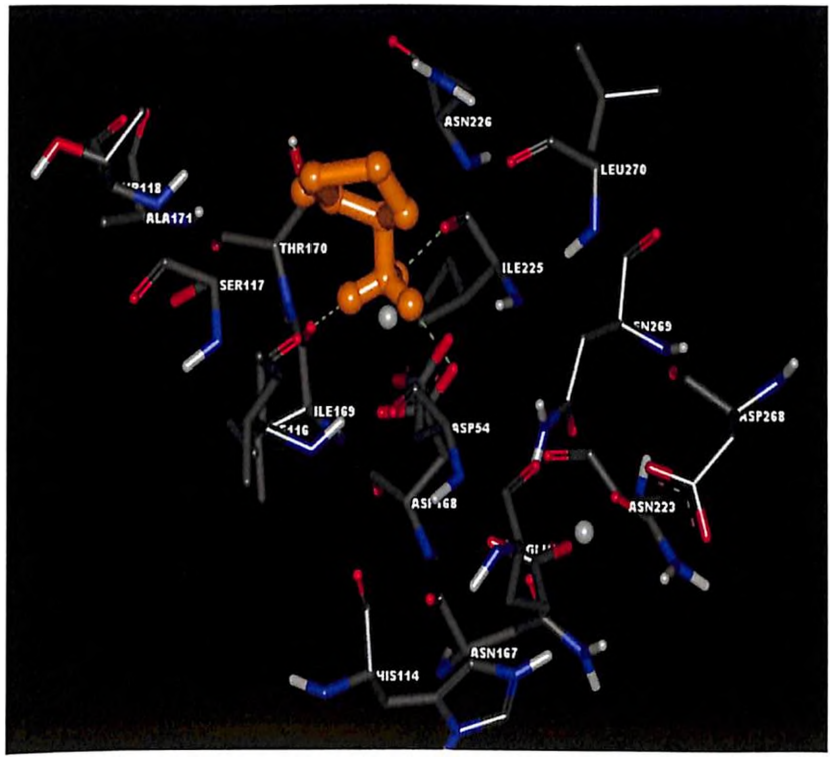
Owing to the high degree of similarity between recombinant variant PON 1 and human PON2, as expected, after homology modeling, the folds of its ground state model remained very similar to that of its template with an RMSD of 0.4 Å computed over the backbone atomic coordinates. Superposition of the 3D structures of the two isoforms reveals non-conserved residues at the N- terminal region. The analysis of human PON2 model revealed that, the six bladed β- propeller scaffolds with each blade containing four strands and the Velcro closure of N and C termini typical of PON1, was conserved in PON2 also. Two calcium ions, 7.3 Å apart, are seen in the central tunnel of the propeller, one at the top interacting with Asp 168, Asp 54 and Ile 116 residues of PON2 and one in central section interacting with Glu 53, Asp 268, Asn 269, Asn 167 and Asn 223 residues of PON2. (Fig 7.2)

The hydrogen bond donors, acceptors, bond length, van der Waals interaction residues, binding free energy and docking energy of each ligand with PON2 is given in Table 7.2

7.3.2: Docking Homocysteine thiolactone into PON2

After docking HCTL into PON2, hydrogen bond interactions between hydrogen atom of the hydroxyl group of Thr170, oxygen atom of carboxyl group of Ile169, oxygen atom of carboxyl group of Ile225 and oxygen atom of carboxyl group of Asp168 with oxygen and hydrogen atoms of HCTL were observed. The residues involved in van der Waals interaction were Glu53, Asp54, His114, Ile116, Ser117, Asn167, Asp168, Ile169, Ala171, Asn223, Asn226, Asp268, Asn269 and Leu270 and Ca^{2+} ion was found to be involved actively. The binding free energy and docking energy of the complex was observed to be -6.63 and -7.12 Kcal/mol, respectively which was found to be the lowest amongst all the ligands used in the study.

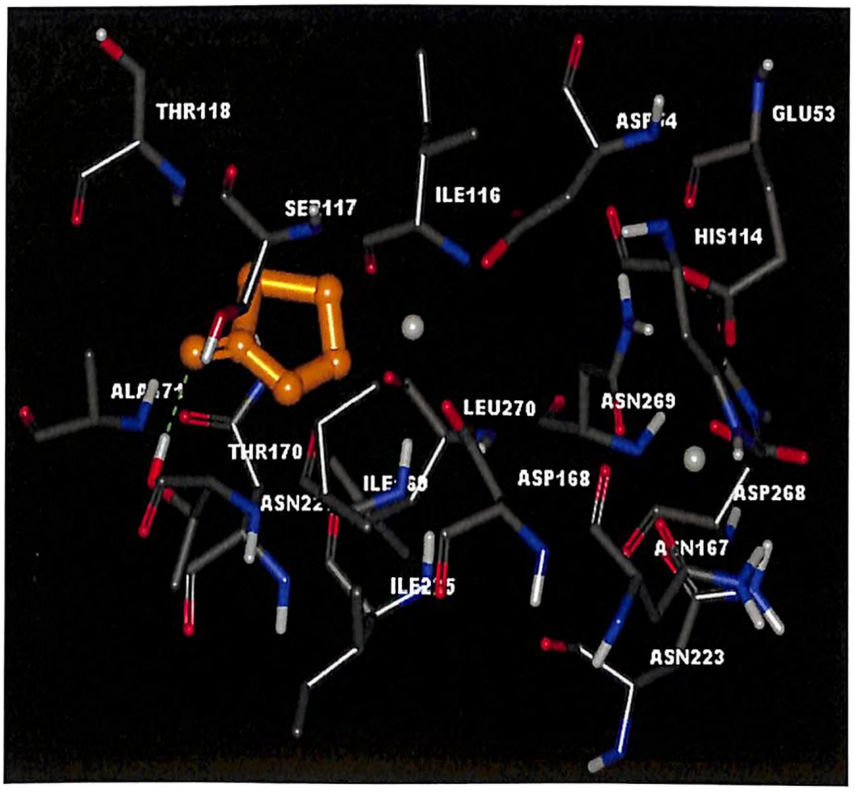
Figure 7.3: Illustration of docked complex for HCTL into PON2



7.3.3 Docking γ- thiobutyro lactone into PON2:

Docking simulation of GTBL into PON2 resulted in the formation of single hydrogen bond with the bond distance of 2.00437 Å and it was observed that hydrogen atom of hydroxyl group of Thr170 acts as hydrogen bond donor to interact with oxygen atom of GTBL. The residues taking part in van der Waals interactions are Glu53, Asp54, His114, Ile116, Ser117, Thr118, Asn167, Asp168, Ile169, Ala171, Asn223, Asn226, Asp268, Asn269 and Leu270. Also, a Ca^{2+} ion was found to be actively involved in the interaction. The binding free energy and docking energy of the complex was observed to be -3.69Kcal/mol each.

Figure 7.4: Illustration of docked complex for GTBL into PON2:

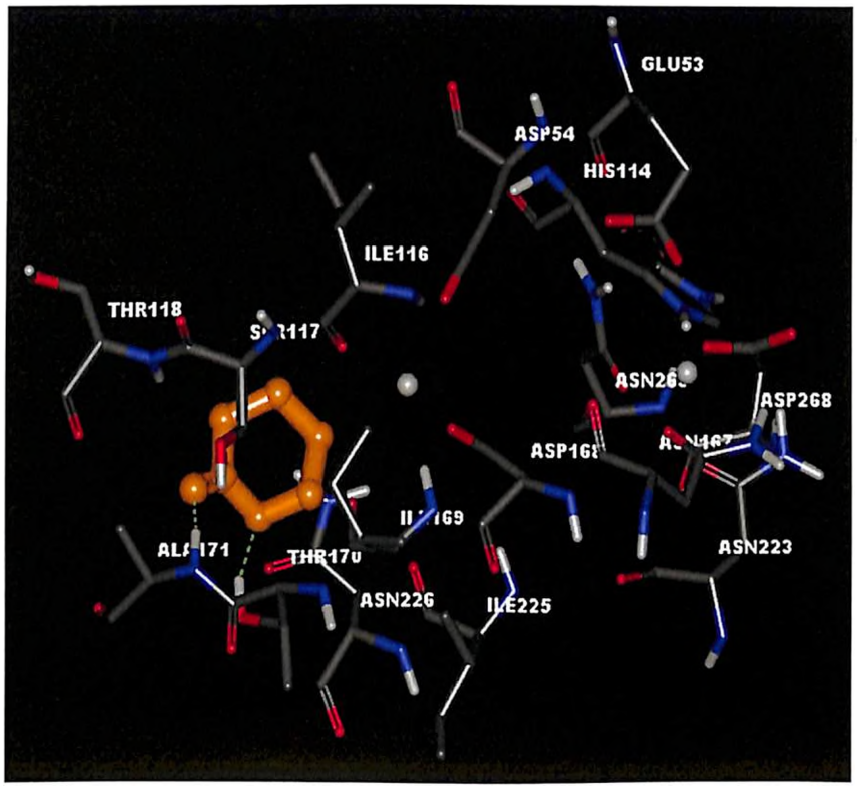


7.3.4: Docking Δ- valero lactone into PON2:

Upon assessing position and orientation of PON2- DVL docked complex, hydrogen atom of amine group of Ala171 interacts with an oxygen atom of DVL and hydrogen

atom of hydroxyl group of Thr170 of PON2 interacts with oxygen atom of DVL. The van der Waals interactions between PON2 and DVL were also observed in the residues Glu53, Asp54, His114, Ile116, Ser117, Thr118, Asn167, Asp168, Ile169, Asn223, Asn226, Asp268, Asn269 and Leu270and a Ca^{2+} ion was found to be actively involved in the interaction. Both, binding free energy and docking energy of the complex was observed to be -4.06Kcal/mol.

Figure 7.5: Illustration of docked complex for DVL into PON2:

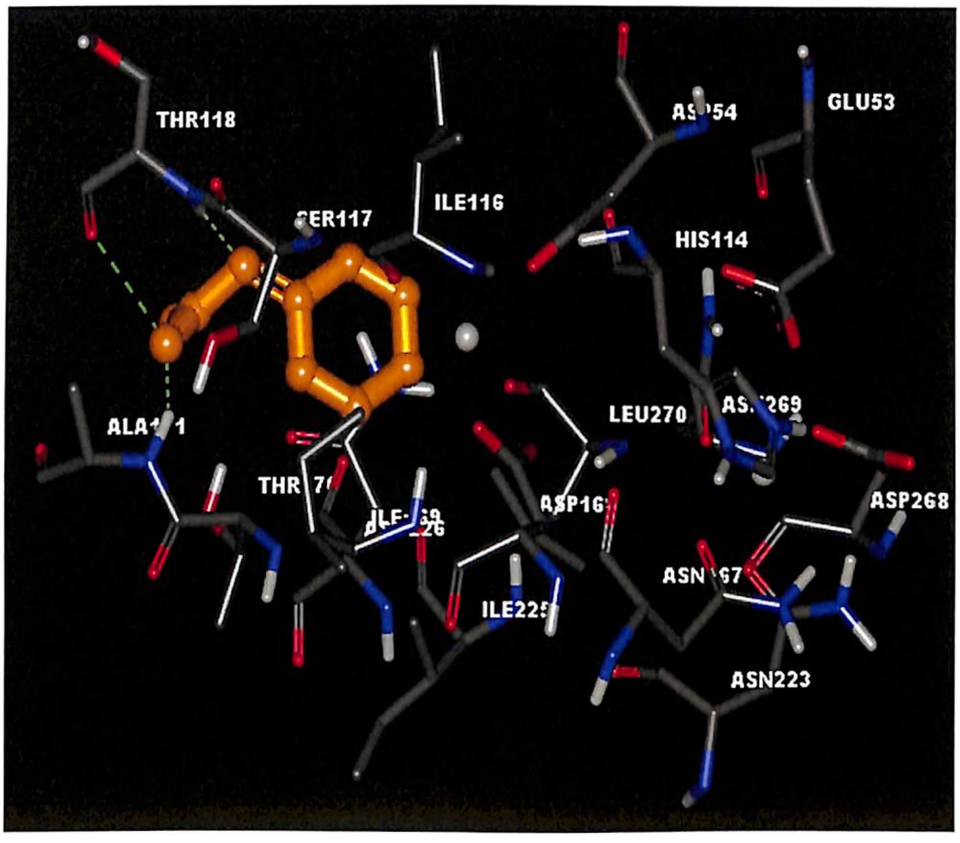


7.3.5 Docking phenyl acetate into PON2:

Phenyl acetate (PA), arylester substrate for PON2, is hydrolyzed by PON2. The binding modes of PA with PON2 were analyzed through docking studies. On examining the position and orientation of PA in PON2 predicted by our docking procedure, it was observed that hydrogen atom of the amine group of Thr118 and hydrogen atom of Ala171 acts as hydrogen bond donors to make hydrogen bond interaction with oxygen atoms of PA with bond length of 2.06227 Å and 1.94718 Å,

respectively. Also, the oxygen atom of Thr118 formed a covalent bond with oxygen atom of PA. In addition, the residues Glu53, Asp54, His114, Ile116, Ser117, Asn167, Asp 168, Ile169, Thr170, Asn223, Ile225, Asn226, Asp268, Asn269 and Leu270 were involved in van der Waals interactions. The Ca^{2+} ion at the top of the central tunnel has greater accessibility to the ligand binding site for making van der Waals contacts into PA. The binding free energy and docking energy calculated by AutoDock for PA was -4.73 and -5.38 KCal/mol, respectively.

Figure 7.6: Illustration of docked complex for PA into PON2:

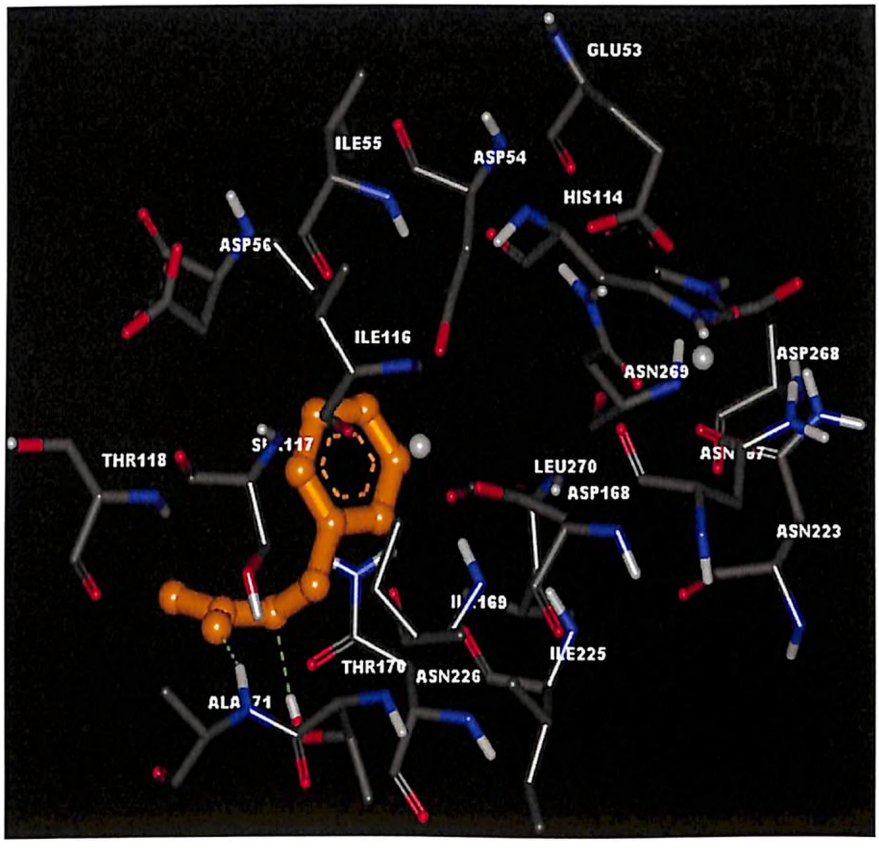


7.3.6 Docking benzyl acetate into PON2:

Docking simulation of BA into PON2 resulted in the formation of two hydrogen bonds and it was observed that hydrogen atom in the hydroxyl group of Thr170 and hydrogen atom of the amine group of Ala171 acts as hydrogen bond donor to interact with oxygen atom of BA. The amino acid residues Glu53, Asp54, Ile55, Asp56, His114, Ile116, Ser117, Thr118, Asn167, Asp 168, Ile169, Thr170, Asn223, Ile225,

Asn226, Asp268, Asn269 and Leu270 were involved in van der Waals interaction with active participation of Ca^{2+} ion. The binding free energy and docking energy of the complex was observed to be -5.21 and -6.16 Kcal/mol, respectively.

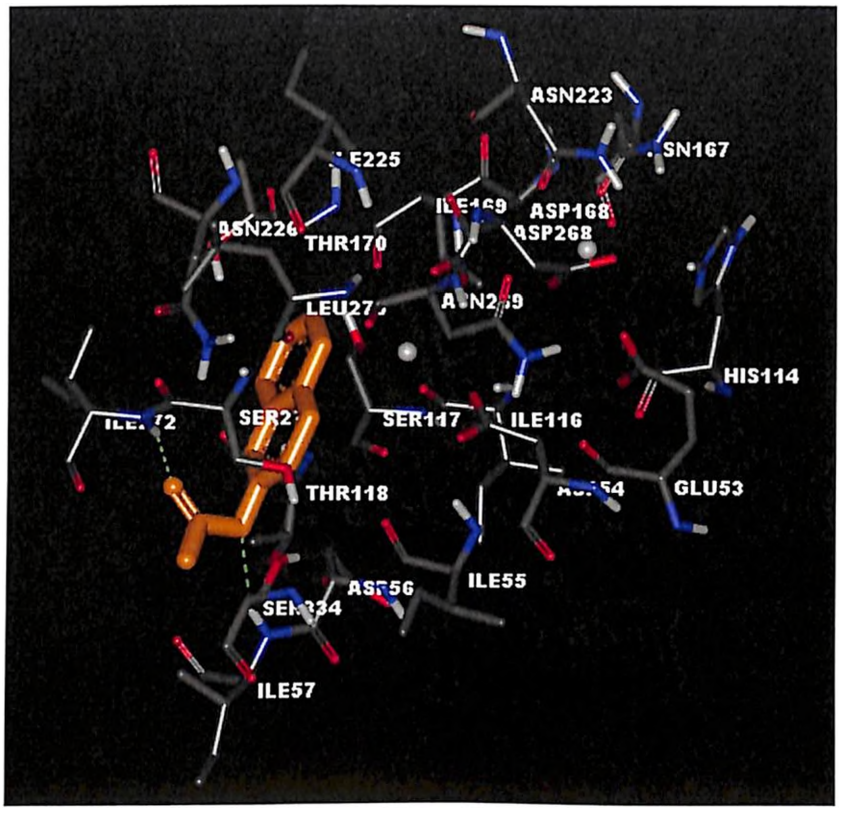
Figure 7.7: Illustration of docked complex for BA into PON2:



7.3.7 Docking 2- naphthyl acetate into PON2:

Docking of 2-naphthylacetate into PON2 resulted in the formation of two hydrogen bonds and it was observed that hydrogen atom of amine group of Ile57 and hydrogen atom of amine group of Ile272 were the hydrogen bond donors to oxygen atoms of 2-NA and residues involved in van der Waals interaction were Glu53, Asp54, Ile55, Asp56, His114, Ile116, Ser117, Thr118, Asn167, Asp 168, Ile169, Thr170, Asn223, Ile225, Asn226, Asp268, Asn269, Leu270, Ser271 and Ser334 and Ca^{2+} ion was found to be involved actively. The binding free energy and docking energy of the complex was observed to be -6.34 and -7.08 Kcal/mol, respectively.

