In vitro Studies on the Mechanism of Glucose Lowering Effect of Amino Acids in CHO-K1 and Adipocytes and *In vivo* Studies on the Amelioration of Molecular Changes in Retina of Streptozotocin Induced Diabetic Sprague Dawley Rats

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CERTIFICATE

This is to certify that the thesis entitled "*In vitro* Studies on the Mechanism of Glucose Lowering Effect of Amino Acids in CHO-K1 and Adipocytes and *In vivo* Studies on the Amelioration of Molecular Changes in Retina of Streptozotocin Induced Diabetic Sprague Dawley Rats" submitted by **Ms. R. Selvi**, ID. No. 2006PHXF005 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 acids mixture
ACE	Angiotensin-I converting enzyme
AFGP	Alkyl formyl glycosyl pyrrole
AG	Aminoguanidine
AGE	Advanced glycation end-products
AGT	Angiotensinogen
AGTR1	Angiotensin II type 1 receptor
AKT	Protein kinase B (Akt/PKB)
ALI	Cross-links and arginine– lysine imidazole
ALX	Alloxan
AMP	Adenosine monophosphate
AMPK	5' Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance between groups.
aPKC	Atypical protein kinase C
APS	Ammonium per sulphate
AR2	Aldose reductase
ARVO	Association for research in vision and ophthalmology
ATP	Adenosine triphosphate-activated protein kinase
BCA	Bicinchoninic acid;
BCAA	Branched chain amino acids
BCKD	Branched chain a ketoc acid
BD FACS	Calibur four-color flow cytometry
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
C2C12	Mouse myoblast cell line
Ca2+	calcium
cAMP	Cyclic adenosine monophosphate
CAP	c-Cbl-associated protein
CAT	Cytoplasmic catalase
Cbl	Cannabinoid
CD	Cluster of difference

CHO	Chinese hamster ovary
CHO-K1	Chinese hamster ovarian-K1
CML	Carboxymethyllysine
CO2	Carbondioxide
CREB	cAMP response element-binding
CRKII	Containing adapter protein
$CuSO_4 5H_2O$	Copper(II) sulfate pentahydrate
DAPI	4',6-diamidino-2-phenylindole
DCF	Dichlorodihydo fluorescence diacetate (DCF) method
DCFDA	2', 7' Dichlorodihydrofluorescein Diacetate
DDC	Di-ethly dithiocarbamate
DEPC	Diethylpyrocarbonate
DM	Diabetes mellitus
DMEM	Dulbecco's modified eagle's m
DMO	Diabetic macular oedema
DMSO	Dimethyl sulfoxide
DR	Diabetic retinopathy
DTNB	Sodium dodecyl sulfate
dUTP	2′-Deoxyuridine, 5′-Triphosphate
EDTA	Ethyl diamine tetra acetic acid
EGM™-2 MV,	Endothelial cell growth medium BulletKito-**2**
eNOS	Endothelial nitric oxide synthase
ERC	Endosomal recycling compartment
F_{12}-K_1	Nutrient mixture HAM 12
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FS	Forward scatter
G	Glucose
G6P	Glucose 6-phosphate
GAP	GTPase-Activating Protein
GLUT1	Glucose transporter1
GLUT3	Glucose transporter 3
GLUT4	Glucose transporter 4

GPx	Glutathione peroxidase
GS	Glycogen synthase
GSH	Glutathione
GSK3	Glycogen synthase kinase 3
GSSG	Glutathione disulfide (oxidized)
GSV	GLUT4 storage vesicle
H2O	Water
HbA1C	Glycosylated hemoglobin *A1c*
HCL	Hydrochloric acid
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HGF	High-fat diet
HPLC	High performance liquid chromatography
HRP-	Horse raddish peroxidase
IBMX	3-Isobutyl-1-methylxanthine
ICAM-1	Intercellular adhesion molecule- 1
IF	Immunofluorescence
IGF- I	Insulin-like growth factor 1
IgG	Immune globulin G
IHC	Immuohistochemistry
IKK	IκB kinase
IL-1a	Interleukin-1a
IL-6	Interleukin- 6
iNOS	Inducible nitric oxide synthase
IR	IR -insulin receptor
IRAP	Insulin-regulated amino peptidase
IRS	IRS -insulin receptor substrate
IRS- I	Insulin receptor substrate- 1
IRTK	Insulin receptor tyrosine kinase
JNK	c-jun N-terminal kinase
KATP	ATP-sensitive K+
KCl	Potassium chloride
kDa	kilo Dalto
KH_2PO_4	Potassium di hydrogen phospatae