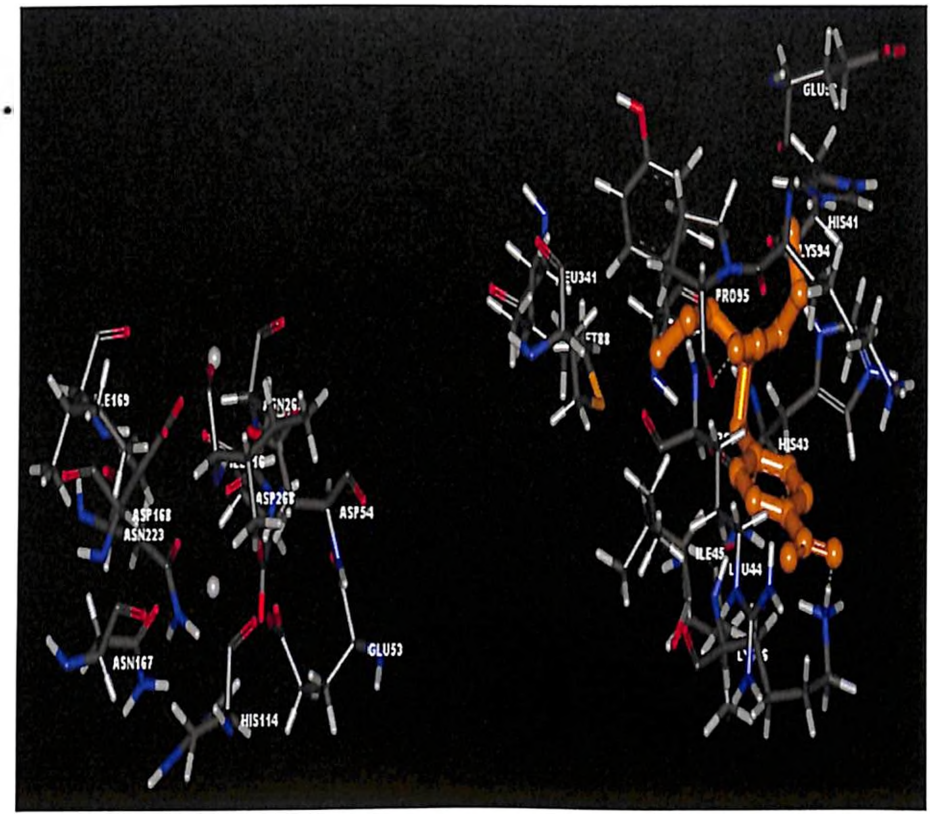
Figure 7.8: Illustration of docked complex for 2- NA into PON2:



7.3.8 Docking paraoxon into PON2:

The binding of paraoxon into PON2 resulted in the formation of hydrogen bonds and it was observed that hydrogen atom of amine group of Lys46 acts as hydrogen bond donor and oxygen atom of Pro95 acts as hydrogen bond acceptor. Van der Waals interaction with PAR was formed by His43, Leu44, Ile45, Met88, Glu93, Lys94, Arg96, Leu341 and Tyr351 residues of PON2. The binding free energy and docking energy of the complex was observed to be -5.56 and -7.95 Kcal/mol, respectively. The binding mode of PAR to PON2 was different from the other substrates. PAR exhibited binding, in a region outlying from the substrate binding site which was in coherence with the already documented experimental report wherein, PON2 was also shown to exhibit negligible paraoxonase activity (Draganov and La Du 2004).

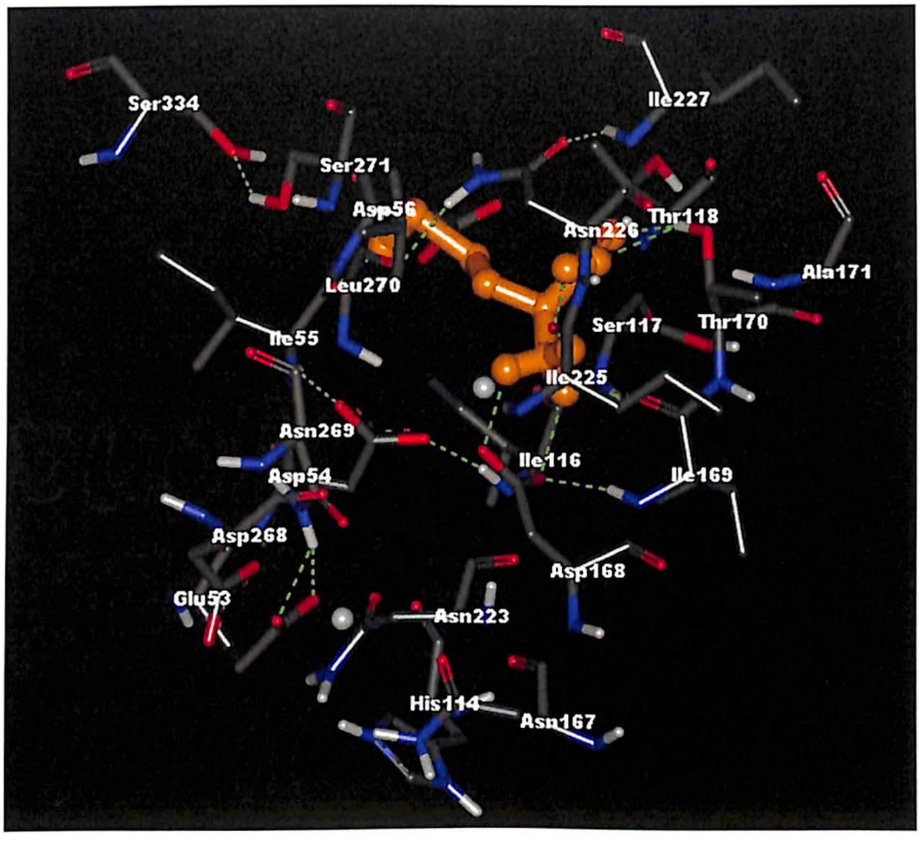
Figure 7.9: Illustration of docked complex for PAR into PON2:



7.3.9 Docking Homocysteine into PON2:

Upon assessing position and orientation of PON2- Hcys docked complex, hydrogen atom of hydroxyl group of Thr170 in PON2 interacts with an oxygen atom of Hcys and oxygen atom of Asp168, Ile169, Ile55 and Ile225 of PON2 interacts with hydrogen atoms of Hcys. The van der Waals interactions between PON2 and Hcys were also observed in the residues Glu53, Asp54, Ile116, Asn226, Tyr235, Ile227, Asn269, Leu270, Ser271, Ala333, Ser268, His284, Ser334 and a Ca^{2+} ion of PON2 were found to be actively involved in the interaction. Both, binding free energy and docking energy of the complex was observed to be -5.08Kcal/mol and -7.64Kcal/mol.

Figure 7.10: Illustration of docked complex for HCYS into PON2:



7.3.10 Comparing PON 1 and PON2:

The residues Glu 53, His 114, Asn168, Asn 223, Asp 270 and Asn269 exhibit hydrogen bonding interaction for both lactonase and aryl esterase activity in PON1,(Hu, Jiang et al. 2009) including the natural substrate HCTL, whereas, in PON2 the same residues Glu 53, His 115, Asn167, Asn 224, Asp 269 and Asn270 were found to have hydrophobic interactions for the same activity. The paraoxon binding site was found to overlap with the lactonase and aryl esterase binding sites of PON1, but similar interaction was not observed in PON2 and it was found to bind to a region away from the central Ca^{2+} ion, at residues His43, Leu44, Ile45, Lys46, Met88, Glu93, Lys94, Pro95, Arg96, Leu341 and Tyr351 forming the hydrogen bonding and hydrophobic interactions. It was observed that the PON2 had lowest binding energy

and highest affinity for the physiological substrate HCTL (-6.63Kcal/mol) than PON1 which showed a binding energy of (-5.72Kcal/mol).

The docked structures of PON2 exhibit relatively a large number of amino acid residues involved in ligand interactions and the involvement of one of the Ca^{2+} ions were observed in each PON2- ligand interaction except paraoxon. This shows that the calcium present in the central section of the central tunnel has catalytic activity. As shown in the table7.2, it is obvious that out of the 7 ligands tested, six ligands share the same binding modes in PON2 enzyme whereas; paraoxon exhibits a completely different binding mode. In addition to the known ligands, this study also gives the molecular interaction for the physiological substrate namely, HCTL which is formed in all cell types when there is excess homocysteine, as a result of error editing met-tRNA synthetase(Jakubowski, Zhang et al. 2000)

CHAPTER 8: BENEFICIAL EFFECT OF AMINO ACID IN MITIGATING THE ANGIOGENIC EFFECT OF ADVANCED GLYCATION END PRODUCTS

8.1: INTRODUCTION

8.1.1 Regulation of angiogenesis

Angiogenesis occurs in the healthy body as a part of wound healing process in order to restore the blood flow to tissues after injury or insult. In females, angiogenesis also occurs during the monthly reproductive cycle (to rebuild the uterus lining, to mature the egg during ovulation) and during pregnancy (to build the placenta, the circulation between mother and fetus). Angiogenesis is the continued expansion of the vascular tree as a result of EC sprouting from existing vessels, occurs in vascular regions of the embryo. It is an important natural process occurring in the body, both in health and in disease which is regulated by factors inducing and inhbiting angiogenesis. When angiogenic growth factors are produced in excess of angiogenesis inhibitors, the balance is tipped in favour of blood vessel growth and vice versa.

8.1.2 Mechanism of angiogenesis

The angiogenic growth factors bind to specific receptors located on the EC. Once growth factors bind to their receptors, the ECs become activated and start secreting enzymes. These Enzymes (matrix metalloproteinase's or MMP) dissolve the basement membrane and pave way for the EC proliferation and migration. Adhesion molecules, especially integrins (avb3, avb5) help in the migration of EC. As the vessel extend, the tissue is remolded around the vessel. The sprouting ECs roll up to form a blood vessel tube, individual blood vessel tubes connect to form blood vessel loops that can circulate blood. Finally, newly formed blood vessel tubes are stabilized by specialized muscle cells (smooth muscle cells, pericytes) that provide structural support(Risau 1997; Gale and Yancopoulos 1999).

8.1.3 Factors promoting angiogenesis and inhibiting angiogenesis

Angiogenic mediators can be broadly divided into two groups: Promoters (Table 8.1) and inhibitors (Table 8.2) (Polverini and Leibovich 1984; Folkman and Klagsbrun 1987; Polverini 1989; Klagsbrun and D'Amore 1991; Moses and Langer 1991; DiPietro and Polverini 1993) The majority of the stimulatory molecules are proteins, and many of them are growth factors that induce ECs to divide, migrate directionally toward the inducing stimulus, and differentiate into tubular structures. Most are secreted by a variety of cells, including ECs themselves, in response to exogenous or endogenous stimuli and are produced locally and function in an autocrine and/or paracrine manner. These mediators can stimulate angiogenesis directly by interacting with receptors on the EC surface, or indirectly by attracting and activating accessory cells, *i.e.,* inflammatory macrophages, and inducing them to produce angiogenic mediators (Polverini and Leibovich 1984). Elements like copper, function as co-factors in key interstitial enzyme systems or, in the case of plasminogen activator, can activate latent enzymes such as transforming growth factor to reveal its angiogenic (or angiostatic) activity (Roberts and Sporn 1989).

Table 8.1: Promoters of Angiogenesis

S.No	Factors
1	Angiogenin
2	Angiopoietin-1
3	Del-1
4	Fibroblast growth factors: acidic (aFGF) and basic (bFGF)
5	Follistatin
6	Granulocyte colony-stimulating factor (G-CSF)
7	Hepatocyte growth factor (HGF) /scatter factor (SF)
8	Interleukin-8 (IL-8)
9	Leptin
10	Midkine
11	Placental growth factor
12	Platelet-derived EC growth factor (PD-ECGF)
13	Platelet-derived growth factor-BB (PDGF-BB)
14	Pleiotrophin (PTN)
15	Progranulin
16	Proliferin
17	Transforming growth factor-alpha (TGF-alpha)
18	Transforming growth factor-beta (TGF-beta)
19	Tumor necrosis factor-alpha (TNF-alpha)
20	Vascular endothelial growth factor (VEGF)/ vascular permeability factor (VPF)

A common property of these inhibitors is that almost all of them can influence the ability of cells to produce, interact with, or degrade their extracellular matrix(Canfield, Schor et al. 1986; Ingber and Folkman 1988; Madri, Pratt et al. 1988; Maragoudakis, Sarmonika et al. 1988; Ingber and Folkman 1989). Alterations in the organization and composition of the extracellular matrix have been shown to have a profound effect on the growth and function of ECs and in determining whether ECs will differentiate and organize into a three-dimensional capillary network(Maragoudakis, Sarmonika et al. 1988).

Table 8.2: Inhibitors of angiogenesis:

S.No.	Factors
1	Angioarrestin
2	Angiostatin (plasminogen fragment)
3	Antiangiogenic antithrombin III
4	Cartilage-derived inhibitor (CDI)
5	CD59 complement fragment
6	Endostatin (collagen XVIII fragment)
7	Fibronectin fragment
8	Gro-beta
9	Heparinases
10	Heparin hexasaccharide fragment
11	Human chorionic gonadotropin (hCG)
12	Interferon alpha/beta/gamma
13	Interferon inducible protein (IP-10)
14	Interleukin-12
15	Kringle 5 (plasminogen fragment)
16	Metalloproteinase inhibitors (TIMPs)
17	2-Methoxyestradiol
18	Placental ribonuclease inhibitor
19	Plasminogen activator inhibitor
20	Platelet factor-4 (PF4)
21	Prolactin 16kD fragment
22	Proliferin-related protein (PRP)
23	Retinoids
24	Tetrahydrocortisol-S
25	Thrombospondin-1 (TSP-1)
26	Transforming growth factor-beta (TGF-β)
27	Vasculostatin
28	Vasostatin (calreticulin fragment)

Either insufficient vascularization or excessive vessel formation can contribute to disease pathogenesis.

8.1.4 AGE and RAGE: Angiogenesis can also be addressed at the level of AGE-RAGE interaction that can elicit VEGF elaboration. Previous studies have identified several downstream signaling pathways responsive to RAGE ligation (Stern, Yan et al. 2002; Huttunen and Rauvala 2004; Wautier and Schmidt 2004).The engagement of RAGE by AGE has been reported to induce cellular oxidant stress, activating the transcription factor nuclear factor-αB (NFkB) resulting in the perturbation of a variety of homeostatic functions of the vasculature. RAGE-mediated NFkB activation depends on activation of mitogen-activated protein kinase (MAPK) involving the small GTPase, Ras and extracellular signal-regulated kinase 1 and 2 (ERK1/2) leading to angiogenesis (Yonekura, Yamamoto et al. 2005). The engagement of RAGE by AGE induces expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human EC (Schmidt AM, et al 1995). RAGE has been also reported to be involved in leukocyte adhesion to EC (Chavakis, Bierhaus et al. 2003) and monocyte transendothelial migration (Rouhiainen, Kuja-Panula et al. 2004) Those activities may induce immune/inflammatory responses, leading to aggravation of diabetic vascular complications.

Proliferative diabetic retinopathy is a major complication of diabetes characteristically showing growth of new blood vessels in the retina. The initial loss of capillaries in the retina trigger the hypoxia induced elaboration of growth factors that promote the angiogenesis. Though photo laser coagulation has been widely used successfully, many pharmacological agents are in clinical trial for the treatment of Diabetic retinopathy (Furlani, Meyer et al. 2007).At present the Diabetic Retinopathy Clinical Research (DRCR) Network has 200 participating sites and is evaluating monotherapies and combination treatments for both DME(diabetic macular edema) and PDR (proliferative diabetic retinopthy) (Schwartz, Flynn et al. 2009). VEGF (vascular endothelial growth factor) and its receptors play pivotal roles in the cascade of events such as proliferation, survival and migration of ECs that are associated with physiological and pathological angiogenesis (Olsson, Dimberg et al. 2006) Our previous study has shown that supplementation of the amino acid mixture for the Type 2 Diabetes Mellitus patients had an increased sensitivity for the insulin signaling and

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also decreased the levels of the glycosylated haemoglobin (Natarajan Sulochana, Lakshmi et al. 2002). Free lysine, Alanine, Aspartic acid, or Glutamic acid have been shown to reduce the glycation of human lens proteins, *in vitro* (Ramakrishnan and Sulochana 1993; Ramakrishnan, Sulochana et al. 1996; Ramakrishnan, Sulochana et al. 1997). Therefore the objective was to study the effect of amino acids on angiogenesis in ECs, pericytes and retinal pigment epithelial cells. Five amino acids namely glycine (simple), glutamic acid (acidic), leucine (branched chain), lysine (basic) and cysteine (sulphur containing) were used.

8.2: MATERIALS AND METHOD

8.2.1*In vitro* experiments in BREC

The primary BREC were cultured from the retina and characterized as ECs using Factor VIII and VE–Cadherin. The cells were exposed to AGE (100 µg/ml) for 24 hrs with and with out amino acids namely glycine, glutamic acid, leucine,lysine and cysteine at varying concentration (0.5, 1 and 2.5mM)

8.2.2 Ca^{2+} imaging The VEGF induced calcium fluorescence was recorded as per the method mentioned in materials and method.

8.2.3 Transendothelial resistance measurement (TER)

The cells were grown in electrode containing chamber slides. To each well added 380 µl basal serum free media was added and observed for 1 hour, to it added 20 µl of media containing 50ng/ml VEGF was added and observed for 2-4hrs. The change in the resistance was recorded.

8.2.4 Test for cytotoxicity

MTT assay was done to test for the cytotoxic effect of the amino acids Glycine, Lysine , Glutamic acid, Leucine and Cysteine on the BREC cells . Briefly the cells were grown in 96 well plate and exposed to 5 mM concentration of the amino acids at varying time points (3,6&12hrs) in DMEM/F12 media. The formazan crystals formed after treatment with MTT was dissolved in DMSO and read at 570 nm in ELISA reader.

8.2.5 Matrigel assay (tube formation assay)

The cells were grown in 30mM glucose for 3days and used as the positive control. Tube formation of BREC cells was stimulated by exposing them to AGE for 24hrs. For the matrigel assay the growth factor reduced ECM was used (ECM625 Chemicon kit). The ECM was coated on a 96 well plate, 50µl per well and allowed to solidify at 37°C. The BREC cells were then plated on the matrix along with AGE and with and without the amino acids(5mM initially and then with lowered concentrations of the amino acids glycine and glutamic acid) and assessed for their inhibition of tube formation. The assay was quantitated by screening 5 different areas in a plate and grading the tube formation with the following guidelines.

Pattern	Value
Individual cells, well separated	0
Cells begin to migrate and align themselves	1
Capillary tubes visible. No sprouting	2
Sprouting of new capillary tubes visible	3
Closed polygons begin to form	4
Complex mesh like structures develop	5

The experiment was repeated twice and the mean and standard deviation obtained was plotted as a graph.

8.2.6 Migration assay

For the migration assay bFGF was used as the positive control. The BREC cells were grown to confluence in gelatin coated 6 well plates in DMEM/F12 media. An wound was created with a sterile pipette tip in the middle of the confluent layer of the cells and then the cells were treated with AGE and the inhibition of migration was tested with the amino acids.

8.2.7 Adhesion assay

For the adhesion assay the lymphocytes were isolated from the human blood using the histopaque gradient. The retinal ECs were plated in 6 well plates coated with gelatin and allowed to grow to confluence. The cells were exposed to 100 µg/ml of AGE for

24 hrs with and without amino acids. To the treated cells 1500 lymphocytes/well lymphocytes were added and incubated for 4 hrs, the excess which were not adhered was removed by PBS wash. The number of cells adhered was counted in four different fields.

8.2.8: Fluorescent labeling of VEGF in BREC, BRP and BRPE

The induction of the growth factor VEGF (Vascular endothelial growth factor) in the BREC cells was done by exposing them to AGE (100μg/ml) for 24hours. Briefly the BREC cells were grown in cover slips in a 24 well plate and exposed to AGE (100μg/ml) for 24 h with and without amino aicd. The cells were fixed in 100% methanol and stained for the VEGF protein with primary antibody raised in rabbit and FITC conjugated secondary antibodies (Santa Cruz). The cells were captured under the zeiss fluorescent microscope.

8.2.9: Expression of VEGF in BREC, BRP and BRPE cells by RT-PCR

RNA was extracted (sigma Genelute mammalian total RNA mini prep kit) according to the manufacturer's instructions. RT-PCR (reverse transcription polymerase chain reaction) was performed as follows, 1μg of RNA was treated with Dnase I (Invitrogen), Reverse transcription was carried out using random hexamer (Thermoscript, Invitrogen) using the manufacturers protocol. The PCR was carried out using the following primers. For this assay 0.5 mM and 1 mM concentration of the amino acids glycine and glutamic acid were used.

GAPDH	Forward primer: 5'-TGTTCCAGTATGATTCCACCC-3' Reverse primer: 5'-GTCTTCTGGGTGGCAGTGAT-3'
VEGF	Forward primer: 5'-CGAAACCATGAACTTTCTGC-3' Reverse primer: 5'-CCTCAGTGGGCACACACTCC-3'

All the experiments were done in duplicates.

8.2.10 RAGE immunoflourecence in BREC, BRP and BRPE

Briefly the BREC cells were grown in cover slips in a 24 well plate and exposed to AGE (100μg/ml) for 24hrs with and without aminoacid. The cells were fixed in 100% methanol and stained for receptor for advanced glycation end product (RAGE Santa cruz) protein with primary antibody raised in goat and FITC conjugated secondary antibodies (Santa Cruz). The cells were captured under the zeiss fluorescent microscope.

8.2.11: Actin stress fibers by IF in BREC, BRP and BRPE

The phalloidin was conjugated with FITC in an acidic environment and this conjugate stains the active actin. The phalloidin conjugate was used to stain all the three cells by incubating them for 2-4 h with it. The green fluorescence of the cells represents the active actin. The cells were counter stained with DAPI for nuclear stain.

8.2.12 : Statistics

Results are expressed as means ± SD unless otherwise indicated. Statistical analysis used the unpaired Student's *t test* to compare quantitative data populations with normal distributions and equal variance. A p value of <0.05 was considered statistically significant.

8.3 : RESULTS

8.3.1: Ca $^{2+}$ imaging In BREC cells

The VEGF increased the calcium release from the BREC about 2 folds from the basal level. Figure 8.1. This indicates the VEGF can induce permeability changes in the cell.

Figure 8.1: VEGF induced increase in intracellular calcium in BREC

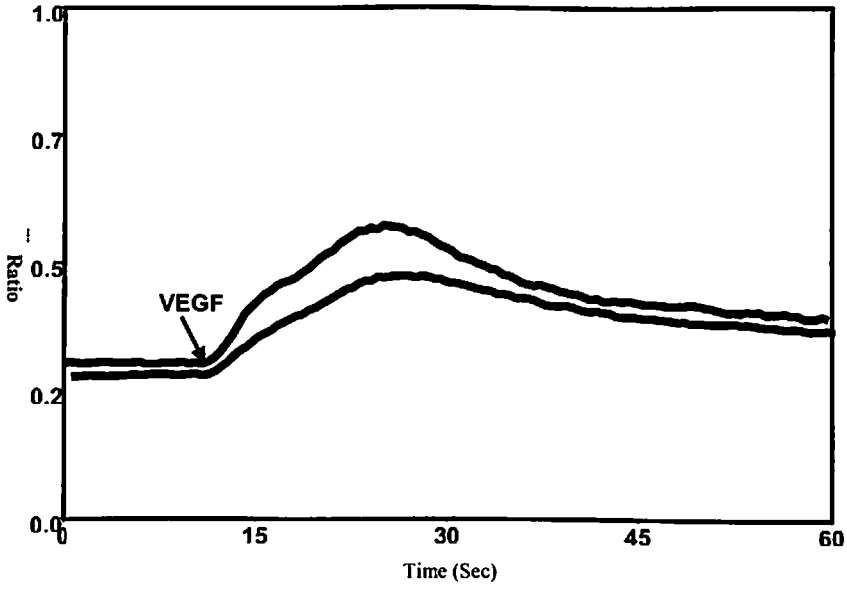


Figure 8.1 : The figures shows the increase in calcium at the point of addition of VEGF indicated by the arrow.

8.3.2: Transendothelial resistance measurement in BREC after induction of VEGF:

To asses the permeability changes confluent BREC cultures were incubated in the presence and absence of VEGF and the transendothelial ressistance was measured upto 3 hrs. There was a decrease in the TER from 30 min which decreased with increasing concentration of VEGF Figure 8.2.

Figure 8.2: VEGF induced Transendothelial resistance changes in BREC

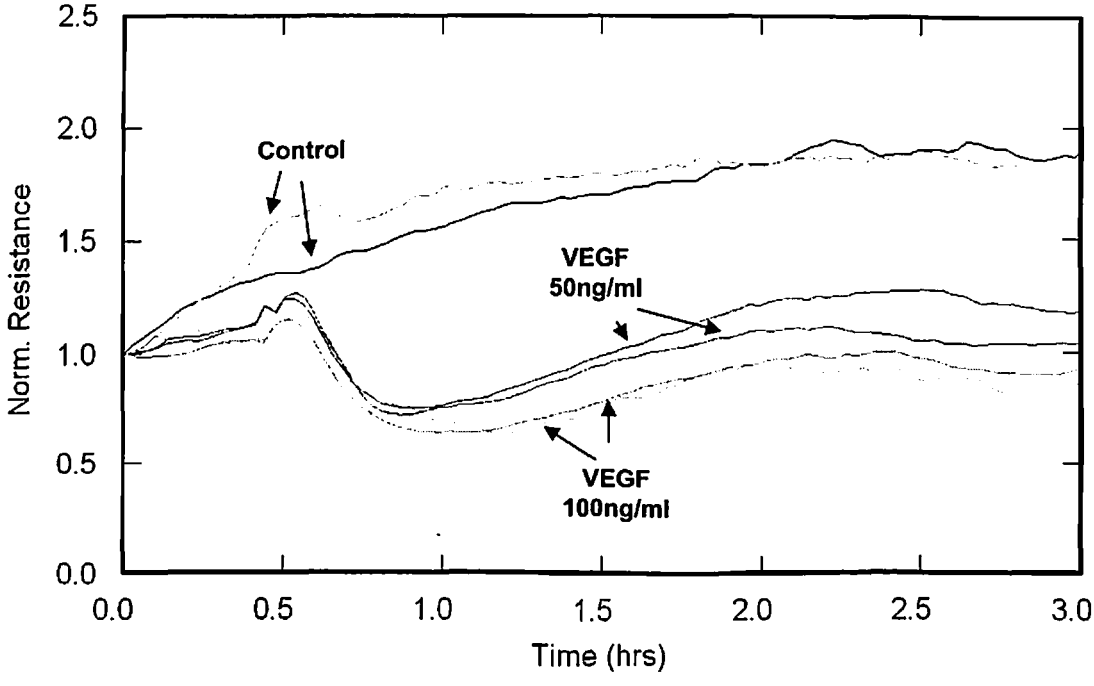
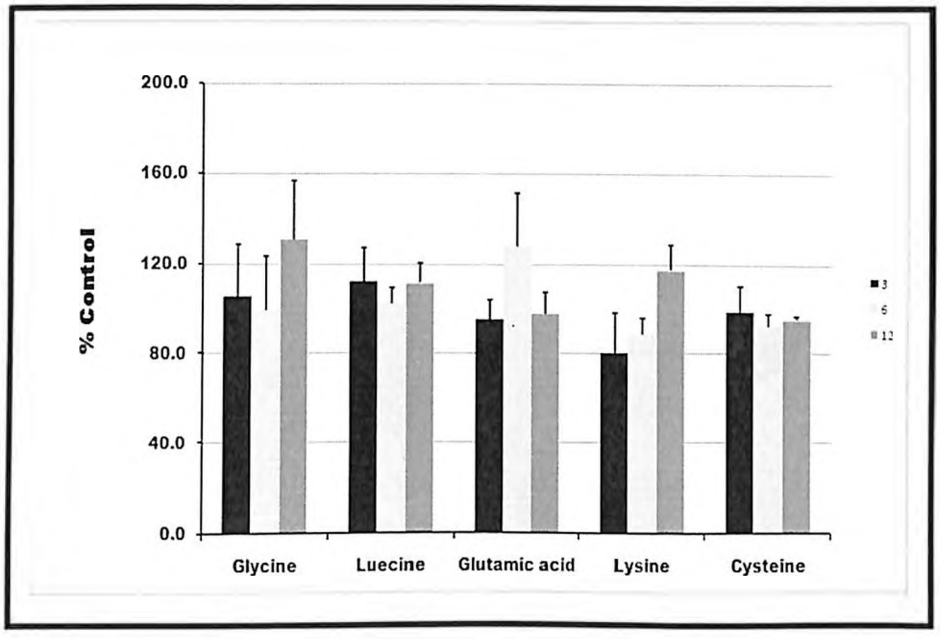


Figure 8.2: The figure shows two controls without addition of VEGF, two with 50ng VEGF and two with 100ngVEGF.There is a dose dependent decrease in the TER with increasing concentrations of VEGF.

8.3.3: The cytotoxic effect of High glucose and amino acids in BREC

5mM concentration of all the amino acids was added to BREC cells to test for the cytotoxixcity. It was found that none of them had any cytotoxic effect at this concentration as seen at the end of 24 h (figure 8.3).

Figure 8.3: The cytotoxic effect of amino acids on the BREC



8.3.4: Tube formation assay

The effect of amino acid on the tube formation assay was tested in the presence of 30mM glucose and it was found that Leucine, cysteine and lysine were not effective but, glycine and glutamic acid were anti angiogenic at 5 mM concentration. (Figure 8.4). The cells were grown in 30mM glucose for 3 days and used as positive control for the matrigel assay.

Figure 8.4: Matrigel assay of amino acids with 30mM glucose.

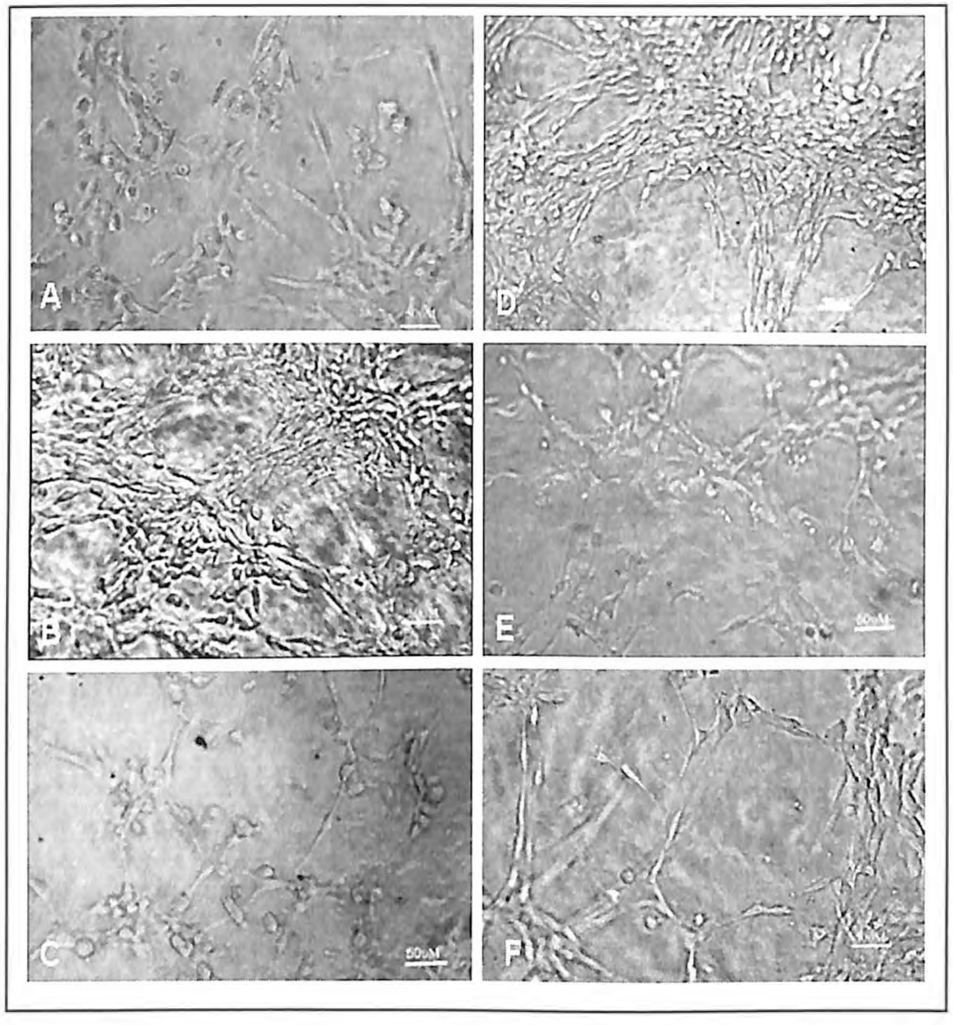
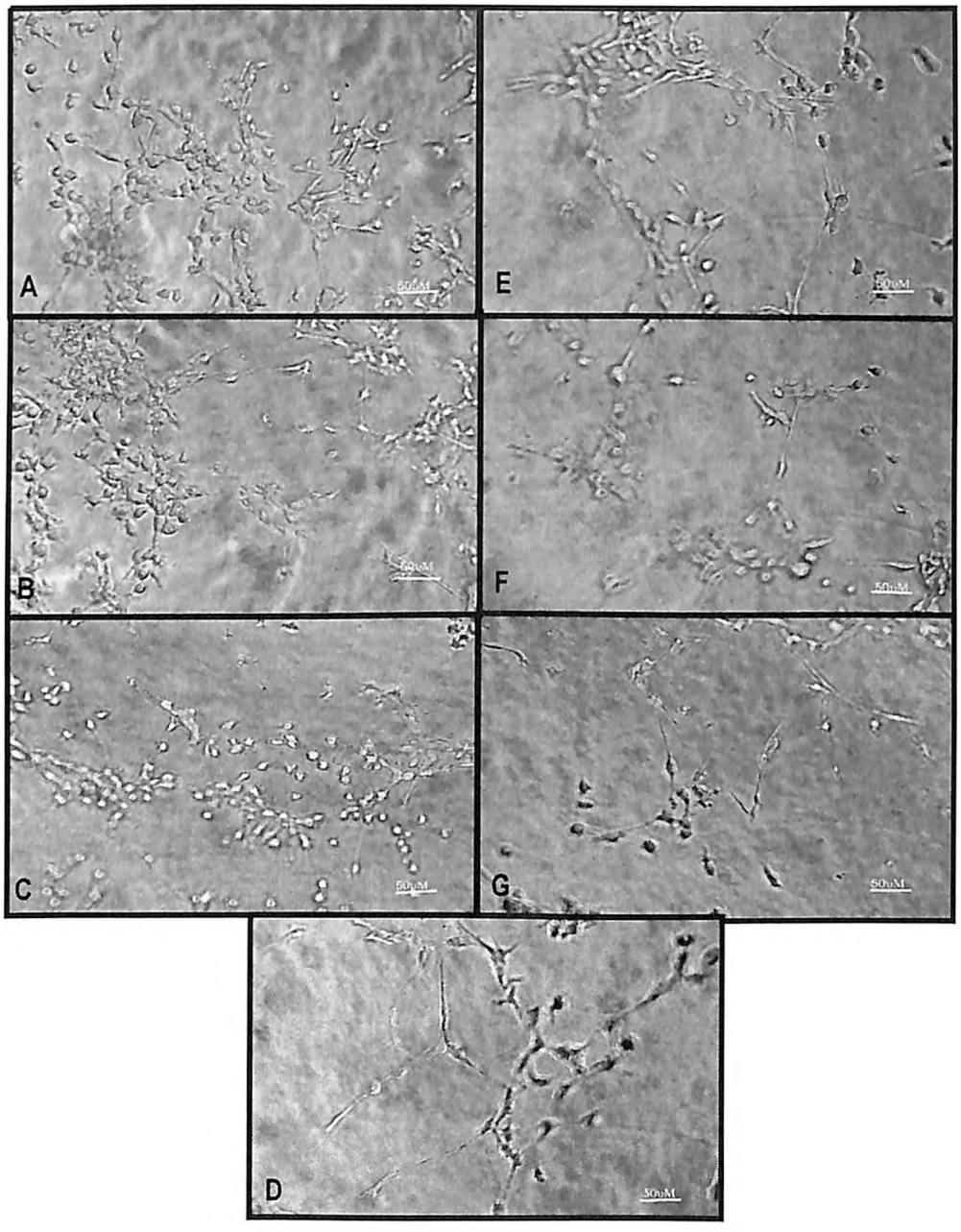


Figure 8.4 A: 30mM glucose shows tube formation positive control, B: 5mM Luecine shows mesh formation, C: 5mM glutamic acid shows reduce number of tubes, D: 5mM Lysine shows mesh formation E: 5mM cysteine shows mesh formation of the tubes, F: Glycine shows reduced number of tube formation.

Lower concentrations of the amino acids glycine (0.5 mM,1mM & 2.5 mM) and glutamic acid (0.25,0.5 mM, 1 mM & 2.5 mM) were tested for the same effect and it was found that these amino acids even at a lower concentration had an dose dependent effect in the tube formation assay(Figure 8.5) showing an inhibition of 47, 58 and 76% for glycine and 52, 64, 64 & 70% for glutamic acid with 30mM glucose.

Figure 8.5: Tube formation assay after treatment with various concentrations of glycine and glutamic acid in the presence of 30mM glucose



A-D : Varying concentration of glutamic acid (0.25 mM, 0.5mM,1mM & 2.5mM)
E- G: Varying concentration of glycine (0.5mM, 1mM & 2.5mM). In the presence of 30 mM glucose.

The number of tubes tube formed were counted for each experiment in 5 different fields and compared with the tube formation with 30mM glucose. The number of tubes formed was significantly decreased in the amino acid treated cells. (Figure 8.6)

Figure 8.6: Bar diagram showing the dose dependent inhibition of tube formation by amino acids glycine and glutamic acid in the presence of 30mM glucose.

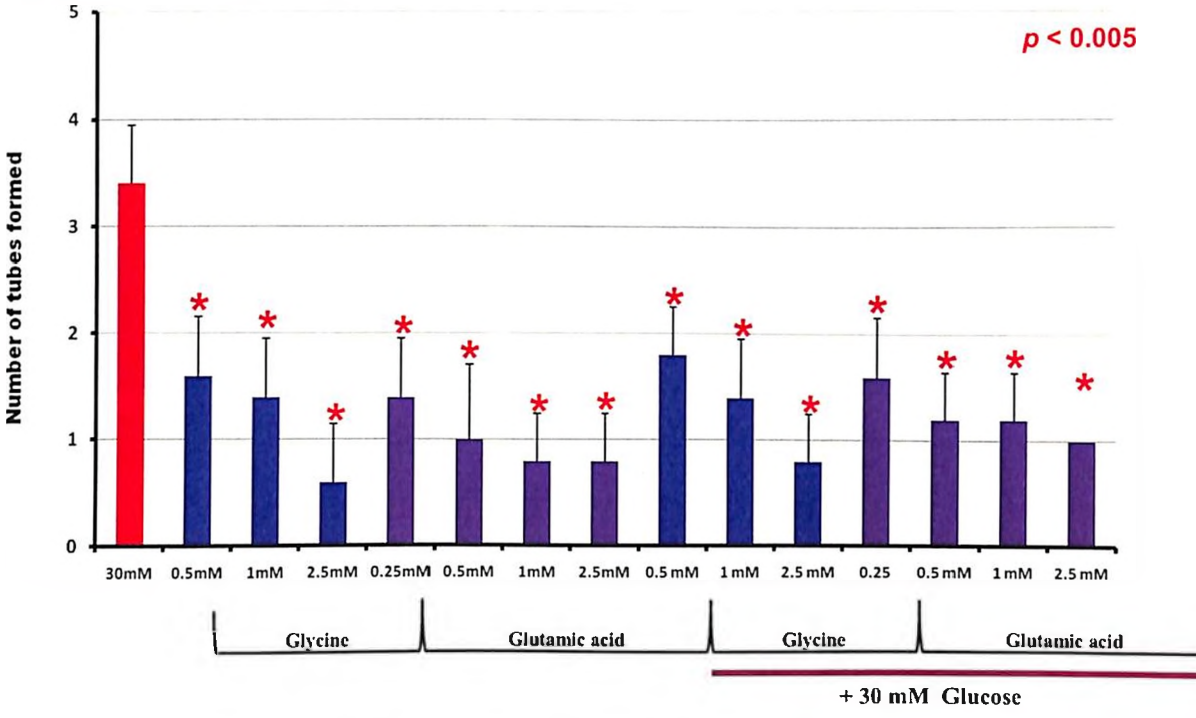
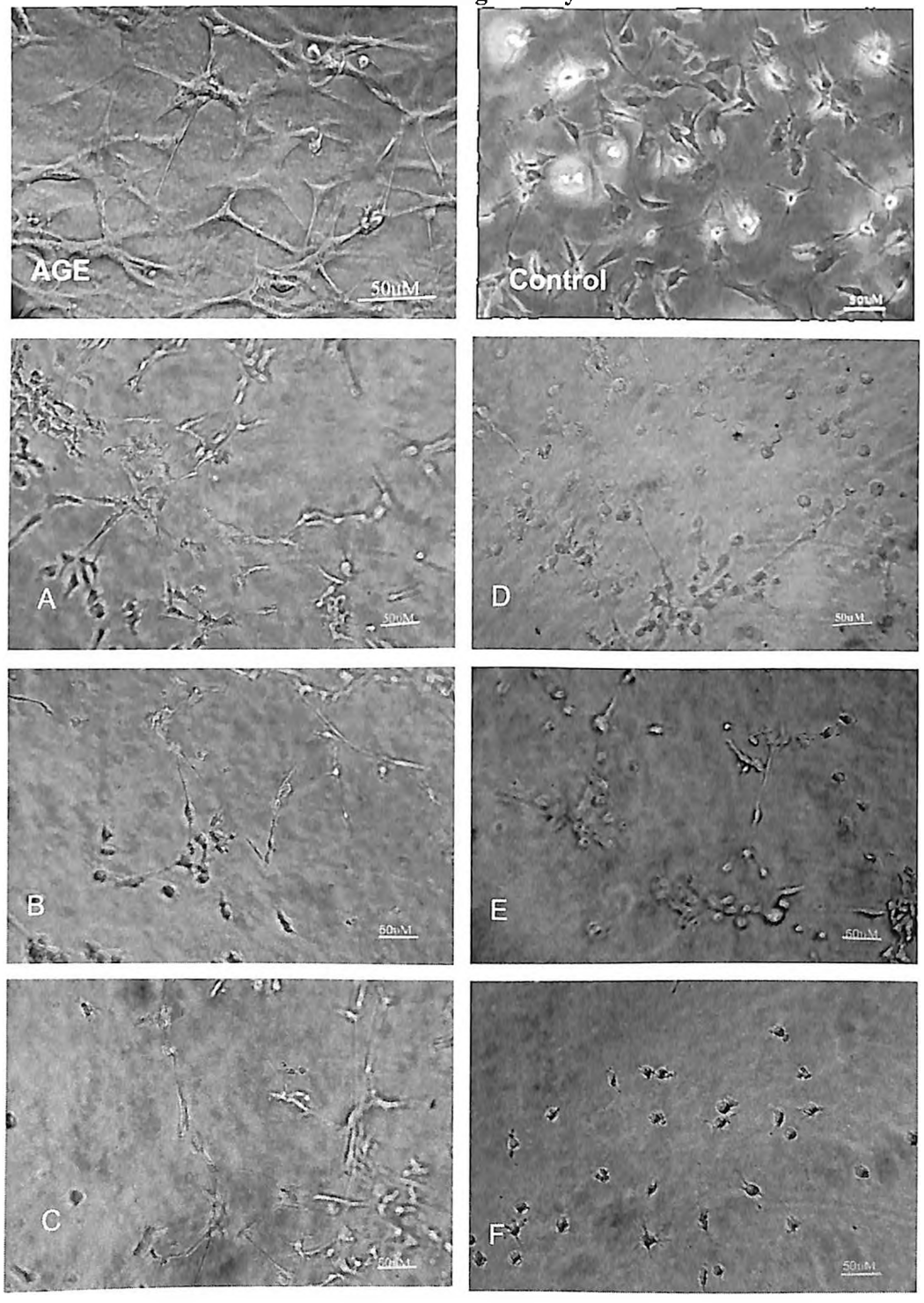