LDL	Low density lioprotein
LSS	Liquid scintillation
MAP	Mitogen-activated protein
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemotactic protein
MEK	Mitogen activated/extracellular signal regulated kinase
mg	Microgram
mg	Milligram
$MgCl_2$	Magnesium chloide
MNT	Medical nutrition therapy
mPER	Mammalian protein extraction reagent.
mPER	Mammalian Protein Extraction Reagent,
MQ water	Milli-Q-water
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl) 2,5diphenyl tetrazoliumbromide
Na^+	Sodium ions
Na_2HPO_4	Disodium hydrogen phosphate
NAC	N-acetyl cysteine
NaCl	Sodium chloride
NADPH	p-nicotinarnide adenine dinucleotide phosphate (reduced fom)
NaOH	Sodium hydroxide
NCCS	National Centre for Cell Science
NFK-B	Nuclear factor kappa-B
NIDDM	Non-insulin dependent diabetes mellitus
NO	Nitric oxide
NOS	Nitric oxide synthase
NOS2	Nitric oxide synthase 2
NOS2A	Nitric oxide synthase 2 (inducible)
NOS3	Nitric oxide synthase 2
NPDR	Non-proliferative diabetic retinopathy
PAGE	Polyacrylamide Gel electrophoresis
PBM	Preadipocyte basal medium
PDK1	Plasma membrane by activating kinases

PDR	Proliferative Diabetic Retinopathy
PH	Pleckstrin homology
PHLPP	PH domain Leucine-rich repeat protein phosphatase
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-OH-kinase
PI3K	Phosphoinositol 3 kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol(3,4,5) phosphate
PKB	Protein kinase B
PKC	Protein kinase C
PM	Plasma membrane
PMSF	Phenylmethanesulfonylfluoride
PMT	Photomultiplier tube
POP	Persistent organic pollutant
POPOP	1,4-Bis(5-phenyloxazol-2-yl)benzene
PPAR	Peroxisome proliferator-activated receptor g
PPO	2,5-diphenyl- oxazole
PRMT1	Protein arginine N-methyltransferase 1
PS	Phosphatidylserin
PTB	Protein tyrosine binding
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
RAGE	Receptor for advanced glycation end products
RIPA	Radio immune precipitate assay
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
RP-HPLC	Reverse phase high performance liquid chromatography
RPM	Revolutions per minute
RT-PCR.	Reverse transcription polymerase chain reaction
SAPK	Stress-activated protein kinase
SC	Specialized compartment‘
SD	Sprague dawely
SDS	Sodium dodecyl sulfate
SDS	Sodium dodecyl sulphate

ABSTRACT

Type 2 diabetes mellitus (T2DM) is a heterogeneous group of disorders characterized by variable degrees of impaired insulin secretion, insulin resistance and decreased glucose uptake. The worldwide prevalence of DM has risen dramatically over the past two decades. Oral glucose lowering drugs lose their effectiveness in a significant percentage of patients. Alternative or combinational therapy along with standard therapies can prove to be beneficial. Amino acids (AA) are considered to be beneficial to people with type 2 diabetes by scavenging the excess glucose in the blood (anti-glycating) or by stimulating the pancreas for insulin secretion (secretogogue) or by up-regulation of the insulin receptor system (insulin sensitizer). However there are controversies over the role of amino acids in the disease as some studies relate it to predictive diagnostics or the disease process of T2DM. Therefore more studies are warranted to understand the mechanism of AA in relation to hyperglycemia. The present study explores if amino acids as mixtures can enhance glucose uptake in CHO-K1 and adipocyte cells under high glucose conditions. Glucose of 7 mM in CHO-K1 and 5 mM in adipocyte cells are considered as normal glucose. A concentration of 12, 17, 27 mM is considered as high glucose in CHO-K1 while it is 33 mM in adipocytes cells. While the mechanistic studies were done in the CHO-K1 cells, the glucose uptake was further evaluated in the human cell line namely adipocytes. Amino acids mixture (AAM) of 2.5, 5, 10 and 20 mM were evaluated and experiments showed that 5 mM to be optimal. The mixture was constituted based on the previous experimental data in the laboratory as well as based on literature. AAM were observed to be non-cytotoxic as up to 20 mM under a high glucose of 27 mM as studied by MTT and trypan blue tests. The AAM of 5 mM increased the 2-deoxyglucose (2DG) uptake at all glucose concentration significantly in CHO-K1 cells. There was also a significant increase in the GLUT4 (glucose transporter) translocation as revealed by flow cytometer in CHO-K1 cells. The AA mixture showed a synergistic effect with insulin and presence of AA alone in the absence of insulin did not promote GLUT 4 translocation. Amino acids, thus increases the glucose (G) uptake under high glucose conditions. The present study explores further, in elucidating the signaling pathway involved in the glucose uptake, in CHO-K1 cells. The addition of 5 and 20 mM AAM increased the IRTK and PI3K activity significantly with increase in glucose concentration (ANOVA $p=0.025$, $p=0.003$ respectively). Addition of 5 mM AAM in the presence of normal glucose

significantly increased the levels of phosphorylated Akt Ser473 *(p=0.02)* with no significant change at high glucose. At 20 mM AAM there was a significant decrease in Akt phosphorylation *(p=0.035)* associated with high glucose concentration showing that the addition of AAM to high glucose exposed cells and does not warrant cell survival signaling unlike the cells exposed to high glucose alone without AAM where the AKT expression was high. In order to substantiate this, apoptosis was evaluated under same conditions. High glucose was found to increase the number of apoptotic cells while addition of 5 mM AAM decreased the same significantly as evaluated by FACS analysis using annexin V staining *(p<0.05)*. Since AAM addition in high glucose is not associated with Akt expression, the increased glut 4 translocation and therefore the glucose uptake is inferred to be independent of Akt pathway. AAM (5 mM) increased the glycogen synthase activity at all concentrations of glucose *(p<0.05)* further showing that there is increased glucose uptake as well as utilization.