Figure 8.6: 30mM glucose is given in red, glycine is given in blue and the glutamic acid is given in violet. The red star indicate significance $p<0.005$

To test if the amino acids could inhibit the effect of AGE: Tube formation was done by exposing the cells to 100 µg/ml AGE for 24 hrs with and without amino acids and the number of tubes formed were counted and recorded. The number of tubes formed in AGE and the amino acid treated were counted in 5 different regions and compared. The number of tubes formed in the amino acid treated was significantly lesser than the AGE treated. Figure (8.7, 8.8)

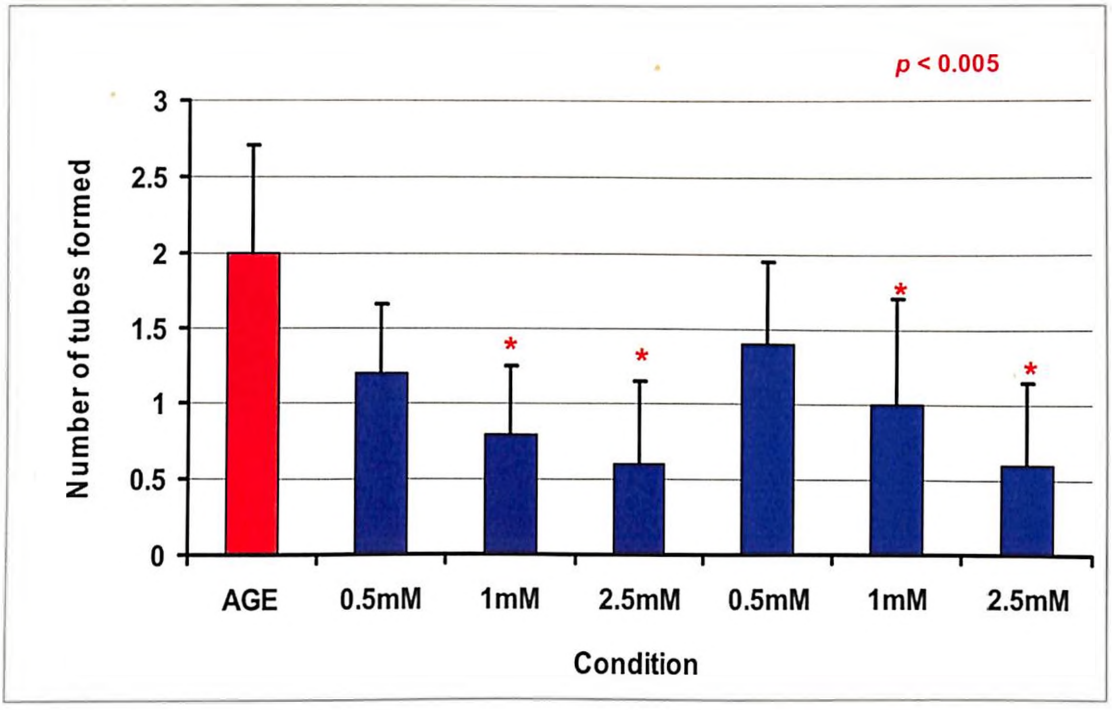
Figure 8.7: The effect of amino acids on the AGE induced tube formation – matrigel assay



A-C: Varying concentration of glutamic acid (0.5 mM, 1 mM & 2.5 mM)

D-F: Varying concentration of glycine (0.5 mM, 1 mM & 2.5 mM). In the presence of AGE.

Figure 8.8: Bar diagram showing the dose dependent inhibition of tube formation by amino acids glycine and glutamic acid in the presence of AGE.

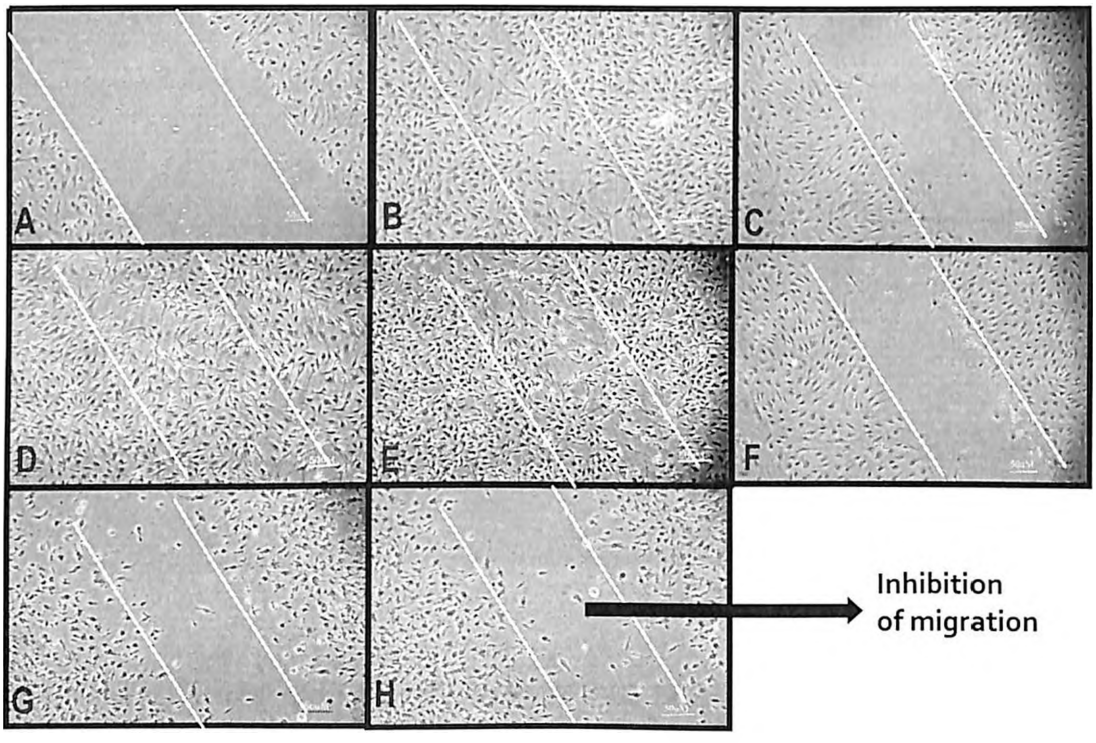


The effect of inhibition was significant for glycine and glutamic acid treated cells both at 1.0 and 2.5mM ($p<0.05$) and therefore the effect of these amino acids at the level of migration and adhesion which are the major steps involved in tube formation was looked into

8.3.5: Migration assay in BREC cells with bFGF and amino acids

Glutamic acid/Glycine inhibits the bFGF induced migration of retinal endothelial cells: Migration is the initial step required by the cell before it goes for formation of new tubes. There was an inhibition of migration by both the amino acids glycine and glutamic acids but not by cysteine at 5 mM concentration (Figure 8.9).The inducer of migration was bFGF (positive control) in this experiment.

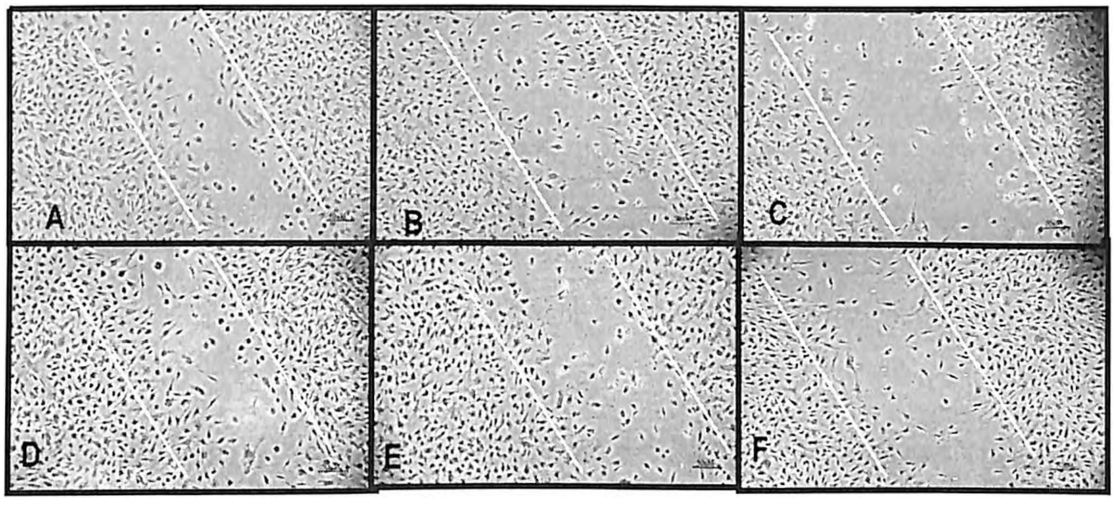
Figure 8.9: EC migration assay: Effect of the amino acid glycine and glutamic acid



A: Control, B: Cysteine 5mM, C: Glutamic acid 5mM, D: bFGF 1ng(positive control)
E: bFGF + Cysteine 5mM, F: bFGF + glutamic acid 5mM,G: glycine 5mM and
H: bFGF + glycine5mM

The effect of amino acids at lower concentration was tested and found that they had an inhibitory effect even at 1mM concentration (Figure 8.10).

Figure 8.10: Inhibition of migration by amino acids dose dependently

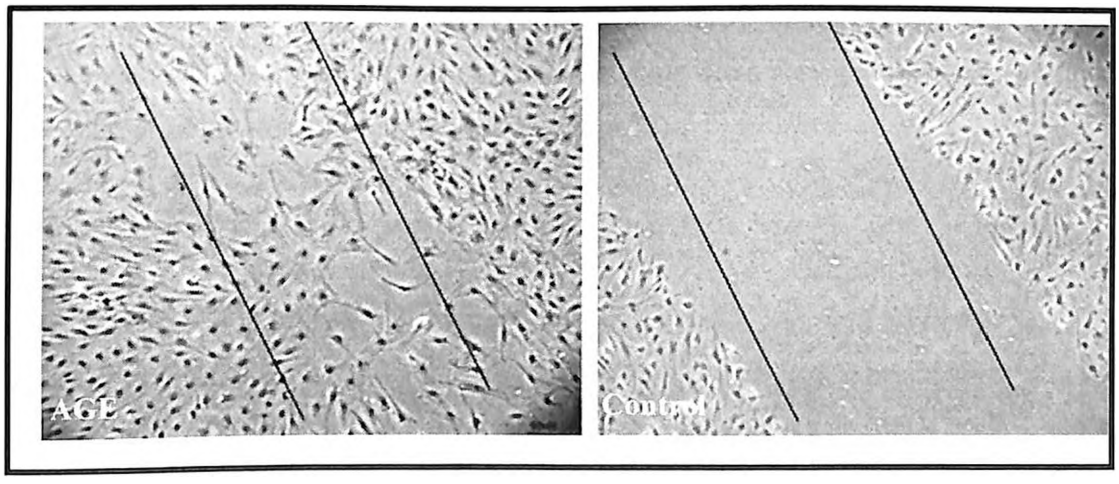


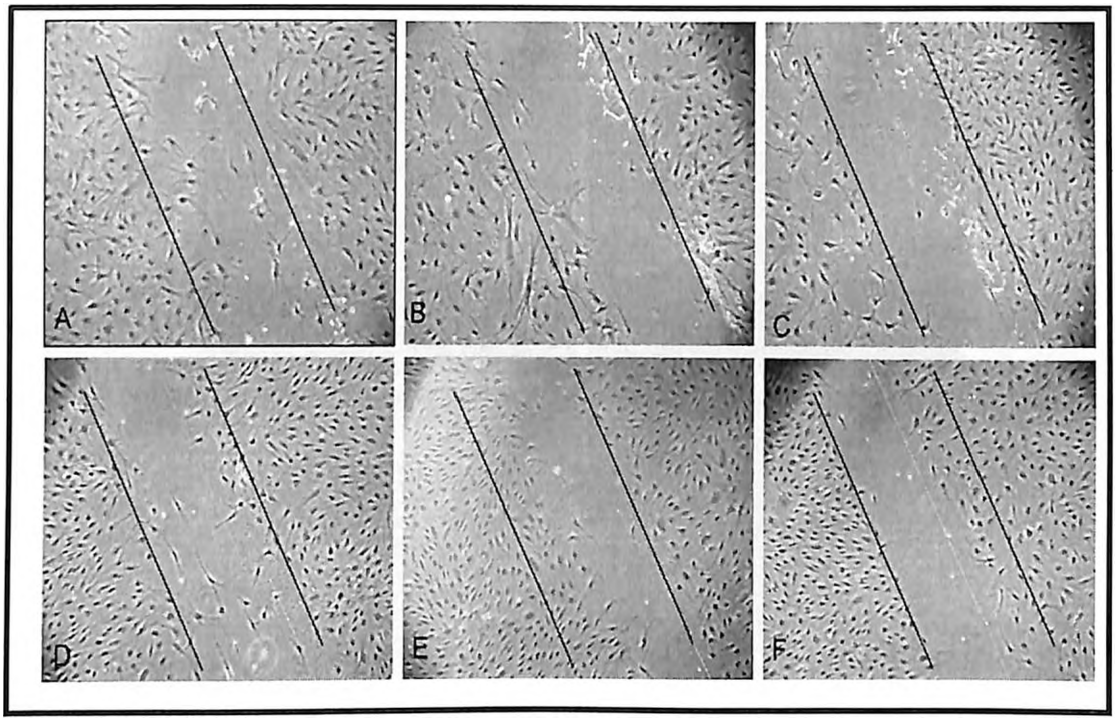
A-C: 0.5mM to 2.5mM concentration of glycine with bFGF
D-F: 0.5mM to 2.5mM concentration of glutamic acid with bFGF

8.3.6 : Migration assay in BREC with AGE and amino acids

AGE also induces the migration of cells. Inhibition of this migration by amino acids was found to be a dose dependent (Figure 8.11).

Figure 8.11: Effect of amino acids in the migration effect of AGE in BREC cells





A-C: 0.5mM to 2.5mM glycine + AGE

D-F: 0.5mM to 2.5mM glutamic acid + AGE

8.3.7.: Adhesion assay in BREC cells with AGE and amino acids

In diabetic complications the initial step is said to be the inflammation and adhesion of monocytes on the EC layer.The effect of AGE in the adhesion of monocytes to the ECs was also studied and the effect of amino acids in inhibiting the adhesion of monocytes was also tested. It was found that glycine had a very good inhibitory effect on adhesion of monocytes on the cells(Figure 8.12).

Figure 8.12: The effect of amino acids on the adhesion of monocyte to the endothelial cells after treatment with AGE

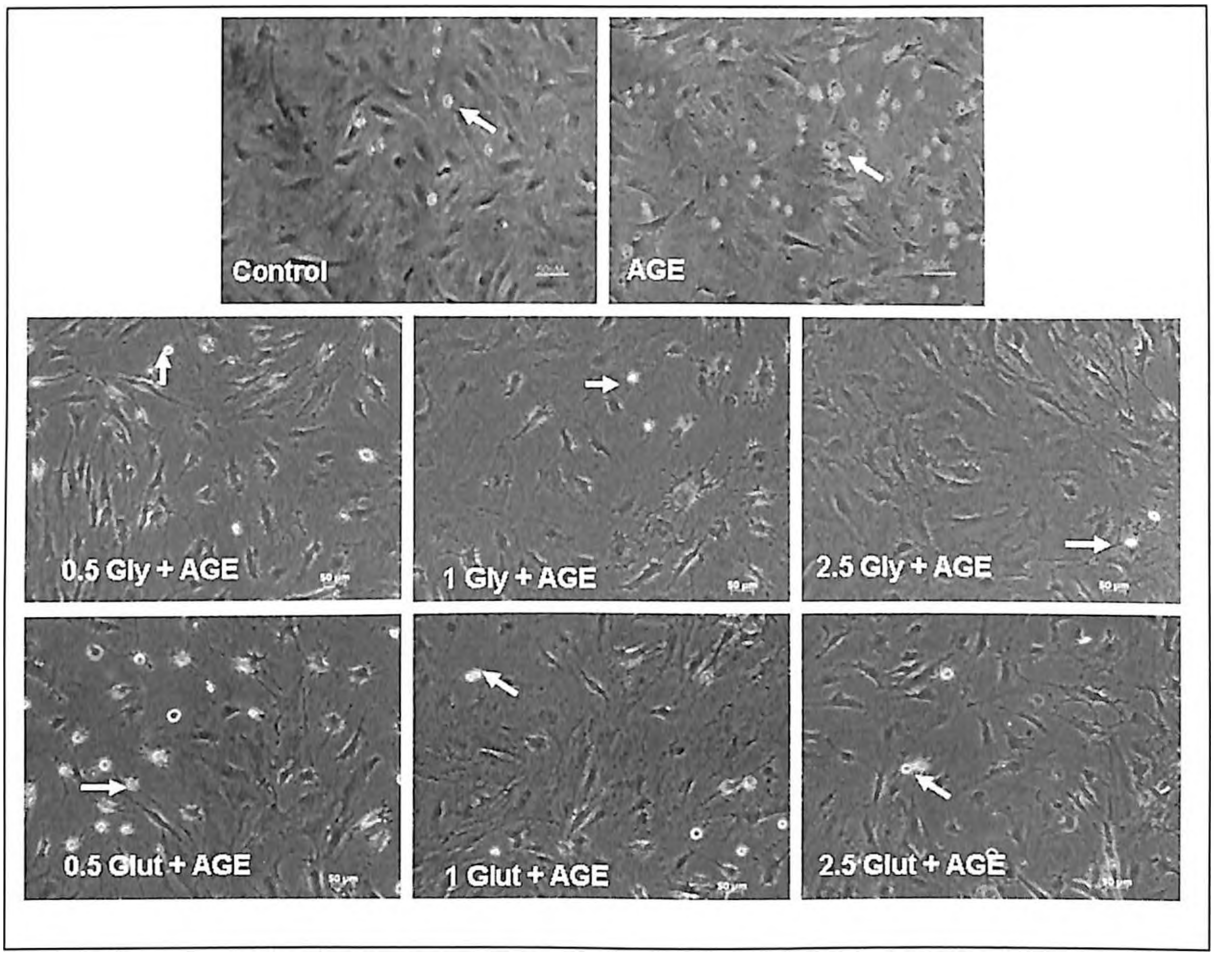


Figure 8.12: The white arrow indicates the monocytes adhered to the endothelail cells.

8.3.8 : Immunoflourescence for VEGF in BREC, BRP and BRPE

Since there was an inhibition of tube formation and migration by these amino acids in the retinal ECs we wanted to test the effect of these amino acids on the angiogenic protein VEGF in all the three cells by immunofluorescence. The VEGF expression in all the cells was induced by 100µg/ml AGE for 24 hrs. When the amino acids were added along with AGE there was a decrease in the VEGF expressions as the concentration of amino acids were increased from 0.5 mM to 2.5 mM. (Figure 8.13 , 8.14 , 8.15).

Figure 8.13: VEGF expression in BREC in AGE treated and with varying concentrations of amino acids glycine and glutamic acid.

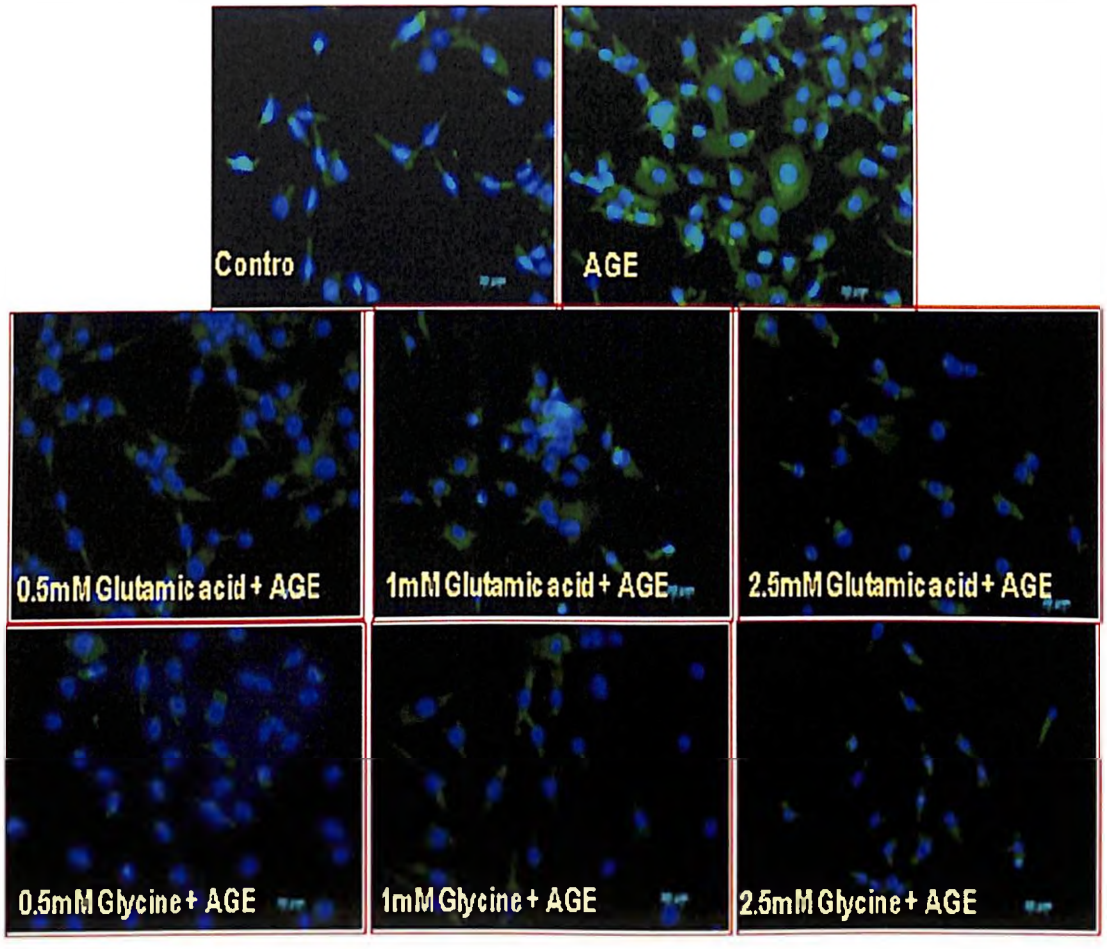


Figure 8.13: The green fluorescence indicates the VEGF expression and the cells are counter stained with DAPI for nuclear stain

Figure 8.14: VEGF expression in BRP cells in AGE treated and with varying concentrations of amino acids glycine and glutamic acid.

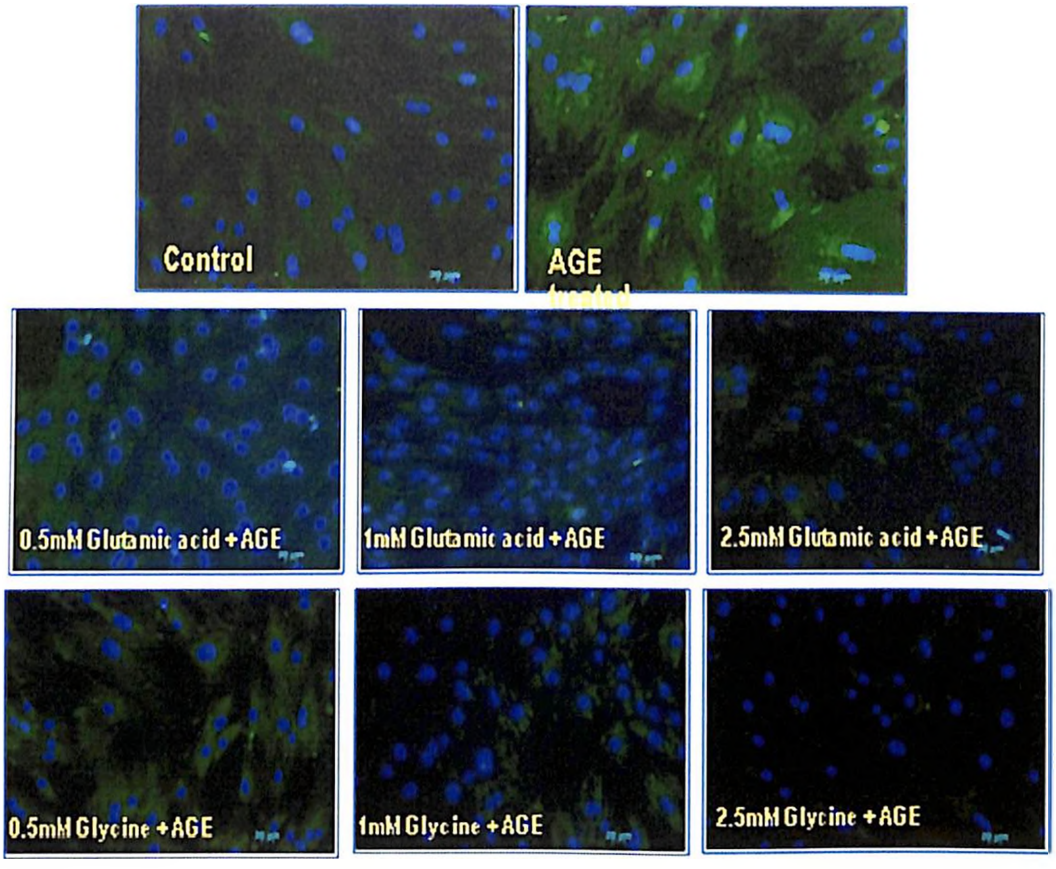


Figure 8.14: The green fluorescence indicates the VEGF expression and the cells are counter stained with DAPI for nuclear stain

Figure 8.15: VEGF expression in BRPE cells in AGE treated and with varying concentrations of amino acids glycine and glutamic acid.

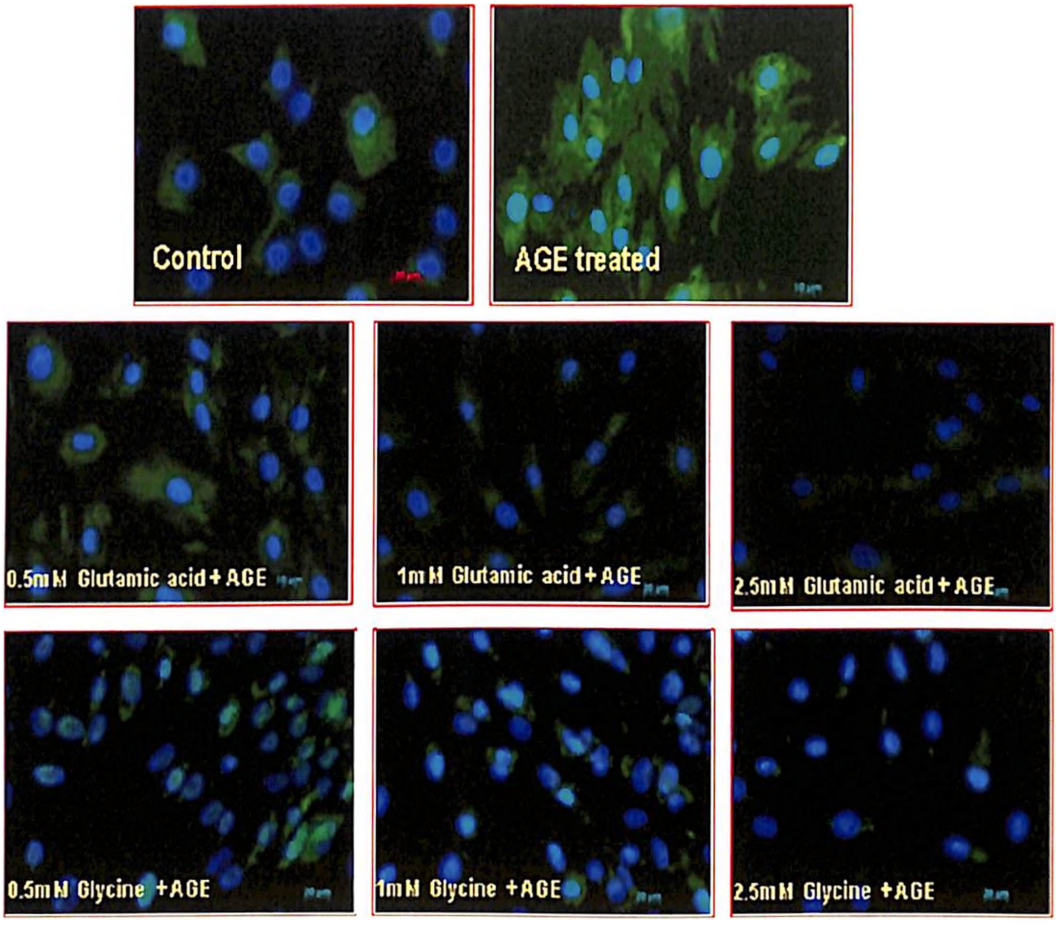


Figure 8.15: The green fluorescence indicates the VEGF expression and the cells are counter stained with DAPI for nuclear stain

8.3.9: RT PCR forVEGF in BREC, BRP and BRPE cells

To confirm the observation made by the IF assay, the VEGF expression was done by RT-PCR in the presence of AGE and the effect of amino acids glycine and glutamic acid at varying concentrations was tested. There was a decrease in the expression as the concentration of the amino acid was increased. The mRNA expression of VEGF and the variation in the band intensity is represented as a histogram in Figure 8.16 (BREC Cells), Figure 8.17 (BRP cells), Figure 8.18 (BRPE cells)

Figure 8.16: mRNA expression of VEGF by RT PCR in BREC cells

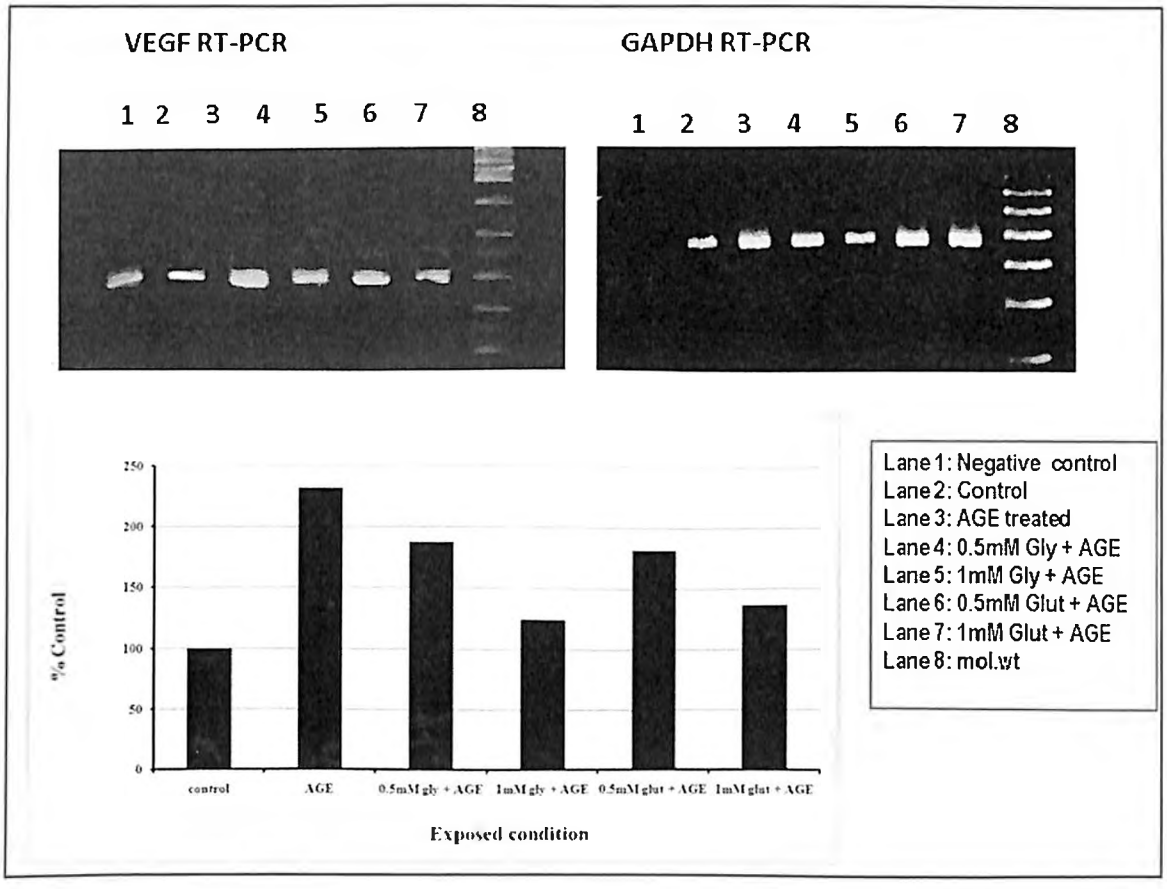


Figure 8.17: mRNA expression of VEGF by RT PCR in BRP cells

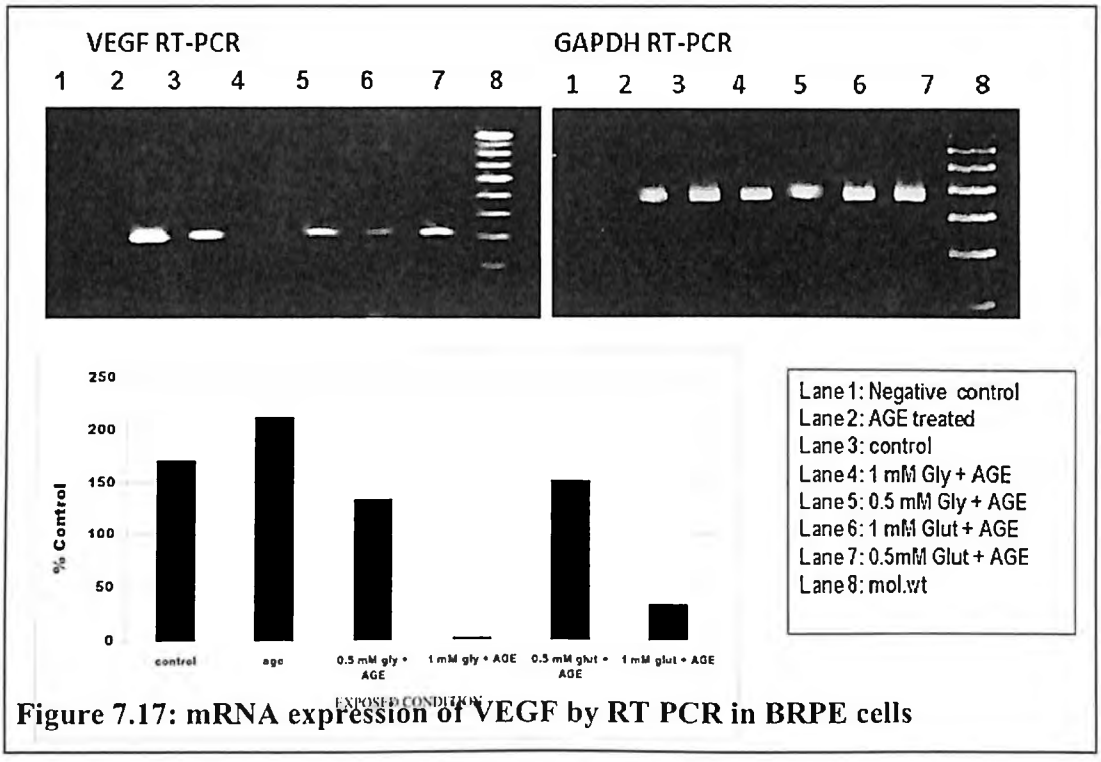
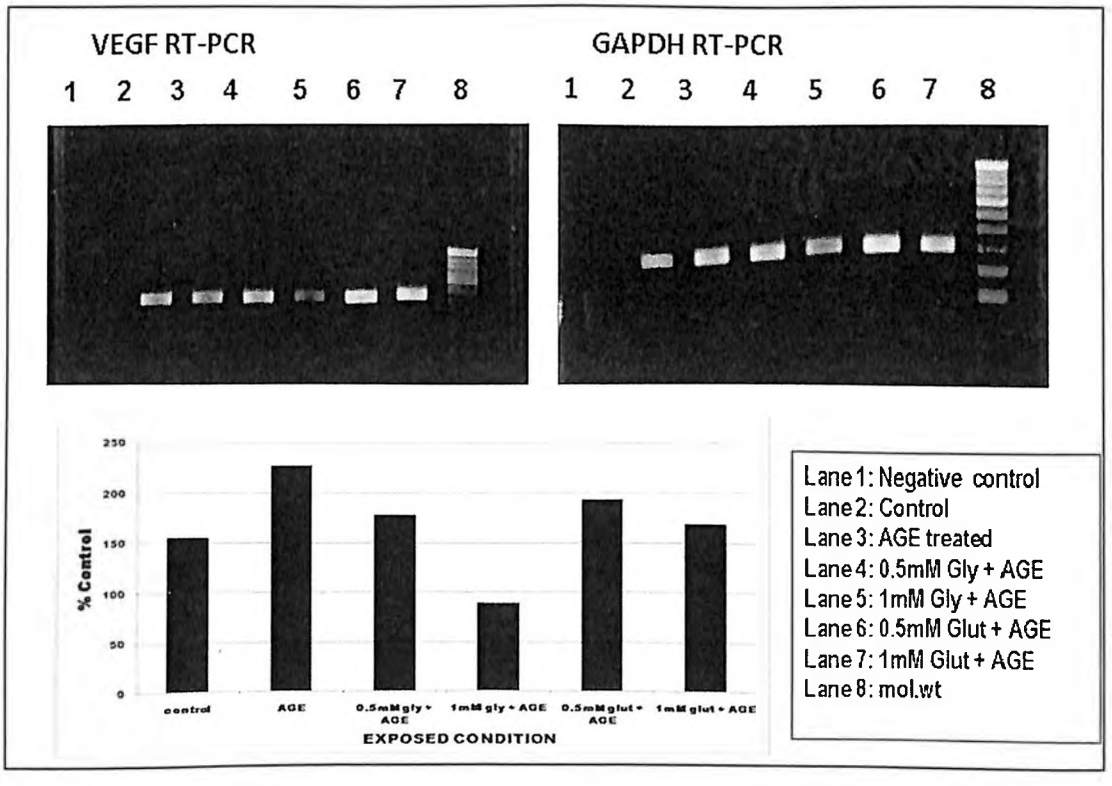


Figure 7.17: mRNA expression of VEGF by RT PCR in BRPE cells

Figure 8.18: mRNA expression of VEGF by RT PCR in BRPE cells



8.3.10 : RAGE expression in BREC, BRP and BRPE:

When a decreased expression of VEGF was observed in all the three cells, the RAGE expression was studied in all the three cells since the AGE binds to RAGE initially which in turn triggers the VEGF expression through NfKB activation. The RAGE expression in all the three cells was studied by IF AGE alone and in the presence of 2.5mM amino acids (Glycine and glutamic acid). Figure 8.19,20 and 21.

Figure 8.19: The RAGE expression in BREC cells

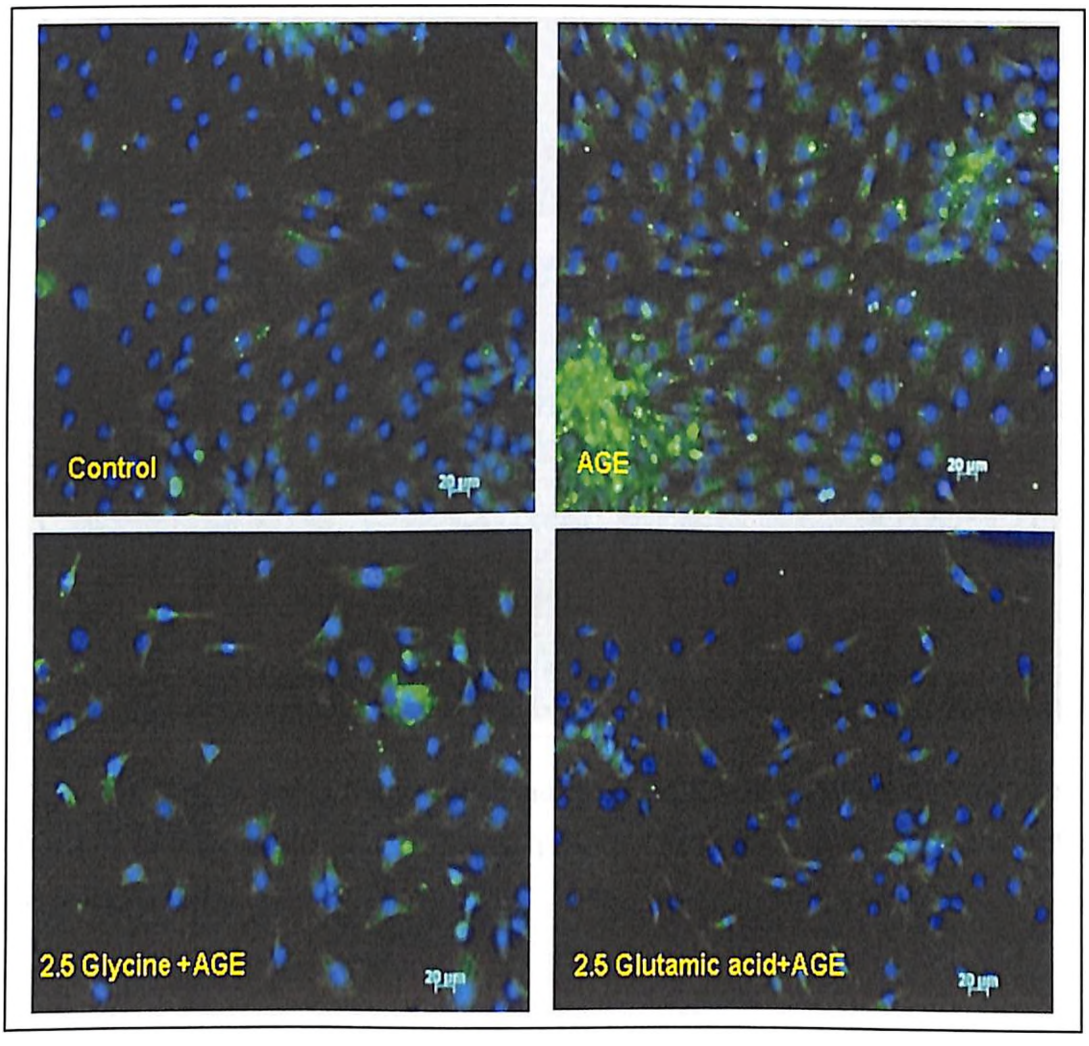


Figure 8.19: The green fluorescence indicates the RAGE expression (which appears as dots as they are membrane proteins) and the cells are counter stained with DAPI for nuclear stain.

Figure 8.20: The RAGE expression in BRP cells

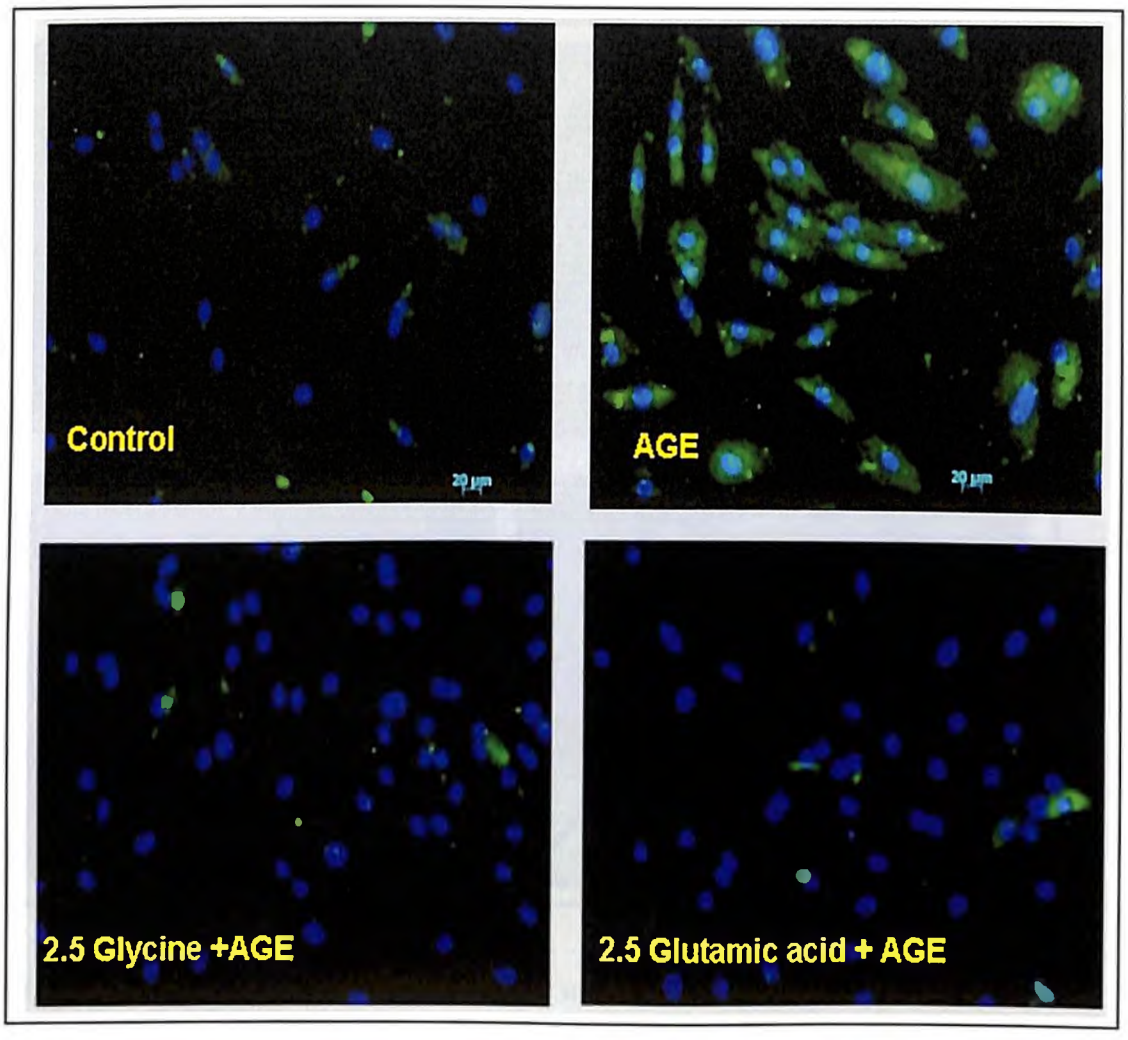


Figure 8.20: The green fluorescence indicates the RAGE expression (which appears as dots as they are membrane proteins) and the cells are counter stained with DAPI for nuclear stain.

Figure 8.21 : The RAGE expression in BRPE cells

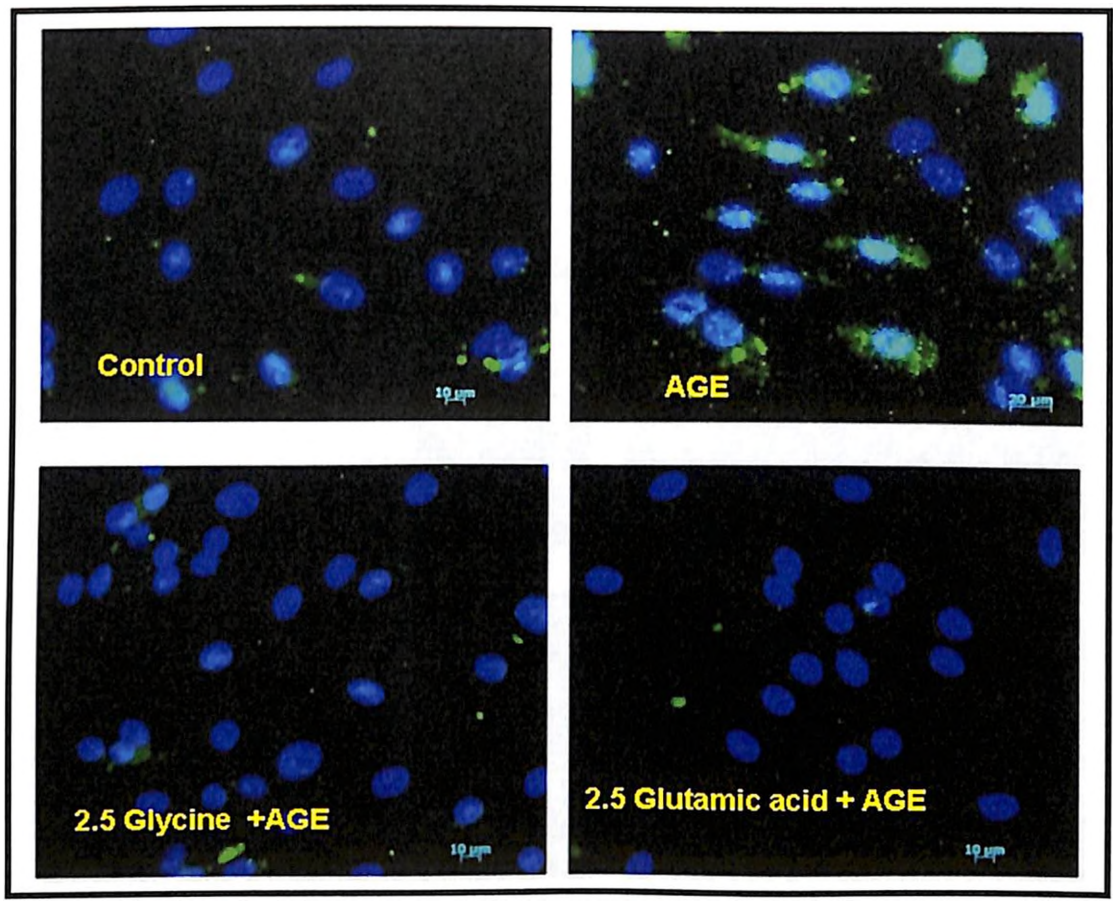


Figure 8.21: The green fluorescence indicates the RAGE expression (which appears as dots as they are membrane proteins) and the cells are counter stained with DAPI for nuclear stain.

8.3.11 :Actin stress fibers by IF in BREC, BRP and BRPE

There was a decrease in the expression of RAGE in all the three cells when treated with amino acids when compared to AGE alone. Out of the 2 amino acids tested it was found that the glycine was having a good effect at the VEGF level and glutamic acid was having a better effect at the RAGE protein expression level.

The effect of both the amino acids at the level of actin stress fibers was also studied in all the three cells. Both the amino acids were found to decrease the actin stress fiber

formation. The functional actin stress fiber was stained with phalloidin conjugated FITC which was prepared in house. The actin stress fiber in all the three cells is shown below. (Figure 8.22,23 and 24)

Figure 8.22: The actin stress fibers in BREC cells treated with AGE and amino acids

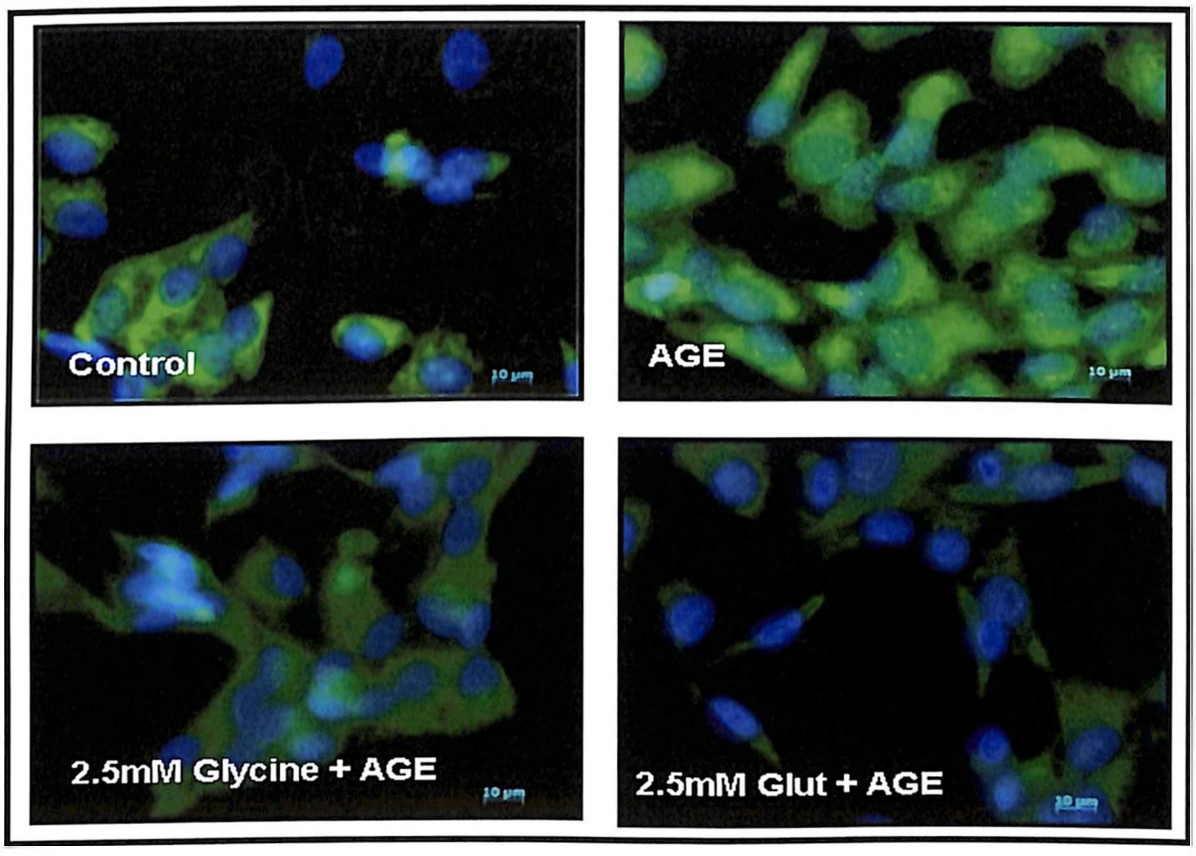


Figure 8.22: The green fluorescence indicates the Actin stress fibers which are stained with phalloidin FITC conjugate which stains the active actin and the cells are counter stained with DAPI for nuclear stain

Figure 8.23: The actin stress fibers in BRP cells treated with AGE and amino acids.

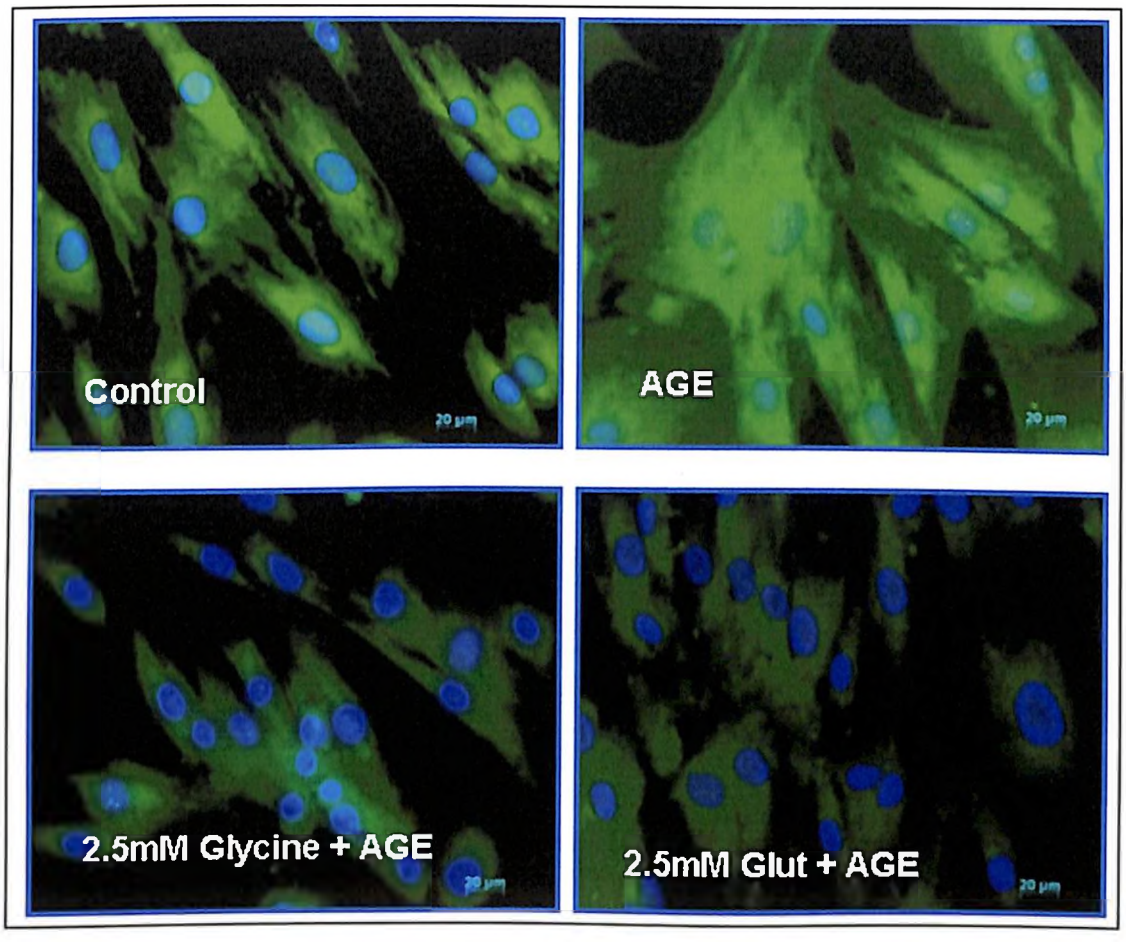


Figure 8.23: The green fluorescence indicates the Actin stress fibers which are stained with phalloidin FITC conjugate which stains the active actin and the cells are counter stained with DAPI for nuclear stain

Figure 8.24: The actin stress fibers in BRPE cells treated with AGE and amino acids.

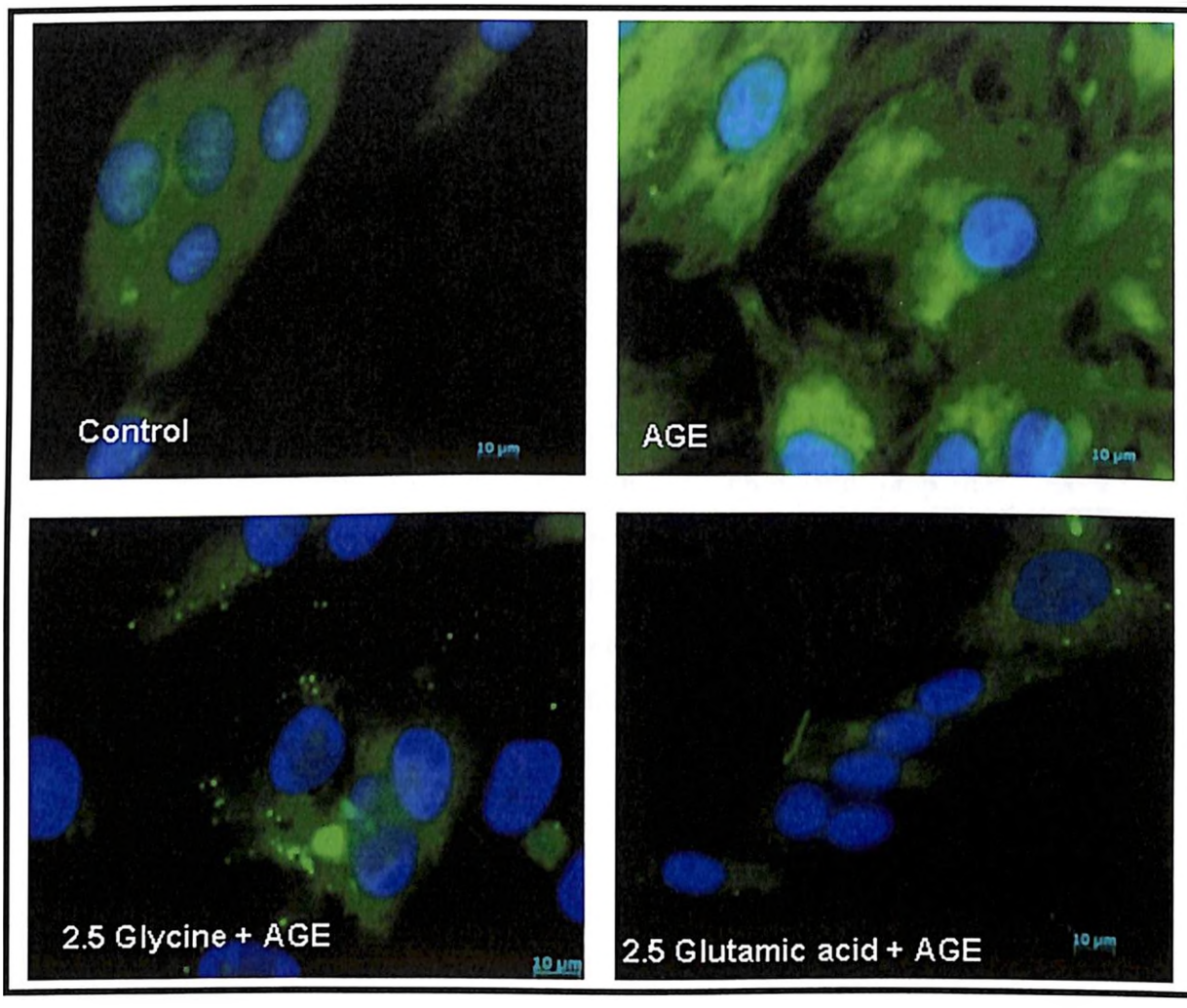


Figure 8.24: The green fluorescence indicates the Actin stress fibers which are stained with phalloidin FITC conjugate which stains the active actin and the cells are counter stained with DAPI for nuclear stain

CHAPTER 9: DISCUSSION

ECs as a single cell layer lines the inner surface of blood vessels, that plays a crucial role in the physiology and pathology of the vascular system. ECs play an important role in vasculogenesis and angiogenesis. This layer of vascular endothelium is susceptible to a variety of stimuli, both local and systemic, and any structural and functional modulation serves as a pathogenic risk factor in vascular diseases, involving inflammation, thrombosis, hypertension, atherosclerosis and apoptosis. Endothelial dysfunction is characterized by a shift of the endothelium toward reduced vasodilation, proinflammatory state and with prothrombotic properties. These processes are thought to modulate the disease process through effects on cellular metabolism, signaling, and growth factors. The severity of endothelial dysfunction has been shown to have prognostic value for cardiovascular events and also a major contributor of diabetic retinopathy.(Hadi, Carr et al. 2005)

ECs and pericytes are in the ratio of 1:1 in the retina and the loss of pericytes is the hall mark of diabetic retinopathy. Therefore the culture of both ECs and pericytes can be used to study complicated pathophysiological processes occurring in the vessel wall *in vivo* during diabetic retinopathy. Therefore the cells were successfully isolated from bovine retina by homogenizing the retina and filtering the homogenate through a 41 μ nylon filter. The cells were then grown in 0.1% gelatin coated petridishes using commercially available enriched medium (Endopan). The time taken for the primary cells to reach confluence varied from 18 – 21 days. Further purity of the cells was obtained by CD 31 coated dynabead isolation which aided us in separating both the cells to purity. These cells were characterized based on marker studies (BREC – Factor VIII and VE Cadherin and pericytes (BRP- NG2 and Actin. Thus the isolated retinal ECs and pericytes upto 4 passages were used as *in vitro* cell culture models to mimic diabetic retinopathy conditions

Advanced glycation end products are a heterogeneous group of compounds formed by two mechanisms which have been proposed. One mechanism involves the transition metal-catalyzed autoxidation of free sugars, which also yields dicarbonyl compounds and superoxide and hydroxyl radicals and the second is the non enzymatic glycation of proteins by glucose. These AGEs have been implicated in micro vascular complications of DR, by various factors like promoting the EC barrier permeability, inducing proliferation and migration of cells and as well as by elaborating the growth factors.(Guo, Huang et al. 2006; Goh and Cooper 2008) in ECs. But in the case of pericytes, AGE has been reported to promote apoptotic signaling thereby leading to pericyte loss, characteristic of DR. (Lecomte, Denis et al. 2004; Chen, Jiang et al. 2007). However, the molecular pathophysiology associated with these changes at the level of retinal capillaries is still not clear. The change in the level of glucose uptake may be the basic mechanism involved which has not been looked into. The glucose transport in BREC and BRP is by the carrier mediated facilitated transport, independent of insulin. Among the five different isoforms of glucose transporters, GLUT-1 and GLUT-3 are the most widely distributed transporters in the ECs and pericytes and they regulate the basal glucose uptake of barrier tissues namely retina and brain ECs. (Maher, Simpson et al. 1993; Olson and Pessin 1996).The GLUT1 expression is reported to differ between retina and cerebral cortex, suggesting that glucose transport is regulated differently in these embryologicaly similar tissues (Takata, Kasahara et al. 1992). Looking at the type of the barrier EC, it has been shown that there is down regulation of GLUT-1 expression in brain EC and there is no such down regulation even after exposure to high glucose for 5 days in retinal ECs. GLUT-1 transporter regulation in response to high glucose conditions in ECs and pericytes has been differently reported with opposing views.(Knott, Robertson et al. 1996; Alpert, Gruzman et al. 2005). In this study we looked into the GLUT-1 mediated transport of glucose under the conditions of accumulating Advanced glycation end products (AGE) in the bovine retinal capillary EC and PC. There were mild alterations in terms of a decrease in the GLUT-1 expression in the EC on exposure to AGE for 6 days that was not statistically significant. There was no such change in the glucose uptake and GLUT-1 translocation in the case of pericytes.

Therefore the deleterious effects could be due to the accumulation of AGE in the retina which is reported in various studies needs attention.(Glenn and Stitt 2009). ROS-dependent process also play a central role in the generation of intracellular AGEs.. Autoxidation of protein-bound Amadori products appears to be the most important source of free radicals under near physiologic conditions (Hunt, Bottoms et al. 1993). and inhibition of oxidant pathways prevents intracellular AGE formation which has important implications in preventing diabetic complications.

Many studies demonstrate that hyperhomocysteinemia (HHcys) as an independent risk factor for atherosclerosis in the coronary, cerebral and peripheral vasculature. (Wenzler, Rademakers et al. 1993; Pianka, Almog et al. 2000; Genser, Prachar et al. 2006). There are reports which states HHcys as a risk factor for coronary heart disease in diabetes mellitus (Mataradzija, Resic et al.; Soinio, Marniemi et al. 2004). Metabolic conversion of Hcy to a chemically reactive metabolite, Hcy-thiolactone is suggested to contribute to Hcy toxicity in humans. Increased levels of Hcys reportedly increase the concentration of HCTL.(Jakubowski, Zhang et al. 2000). Recent reports are indicative of increased Homocysteine thiolactone levels associated with the development and progression of diabetic macrovasculopathy (Gu, Lu et al. 2008), as well as microvascular complications as reported in diabetic nephropathy (Joseph and Friedman 2009) and retinopathy (Brazionis, Rowley et al. 2008). However, the impact of it on the microvascular EC in the retina is still not well understood.

The enzyme PON (paraoxonase) is a calcium dependent 45 kDa protein an antioxidant enzyme with multiple enzyme activity namely organophosphatase, arylesterase and lactonase. This enzyme removes the HCTL from the system thereby preventing HCTL toxicity. The serum PON HCTLase and AREase are reported to be significantly lowered in diabetic patients (Lakshman, Gottipati et al. 2006; Sonoki, Iwase et al. 2009), while the Hcys and HCTL levels were found to be increased in these conditions(Aydemir, Turkcuoglu et al. 2008; Shargorodsky, Boaz et al. 2009). In this study the HCTL and the PON-HCTLase/AREase activity in proliferative diabetic retinopathy cases and control MH cases were measured at the level of vitreous. Thus a first report was made on the significnat increase in vitreous HCTL and PON-HCTLase activity in PDR compared to the disease control Macular Hole. This

increase in the HCTLase activity of PON, is probably a protective mechanism to eliminate the HCTL. To further explore this, the dose and time dependent effect of Hcys and HCTL were also tested on the BREC cells. It was found that the cells were viable at both the physiolgogical and pathological concentrations of Hcys and HCTL even though an increase in the oxidative stress measured in terms of TBARS was observed. It was found that in both the Hcys and HCTL treated cells the HCTLase activity of PON was significantly elevated and was maximam at 200 μM at 24 h. The differential effect seen with Hcys having nearly a 2 fold higher effect than HCTL can be explained by the differential uptake of the molecules and this can be characteristic of the cell type. Hcy transport has higher affinity, lower transport capacity, and predominantly mediated by a sodium/lysosome-dependent system ASC.(Jiang, Yang et al. 2007). However the retinal capillary EC uptake of Hcys and HCTL has so far not been looked into.The mRNA expression of PON 2 was specifically increased in the BREC cells similar to the activity. PON2 which is ubiquitously present in all tissues, has the highest lactonase activity, though all the three isoform exhibits it.(Stoltz, Ozer et al. 2009). The increase of PON in vitreous could be contributed by the inner retinal barrier permeability and as well as the proliferating ECs which are characteristic of PDR. The presence of PON 2 in the vitreous was confirmed by western blot. The *in vitro* study supports this fact wherein exposure of Hcys and HCTL increases the PON 2 mRNA expression also, which is the tissue form of the PON.

One of the interesting observations made was that even though the HTLase activity increased dose dependently with increase of Hcys there was a 50 % drop in the AREase activity in the cell culture system and also in the vitreous of the PDR cases. This shows the molecule Hcys is probably involved in modulating the kinetics of the PON though it is not a substrate unlike HCTL. To further probe this effect we took a bioinformatics approach

PON2 bound to two Ca^{2+} ions was modeled for the first time using MODELER 9V7, based on the crystal structure of PON1 (1V04) as template given by Harel et al. (Harel, Aharoni et al. 2004) .The RMSD between the modeled structure of PON 2 and the template PON1 was calculated using PYMOL and was found to be 0.169Å.

Energy minimization was done for the ligands using Argus Lab. (Thompson). Docking studies were done for PON 2 ligands namely HCTL, Hcys and phenyl acetate (PA) using Autodock 4.0. The docking for the substrates HCTL and PA with PON 2 was done by blind docking as well as active site defined rigid docking based on PON1 active sites. Both yielded the same results. However as the binding of Hcys to the PON 2 protein is not known, blind docking was done for Hcys. The docking study reveals that Hcys binds to the same pocket to which HCTL and PA docks. The ligand Hcys is involved in the same hydrophobic interactions at the active site as for the HCTL and phenyl acetate. Therefore it is quite possible that Hcys can affect the HCTLase and AREase activity of PON2.

Our previous studies have shown that supplementation of the amino acid mixture for the Type 2 Diabetes Mellitus patients had an increased sensitivity for the insulin signaling and decreased the levels of the glycosylated haemoglobin. Free lysine, Alanine, Aspartic acid, or Glutamic acid has been shown to reduce the glycation of human lens proteins, *in vitro* (Ramakrishnan, Sulochana et al. 1996; Ramakrishnan, Sulochana et al. 1997). Based on these previous studies, the antiglycating effect of amino acids namely glycine, glutamic acid, leucine, lysine and cysteine were evaluated in the AGE induced changes in the PON activity in vitro. The effect of AGE on PON activity was tested in the BREC cells by exposing them to various concentrations of AGE for 24 h and it was found that there was a dose dependent decrease in the PON-HCTLase activity with 50% drop at 1 mg/ml concentration. This decrease can be attributed to the glycation of the PON protein (Mastorikou, Mackness et al. 2008). Addition of amino acids , significantly improved the PON activity as seen by glycine and glutamic acid , follweed by cysteine and lysine while Leucine did not show any significant increase. The possible mechanism is that , the the amino acids become preferred substrates of glycation when AGE is added, thereby sparing the PON from glycation and therefore the decrease in its activity.However we need to further probe to establish the same.

AGE signals the cells by binding to its receptor RAGE, which induces cellular signaling via NFkB which ultimately triggers VEGF expression. Angiogenic activity of AGE is mainly mediated through VEGF levels. AGE on the other hand leads to

pericyte dropout, (Yamagishi, Hsu et al. 1995), which in turn relieves the restriction on EC replication and facilitates angiogenesis. AGE also inhibits the prostacyclin-synthesizing ability of microvascular ECs (Yamagishi, Kobayashi et al. 1993), which leads to microthrombus formation, giving rise to hypoxia, the major factor triggering VEGF expression in both ECs and pericytes. (Yamagishi, Yonekura et al. 1997).

Several therapeutic strategies such as inhibition of AGE formation, blockade of the AGE–RAGE interaction, and suppression of RAGE expression or its downstream pathways, are promising for the treatment of vascular complications in diabetes. Some of the drugs which inhibit AGE formation are aminoguanidine, pyridoxamine and LR 90. Metformin blocks the glycation reaction (Rahbar, Natarajan et al. 2000). Blockade of VEGF signal transduction is one of the most well recognized strategies for the development of angiogenic inhibitors.(Nyberg, Xie et al. 2005).VEGF antagonists such as bevacizumab have been used widely while many are in phase III or Phase II clinical trials. Oral antagonist of PKC namely ruboxistaurin is still not FDA approved though the results of the treatment in clinical trials are found to reduce the vision loss (Davis, Sheetz et al. 2009).Apart from photocoagulation either triamcinolone or bevacizumab are being used presently for PDR and DME treatment however triamcinolone have been associated with cataract and raised IOP, also some patients have shown unresponsiveness to bevacizumab (Lux, Llacer et al. 2007). Thus the need for newer compounds either alone or as combinational therapy is still felt. The effect of amino acids which were found to promote the PON activity was also tested for their antiangiogenic effect. While AGE promoted the tube formation significantly, out of the 5 free amino acids tested, glycine and glutamic acid showed inhibition of the tube formation by matrigel assay and this was found to be a dose dependent effect. The inhibition of EC migration and the leucocyte adhesion assay was done to demonstrate the anti-angiogenic nature of the compounds(Carmeliet 2004). The anti angiogenic effect of glycine and glutamic acid was evaluated by migration assay and adhesion assay which showed marked inhibiton of the migration of endothelail cells and adhesion of lymphocytes to the ECs dose dependently. This strengthened our observation of the anti angiogenic effect of the amino acids namely glycine and glutamic acid based on the matrigel assay. This property was further evaluated by the

VEGF expression at the level of protein by IF and mRNA by RT-PCR. The assay revealed lowering of VEGF expression by these 2 amino acids dose dependently. Since there was a drop in the VEGF expression in the ECs by these amino acids, the effect of these amino acids on pericytes and retinal pigment epithelial cells which are influenced by VEGF expression and has a role in the pathogenesis of PDR were also tested. These two cells also showed a decrease in the VEGF expression induced by AGE, on treatment with glycine and glutamic acid treatment. While glycine showed the maximal antiangogemic effect at the level of leucocyte adhesion to the EC, the Gutamic acid showed its effect at the level of inhibition of the emigration, implying that these amino acids function by different mechanism to mediate their antiangiogenic effect. Further experiments needs to be done to explore this. The receptor for AGE (RAGE) which transmits the signal inside the cell to trigger the VEGF expression was also examined with and without amino acids in all the three cells. The glutamic acid follwed by glycine inhibited the RAGE expression as seen by immunoflourascence and it was maximum in the BRPE cells followed by BREC and BRP. Actin cytoskeleton as a structural protgein is involved in multiple processes of the cells including cell shape, motility and cell polarity. To perform all these tasks, the nonmuscle actin cytoskeleton is composed of a number of specialized subtypes of actin networks that communicate with different signaling pathways. One of these is the actin stress fiber network that develops under cellular tension. The actin stress fibers are involved in the cell movement and migration. There was an increase in the actin stress fiber when treated with AGE and an inhibition in the formation of actin stress fiber was observed for both the amino acids as seen by IF.

The literature review shows that dietary glycine is a potent anti-angiogenic agent as seen by CAM assay that can reduce wound healing and tumor growth by fibrin Z chamber assay which was through reduction of iNOS expression. (Amin k et al, 2003.) Glycine has been shown to inhibit VEGF induced cell proliferation and migration by blocking the influx of Ca^{2+}, through the glycine gated chloride channels as studied in bovine pulmonary artery EC line (*CPA*-47) (Yamashina et al, 2001). This study reveals the potemtial anti-angiogenic effect of glycine at the level of retina for the first

time. There are no reports available on the anti-angiogenic role of glutamic acid. This is the first report on the effect of glutamic acid in inhibiting the angiogenesis.

To conclude this is the first report which shows the benefical effects of the amino acids lysine, cysteine, glycine and glutamic acid in promoting the PON activity which is lowered in diabetic retinopathy by AGE formation. In addition the study also reveals potential anit-angiogenic nature of the amino acids glycine and glutamic acid in the retinal capillary ECs, pericytes and in retinal pigment epithelial cells. Future studies will be based on the evaluation of the amino acids in diabetic retinopathy animal models for drug development.

CHAPTER 10: CONCLUSION

- The isolated EC and pericytes had the characteristic morphology as reported in the literature and also showed markers positivity for factor VIII, VE-cadherin and metabolic markers in the case of EC and NG2 and actin positive in the case of pericytes. Therefore these cells were used up to 4 passages for all the experiments.The limitation is that the cells are of bovine origin and the obstacles to the successful culture of human RCEC culture include isolation of microvessel fragments from a small amount of tissue, elimination of pericyte and retinal pigment epithelial contamination and selection of suitable medium and growth factors to maintain long-term culture.

- This is the first report showing the effect of AGE-BSA on retinal capillary EC and the pericytes as studied at the level of glucose uptake. It was found that AGE does not alter the glucose uptake significantly in these cells, though there was a slight decrease in the endothelial cell.

- This is the first report showing the increased HCTL levels and HCTLase activity in the vitreous of the PDR cases. The *in vitro* study also revealed that there is a significant increase in HCTLase activity in the EC lysates treated with Hcys and HCTL. Therefore HCTLase and HCTL levels can be considered as markers for PDR. Both HCTLase activity and the levels of HCTL can be influenced by Hcys levels and Hcys can alos affect the AREase activity of PON. While AGE was found to decrease the paraoxonase activity, addition of aminoacids was found to significantly improve the activity of the enzyme. The amino acids like glycine and glutamic acid were found to increase the activity of PON, by 4 fold which can be of therapeutic importance.

- In this work, molecular modeling and docking studies were performed to explore possible binding modes of known substrates HCTL, GTBL, DVL, PA,

BA, 2- NA, PAR and Hcys into PON2 enzyme. MODELLER 9v7 software was used to model the enzyme PON2 and AutoDock 4.0 software was used to dock the ligands into PON2. The binding pattern of the amino acid residues participating in the hydrolysis was identified and validated by site of the reaction and interaction energies of the docked complexes. It was found that Thr170 made an important contribution in terms of hydrogen bond formation for lactonase activity. The binding energy for HCTL, which is the physiological substrate for this enzyme, is -6.63Kcal/mol and is higher when compared to other lactones (GTBL and DVL) and ester substrates (PA, BA and 2- NA) used for this study. This is indicative of elevated lactonase activity of PON2 when compared to its esterase activity. The semi-flexible blind docking of Hcys with protein PON2 reveals that the ligand Hcys is involved in the same hydrophobic interactions at the active site as for the HCTL and phenyl acetate. Therefore it is quite possible that Hcys can affect the HCTLase and AREase activity of PON2.

- Blockade of VEGF signal transduction is one of the most well recognized strategies for the development of angiogenic inhibitors (Nyberg, Xie et al. 2005) . This study shows that the amino acids glycine and glutamic acid are antiangiogenic because it inhbits, the tube formation as seen in the matrigel assay, migration of the EC and the leukocyte adhension . The actin stress fibers that are characteristically seen in the presence of AGE, is found to be reduced in the presence of the amino acids. In addition, the VEGF expression induced by AGE in the EC, is reduced in the presence of amino acids.

FUTURE SCOPE

- Recently HCTL is being associated with diabetes and diabetic retinopathy. The PON HCTLase is the major enzyme involved in the cleaving of the HCTL and thereby, decrease in PON is a risk factor for diseases associated with Hyperhomocysteinemia (HHcys) in ocular diseases like ARMD, CRVO. There is Polymorphism reported in all the three isoforms of PON and the functional association of this polymorphism to enzyme activity of PON is not elucidated in this ocular disease and this analysis would help, as a prognostic marker of these diseases.

- Therapeutic implication of amino acids in DR by improving PON activity and in mitigating angiogenesis needs to be evaluated in the animal models of DR

- Crystallization of PON 2 has to be done and thereby the Structure and function relationship has to be looked into.

- Recent reports show the increased levels of Hcys and HCTL in the serum and as well as in the vitreous of DR patients.The effect of Hcys and HCTL on pericytes which are regulators of EC growth has not been looked into. This could help us in finding out new mechanisms of pericytes drop out in DR.

- The pharmacokinetics and the pharmacodynamics of the amino acids entry inside the various tissues of the eye has to be analysed and the most effective composition can be used for treatment of retinal complications.

APPENDIX I

Materials for procurement and processing of bovine eye ball:

1. Sterile stainless steel containers.
2. Phosphate Buffered Saline (pH 7.4)

NaCl	8.0g
KH_2PO_4	0.2g
Na_2HPO_4	1.15g
KCl	0.2g
DH_2O	1000 ml

2. Antibiotics – Ciprofloxacin, Penicillin, Amphotericin B (Himedia)
3. Ice pack.
4. Sterile Dissection Set (Scissors and forceps)
5. Sterile Petri dishes

Materials for cell Isolation and culture:

1. Sterile cotton tipped swab
2. Sterile plastic centrifuge tube (Tarsons)
3. M199 Medium (GIBCO) commercially available with 1.2 % glutamine with phenol red

M199 Medium base	90 ml
10%FBS	10 ml

Cell culture reagents and preparation of media

DMEM/F12 Medium:

1 vial of DMEM/F12 powder dissolved in 900 ml of double sterilized milli Q water and filter sterilized under vacuum (sterility check-up – Incubation at room temperature, (overnight). After sterility check the growth media was prepared by mixing

DMEM/F12 Medium base	90 ml
10%FBS	10 ml

Gibco Trypsin: The trypsin was diluted to 0.1% with Dulbecos' Phosphate Buffered Saline (PBS) (Himedia)

M199 Medium (GIBCO) commercially available with 1.2 % glutamine without phenol red

The growth media was prepared by mixing,

M199 Medium base	90 ml
10%FBS	10 ml

Viability count:

0.4 % Trypan Blue (Sigma) dissolved in Phosphate buffered saline (pH 7.4)
Improved Neubaeur Chamber (haemocytometer)

Marker studies:

Immunoflourescence staining:

Pan cytokeratin antibody (1:400) (DAKO)
Cytokeratin 8.13 (sigma)
Vimentin (DAKO)
Anti mouse antibody FITC conjugated (1:15)
0.05% Evan's blue

Immunoperoxidase staining:

Tris buffered Saline (pH 7.6)
Tris: 1.2 g
NaCl: 1.6g
DH2O: 200ml

Protein estimation:

Reagents

Standard BSA	0.1g/dL
Na_2CO_3	2% in 0.2 N NaOH
$CuSO_4$	0.5% in 1% Trisodium citrate
Alkaline copper reagent	49 ml of (2) + 1 ml of (3)
Follin's Ciocalteau Reagent	1:1 dilution

Western blotting Reagents

1. Acrylamide (30%):

Acrylamide-14.6gm
Bis acrylamide-0.4gm
Dissolved in 30ml of water made up to 50ml.

2. Tris-HCl Buffer (PH 8.8):

Tris-9.0gm
Water-25ml
Adjust the PH to 8.8 with1N HCl and made up to 50ml with Distilled water.

3. Tris-HCl Buffer (PH6.8):

Tris-3.0gm
Water-25ml
Adjust the PH to 6.8 with 1N HCl and made up to 50ml with Distilled Water.

4. 10% APS:

Polymerization catalyst required for gel formation. 10% solution used – always prepared fresh.

Increasing APS concentration will make the gel set quicker.

Ammonium per sulphate-10gm
Distilled water-100ml.

5. TEMED (readily available):

Polymerization catalyst. Catalyses the formation of persulphate free radicals from the APS, which in

turn initiates polymerization.Always the last reagent added to the gel.

6. 10% SDS: Binds proteins so they all become negatively charged, therefore separation is on the basis of

size alone and not the intrinsic protein charges.

SDS-10gm
Distilled water-100ml
Stored at room temperature.

Separating gel preparation: Sieves and separates the proteins by size. Percentage gel depends upon the

size of target proteins.

Acryl amide %	Range of separation (kDa)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

10%gel:

30%Acrylamide- 4ml
TrisHCl PH 8.8-2.5ml
10%APS-50µl & TEMED-5µl
10%SDS-100µl
Distilled water-3.344ml

Stacking gel: Large pore size gel with little or no molecular sieving on the sample. Standard 4%

Acrylamide – large enough pore size for most samples.

30% Acrylamide-1.33ml
TrisHCl- (PH 6.8)-2.5ml
10%APS-50µl
TEMED-10µl
10%SDS-100µl
Distilled water-6.01ml.

Western blotting transfer buffer:

Tris-3.3gm
Glycine-14.4gm
SDS-1.0 gm

The contents were dissolved in 800ml of distilled water. The final volume was then made up to 1000ml with methanol.

Ponseau stain:

Ponseau S-0.5gm
Glacial acetic acid-1ml
Made up to 100ml with distilled water. Prepared just before use.

Electrophoretic buffer (5x):

Tris base-15.1gm
Glycine-72.0gm
SDS-5.0gm

Distilled water-made up to 1000ml.Donot adjust the PH of the stock, as the PH will be 8.3 when diluted. Stored at 4^0C until use (up to 1 month).

HEPES buffer (Lysis buffer)

10mMHEPES
1mMEDTA
10mMDTT
Dissolved in 10 ml dH_2O

Protease inhibitor cocktail (PIC)-1mg/ml
HEPES buffer and PIC solution –10ml HEPES+200μl PIC

Sample loading Buffer (3x):

1M Tris HCl (PH 6.8)-2.4ml
20%SDS-3ml
Glycerol-3ml
b-Mercaptoethanol-1.6ml
Bromophenol Blue-0.006gm

Tris buffered saline (pH:7.6) (TBS):

Sodium chloride-8gm
1M tris HCl-20ml
Diluted to 1000ml with distilled water.
Tween- 20-10ml.

5% Skimmed milk: 2.5g of skimmed milk powder was dissolved in 50ml of TBS

PCR:

Gene specific primers: The stock was diluted in 100µl of TE buffer and heated at 65^0C for 5 min.

Taq buffer with $MgCl_2$
100mM DNTP mix (Banglore genei)

Taq DNA polymerase 3U/µl (Banglore genei)
Sterile water

Gel Electrophoresis

Electrophoresis is a method by which the amplification of the gene sequence is confirmed after PCR. DNA, negatively charged moves to the cathode on subjected to electric field and moves according to the olecular weight in a matrix like agarose or polyacrylamide gels. The gels are suitably stained to visualize the product (Fluorescent dyes for agarose gels and silver staining of poly acryl amide gels).

Agarose gel electrophoresis

Preparation of TBE buffer: 54.1 g of Tris, 27.8g of boric acid and 3.65 g EDTA were added in 500 ml water and pH adjusted to 8.0.The stock solution is diluted 1: 10 for further use.

Tracking dye- Bromophenol blue : 0.1gm BPB+100ml 1X TBE buffer in equal volumes of 40%sucrose solution.

Requirements

Molecular weight marker, Agarose, 10 X TBE buffer,Ethidium bromide (2mg/ml), BPB Preparation of agarose gel The gel trough was cleaned with ethanol and the ends were sealed with cellophane tape with the combs placed in the respective positions to form wells. Two percentage of agarose gel was prepared by dissolving agarose in 1X TBE buffer and 8µl of ethidium bromide being finally added and mixed thoroughly and poured on to the trough followed by electrophoresis of the amplified products at 100 V for 30 to 45 minutes. The gel was then captured and analysed using ImageJ software

APPENDIX II

S. No	CONSUMABLES	COMPANY
1	Albumin	Sigma
2	AntiAGE antibody	Allied scientific
3	Antibiotic solution	Sree venkateshwara scientific suppliers
4	Basic fibroblast growth factor	sigma
5	Carboxymethyl lysine	Sigma
6	Carboxymethyl lysine antibody	Allied Scientific
7	Caspase 3	Biotech India
8	Collagnease	Genex
9	Cytochalasin B	Sigma
10	D Glucose-6-Phosphate	Sigma
11	Dimethyl sulfoxide	Sigma
12	DMEM low glucose media	Balajee scientific
13	DMEM with L-glutamine	Balajee scientific
14	DNTP mix	Sanmar specialty centre
15	Dynabead	Joyvel
16	5, 5' dithiobis nitrobenzoic acid	Sigma Aldrich
17	Endopan	Genex
18	Fetal bovine serum	Gibco
19	Fibronectin from human plasma	Sigma
20	Gamma thiobutyro lactone	Sigma Aldrich
21	Goat anti rabbit Ig G FITC	Sanmar specialty centre
22	Human endothelial medium	Joyvel
23	Human sVCAM and sICAM ELISA kits	Biotech India
24	Hydrogen peroxide	Jeyam scientific
25	Immobilon western chemulinescence kit	Servell engineers
26	Insulin	Sigma
27	MTT	Sigma
28	Neural glial 2	Biosource and surgicals
29	Paraoxon	Sigma
30	PEDF and Glut 1 primer	Genetix
31	Ponceau S	Sigma
32	RNAse out	Biocorporals
33	Sample buffer for SDS PAGE	Sanmar specialties chemicals
34	Thermo script	Invitrogen
35	Trypsin (0.25%)	Biocorporals
36	Ve-Cadherin	Allied scientific
37	VEGF antibody	Allied scientific

Table 6.2 : Clinical data of patients with macular hole

Age/ Sex	Past history	Treatment for Systemic Disorder	Other Ocular Dis-order	Duration of Vision Loss	Eye Affected	Macular Hole Grade	Treatment	Baseline Investigations
61/F	G_6PD Deficiency	T. Olvo T.Nayapred T. Oplex	NIL	1 Month	OD	FTMH	NIL	RBS- 103 U- 32 Cr- 1.07
55/M	NIL	NIL	NIL	2 Years	OD	FTMH	NIL	RBS- 133
31/M	HT	NIL	NIL	3 Months	OD	FTMH	NIL	RBS- 100
54/F	Raised BS levels	T. Glyciphage 500mg T. Losar 50	NIL	6 months	OU	FTMH	NIL	RBS- 233
63/M	NIL	NIL	NIL	6 months	OS	FTMH	NIL	RBS- 115 U- 15 Cr- 0.9
65/F	Trigeminal neuralgia	T. tegretal CR T. Baclofen	NIL	4 months	OS	FTMH	NIL	RBS- 138 U- 23 Cr- 0.8
67/M				1 month	OD			
57/M	NIL	NIL	Nil	2 years	OU	OD= FTMH OS= Lamellar MH	NIL	RBS 108

TABLE 6.3: Clinical Data of patients with PDR

Age/ Sex	Past Treat-ment History	Duration of Diabetes (yrs)	Type	DR Quadra nt	Vit Haem	Retinal Detach-ment	Other features	Eye Operated	Treatment	Baseline Investigati ons	Associated Disorder
43/F	None	16	1	4	+	TRD	OU=FVP	OU	T losar, T Femolid,	Rbs-322, BU-22, SCr-0.8	Hypercholester olemia
61/F	None	29	1	4	-	CRD	OD= 360° NVI FVP	OD	T Atenolol, T Glyco phage, T Lipitor, T Pioglitozone	RBS 300, BU-69, Scr-2.5	HT, Hypercholester olemia, CKD Stage IV
54/ M	Laser, Anti VEGF	3	1	4	+	None	OU=PVD	OU	T Glumet,	RBS-251, SCr -1.5 BU-39	HT
52/ M	Laser	26	1	4	+	TRD+ VMT	OS= 360° NVI OD=ERM	OS	T Glucoret Forte, Acarbose	RBS-287, BU-18 ,S cr-1.00	None
47/ M	None	15	1	4	OD =+	TRD	OD=FVP	OU	T Glycidem, T Isinexex, T Ethanbutol	RBS-251, SCr-1.1. BU 26	HT, DN
53/ M	Laser	20	1	4	OS= +	OS=TRD	OU= NVE,NVD , FVP	OU	T Nalop, T Atorva, T Glycalazide, T Metformin T Asprin	RBS-241. BU 35, Scr-1.1	Hypercholester olemia

46/F	Laser	20	1	4	OD =+	CRD	OS=FVP	OD	T Reclide, T Aztor 10mg. T Losor H, T Trioptal	RBS-231, BU -23, Scr-0.9	None
68/ M	Laser, AntiVE GF	6 months	1	4	+	OD=CRD	OS=FVP	OD	T Gemet, T Metpure Xl, T Atorva. T Olmesar H	RBS-159. BU-38, Scr- 1.1	HT
49/ M	Laser	12	1	4	OD =+	OS=TRD	OU+FVP OD=NVE	OS	T Eslo 5, T Glimi H2 T lipicure	RBS-231, BU-33. Scr-1	HT, DN
54/ M	None	20	2	4	OS= +	OS=TRD	OD=FVP, NVE	OU	T Glyclazide, Bisoprolol, T Orlibose, T Mini Press, T Telvas. T Amlodipine	RBS 152. BU 39, Scr-1.4	None
54/ F	Laser	10	2	4	+	OS= TRD	OD=NVD OU= FVP	OU	Inj Mixtard 12-0-12, T Atorva, T Rapace, T Glynace MF. T.Stamlo beta	RBS 287, FBS-210, BU-40, Scr- 1.2	HTN, DN, HYPOTHYROIDISM
48/ F	None	10	2	4	+	None	OS = FVP	OU	T Atocar, T Pyoglit, T Dyotor, T Glynipide T Amtas	BU-51, Scr-1.5. RBS-160	CRF , cardiomyopathy
53/ M	Laser	24	2	4	+	OS = TRD	OS =FVP, TRD OD=CSME	OU	T Glucored, T Envas,	RBS-337, BU-31, Scr-1.1	None

Table 7.2: Molecular interactions of the ligands into PON 2

Ligands	H- bond donor	H- bond acceptor	H-bond length (Å)	vdW interaction residues (Scaling factor= 1.00 Å)	Binding energy (Kcal/ mol)	Docking energy (Kcal/mol)
Homocysteine thiolactone (HCTL)	HCTL::H HCTL::H HCTL::H Thr170:HG1	Asp168: OD2 Ile169: O Ile225: O HCTL: O	1.880 1.785 1.944 2.202	Glu53, Asp54, His114, Ile116, Ser117, Asn167, Asp168, Ile169, Ala171, Asn223, Asn226, Asp268, Asn269 Leu270 Ca 355	-6.63	-7.12
Δ Valero lactone (DVL)	Thr170:HG1 Ala171:HN	DVL: O DVL: O	1.769 2.132	Glu53, Asp54, His114, Ile116, Ser117, Thr118, Asn167, Asp168, Ile169, Asn223, Asn226, Asp268, Asn269, Leu270, Ca 355	-4.06	-4.06
Γ-Thiobutyro lactone (GTBL)	Thr170:HG1	GTBL: O	2.004	Glu53, Asp54, His114, Ile116, Ser117, Thr118, Asn167, Asp168, Ile169, Ala171, Asn223, Asn226, Asp268, Asn269, Leu270, Ca 355	-3.69	-3.69
Phenyl acetate (PA)	Thr118:HN Ala171: HN	PA: O PA: O Thr118:O- PA:O	2.062 1.947 2.767	Glu53, Asp54, His114, Ile116, Ser117, Asn167, Asp 168, Ile169, Thr170, Asn223, Ile225, Asn226, Asp268, Asn269, Leu270, Ca 355	-4.73	-5.38

2- Naphthyl acetate (2-NA)	Ile57:HN Ile272:HN	2-NA: O 2-NA: O	2.364 2.066	Glu53, Asp54, Ile55, Asp56, His114, Ile116, Ser117, Thr118, Asn167, Asp 168, Ile169, Thr170, Asn223, Ile225, Asn226, Asp268, Asn269, Leu270, Ser271, Ser334, Ca 355	-6.34	-7.08
Benzyl acetate (BA)	Thr170:HG1 Ala171:HN	BA: O BA: O	2.062 1.911	Glu53, Asp54, Ile55, Asp56, His114, Ile116, Ser117, Thr118, Asn167, Asp 168, Ile169, Thr170, Asn223, Ile225, Asn226, Asp268, Asn269, Leu270, Ca 355	-5.21	-6.16
Paraoxon (PAR)	Lys46:HZ2 PAR: H	PAR: O Pro95: O	1.741 1.923	His43, Leu44, Ile45, Met88, Glu93, Lys94, Arg96, Leu341, Tyr351	-5.56	-7.95

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LIST OF PUBLICATIONS

Articles published:

1. Barathi S, Angayarkanni N, Aarthi P, Sulochana KN, Rishi P, Maneesh D, Velpandian T, Charanya, M, Muthukumaran S. Homocysteinethiolactone and Paraoxonase - novel markers of Diabetic Retinopathy. Diabetes care. 2010

2. Advanced glycation end product and GLUT-1 expression in Bovine retinal capillary endothelial cells and pericytes. (IOVS) 2010

3. Angayarkanni N, Barathi S, Seethalakshmi T, Punitham R, Sivaramakrishna R, Suganeswari G, Tarun S. Serum PON 1 arylesterase activity in relation to hyperhomocysteinaemia and oxidative stress in young adult central retinal venous occlusion patients. *Eye*. 2008;22(7):969-74.

4. Barathi S, Vadhana P, Angayarkanni N, Ramakrishnan S. Estimation of hydrogen sulphide in the human lymphocytes. *Indian J Biochem Biophys.* 2007; 44(3):179-82.

5. Narayanasamy A, Subramaniam B, Karunakaran C, Ranganathan P, Sivaramakrishnan R, Sharma T, Badrinath SS, Roy J. Hyperhomocysteinemia and low methionine stress are risk factors for central retinal venous occlusion in an Indian population. *Invest ophthalmol Vis Sci.* 2007;48(4):1441-6.

Articles in revision:

1. Comparative modeling of PON2 and analysis of its substrate binding interactions using computational methods **(jobdi)**

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2. The beneficial effect of amino acids in mitigating the effect of AGE induced angiogenesis in BREC cells

3. The role of AGE in pericytes and retinal pigment epithelial cells and the effects of amino acids.

LIST OF PAPERS PRESENTED IN CONFERENCES & AWARDS

NATIONAL:

1. Amino acids Glycine and Glutamic acid function as inhibitors of angiogenesis by down regulating VEGF expression **SBC(I) conference, Nov 2009 at NCCS Pune.**

2. The effect of amino acids on Angiogenesis on Bovine retinal endothelial cells (Oral presentation) Emerging trends in Life sciences research **March 2009 at BITS Pillani.**

3. Cytotoxic effect of Homocysteine and Homocysteine thiolactone in bovine retinal endothelial cells. **SBC [I] conference** at IIT, Chennai on 20 Dec' 08

4. "Glucose uptake in Bovine Retinal Endothelial Cell after exposure to Advanced Glycation End Products" (AGE-BSA), **76th Annual Meeting of the SBC(I)** at Sri Venkateswara University from 25th to 27th Nov' 2007.

5. "To find correlation between oxidative stress and hyperhomocysteinemia in young CRVO cases". IERG conference Hyderabad. **Indian Eye Research Group (IERG),** Hyderabad in 2005.

6. "Correlation between Hyperhomocysteinemia and oxidative stress in Age Related Macular Degeneration" at **Indian Eye Research Group (IERG)** conference, Chennai in 2004.

INTERNATIONAL:

1. Arylestarase and Homocysteine thiolactonase activity of PON1 in the vitreous of patients with proliferative diabetic retinopathy. **ASIA ARVO 2009** at Hyderabad. On 18th Jan'09

2. CRVO and oxidative stress. **Bajaj International Conference** from 4th Sept (08) at Hotel Park Sheraton, Chennai

3. Exposures of Advanced Glycation End Products (AGE) to bovine retinal capillary endothelial cell (BREC) and assess their glucose uptake. **ARVO annual meeting at Fort lauderdale,** Florida from April 27- May1, 2008.

AWARDS

1. **Dr. Ramasamy endowment award** 2004.(Anatomy and physiology)
2. **Travel grant Award** in ARVO ' 08 USA. (2008)
3. **Best Oral presentation Award** presentation in Bajaj International Conference at Chennai (2008)
4. **Travel grant** in the ASIA- ARVO Conference at Hyderabad 2009.
5. **Best poster** of the session as well as the **Best Overall poster –runner** up the ASIA-ARVO at Hyderabad, 2009.
6. **Swarnalatha Punshi Award,** Sankara Nethralaya, 2009
7. BS. Narasinga Rao **best poster award** in SBCI 2009

BRIEF BIOGRAPHY OF THE CANDIDATE

Ms. S.Bharathi obtained her B.Sc Biochemistry degree from Valliammal College, University of Madras, Chennai in 2001. She obtained her M.S. (Medical Laboratory Technology) degree of Birla Institute of Technology and Science, Pilani in 2004 with course work at Medical Research Foundation, Chennai. Then she joined the biochemistry research department, Sankara Nethralaya as Junior Scientist and worked as a junior scientist in Sankara netharalaya for 2 years and was recruited into project in 2006 project funded by Indian council of medical research. She registered for Ph. D in Jan 2006 in Birla Institute of technology & Science, Pilani. She has made presentations in 6 national and 3 international conferences, comprising 8 poster presentations and 1 oral presentation. She has 3 publications. 3 papers are in review related to thesis. 2 more papers are under preparation. She has standardized the cultivation of bovine retinal endothelial cells and pericytes from the bovine retina and she has also tried to characterize them by marker studies.

She has received "Best Paper Award" for paper "CRVO and oxidative stress" (Bajaj International Conference) 2008. She has received the "Best Poster Award" and Best Overall poster –runner up for paper Arylestarase and Homocysteine thiolactonase activity of PON1 in the vitreous of patients with proliferative diabetic retinopathy. ASIA ARVO 2009 International Conference) and also a "B S Narsing Rao Best Poster Award" for paper "Amino acids Glycine and Glutamic acid function as inhibitors of angiogenesis by down regulating VEGF expression SBC(I) conference, 2009.she also received the Best researcher award of Medical Research Foundation for the year 2008-09.She also has done observer ship in university of Illinois department of pharmacology to get trained in special techniques like Ca imaging. Her research interests are in ocular diseases like CRVO, ARMD and diabetic retinopathy.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. N ANGAYARKANNI, is currently the Reader in the Dept of Biochemistry and Cell Biology, Vision Research Foundation, Sankara Nethralaya, obtained her Ph.D. in 1995 from University of Madras in the field of Biochemistry. She had received the UGC fellowship through the GATE score of 91 percentile and had received the CSIR –SRF and the CSIR-Research Associateship. Apart from teaching the under graduate –optometry and post graduate students- MS MLT students for Biochemistry, she is also currently the guide for 3 Ph.D. students. She is currently the principal investigator for 6 major projects from DST, DBT, CSIR and ICMR. She has currently 13 publications. Her areas of interest include the extra cellular matrix changes in association with homocysteine, oxidative stress and antioxidant as well as in angiogenic and antiangiogenic factors in retinal and vitreo-retinal diseases. She also works on *in vitro* disease sequels involving retinal pigment epithelial cells and retinal capillary cells, in addition she works on Tear proteomics in Dry eye syndrome and contact lens wearers. She is also involved in patient care as Biochemist and as Quality manager- NABL. She has visited various universities such as, University of Michigan, Kellogs Eye Institute, Singapore eye research institute, University of Mexico, University of Missouri to gain knowledge in the research interests. She has recently received the ICMR overseas fellowship for young biomedical scientist to get trained in animal models of retinopathies at University of Mexico, USA in the department of cell biology and physiology. She is a permanent member of Society of Biological chemists and a current member of Association for Research in Vision and Ophthalmology and periodically presents number of research papers in the national and international conferences on Eye research.