In order to associate the specific amino acid in the mixture to the increase in glucose uptake, the effect of the same on glucose uptake was studied. Labeled free AA was used to study the AA uptake. AA transport was observed to be influenced by the insulin, presence of the other AA and glucose. With basic amino acids, lysine entry was more than arginine, among branched isoleucine was more than leucine and among acidic glutamic acid was more than aspartic acid as common transporters function for similarly charged amino acids. Glucose uptake was increased with the addition of not only AAM but also free amino acids namely glutamic acid, isoleucine, lysine, alanine and arginine significantly in CHO-K1 cells and the effects vary with the concentration of free AA and the level of glucose. However AAM, irrespective of the levels of high glucose showed significant uptake. It is inferred that AAM is preferable over that of any single free AA as it can derange the metabolism apart from uptake of other amino acids in cells. Evaluation of glucose uptake by free and AAM in adipocytes also revealed increase in uptake of which lysine showed statistical significance *(p=0.05)* when compared to high glucose alone. At 33 mM high glucose, the free amino acids as well as mixture form was observed to promote the translocation of GLUT4 in the high glucose concentration as seen by the fluorescence stain for GLUT4 protein. Enhancement of the expression of GLUT4 is beneficial in the context of insulin resistance.

Uncontrolled hyperglycemia for longer durations results in diabetic complication such as liabetic retinopathy (DR). Hyperglycemia induced ROS generation is one of the major nechanism of development of DR which is characterized by neovascularization attributed to ncreased VEGF. Increased ROS generation under high glucose conditions was observed in CHO cells as reported *(p<0.05)*. Addition of AAM significantly reduced the ROS levels in high glucose *(p<0.05)*. Free AAs also showed lowering of the ROS which varied with the type of AA oncentration and with the glucose concentration. Addition of AAM, rather than free form hows significant *(p<0.05)* decrease of NO levels which could be beneficial in decreasing /EGF. Lysine showed significant increase in NO levels (2 fold), which could be beneficial actor for vasodilation. In order to see if the increase in ROS/ NOS is associated with the changes n SOD activity, the cellular SOD activity was estimated under high glucose condition. With 17 nM glucose there was an increase with 2 fold while in 27 mM glucose there was a 30 % ecrease compare to normal glucose. Addition of AAM showed a fall in SOD activity with espect to glucose (7mM *p=0.01*, 17 mM G *p=0.006*, 27 mM G *p= 0.009*). Thus there is a net xidative stress under high glucose condition with increase in ROS & NO associated with ncrease in SOD which is now reversed in the presence of AA and the anti-oxidant potential of A is implied. Addition of AAM showed significant decrease in the levels of VEGF at all oncentration of glucose (7 mM G: *p=0.004*, 17 mM G: *p=0.037* & 27 mM G: *p=0.004*). ddition of free AA to 17 mM glucose, alanine, leucine, isoleucine showed significant fall *p<0.05)* and addition of alanine, leucine, isoleucine showed significant fall (*p<0.05)*. Addition f arginine & lysine showed fall in VEGF when added to 27 mM G (*p=0.01)*. The study in the HO-K1 cells also hinted on the possible anti-angiogenic effect of AAM as seen by decrease in EGF with increase in glucose. The effects of amino acids in ameliorating the retinal changes in TZ induced diabetic retinopathy rat model were studied. High glucose condition causes ycation of proteins, forming advanced glycation end products (AGE). AGEs relate to their teraction with a variety of cell-surface receptors, leading to cellular pro-oxidant and pro-flammatory events. Based on the previous in vitro studies on the anti-glycating property of AA, is study was extended to animal model. After diabetic induction the diabetic group showed an crease in glucose level with a mean level of 511 ± 60 mg/dl and HBA1C of 7.8 % compared to e control of 84 ± 20 mg/dl with a HBA1C level of 5.1 % as seen at the end of 8 weeks. In the pplemented groups, AAM1 showed the lowest blood sugar level (392 ± 151 mg/dl, 30 % fall)

vith HbA1c of 7.1 %. In addition the retinal homegenate was analysed for the molecular hanges such as in AGE, eNOS, NO, cytokines and ICAM that are characteristic of diabetic etinopathy. A significant increase in AGE level were observed when as expected in diabetes in erum *(p<0.05)*, lens and retina (p<0.001). A significant fall in AGE levels in the treated group f composition AAM1 in serum *(p=0.008)*, lens *(p<0.01)* and retina *(p<0.05)* was observed. Diabetic group shows a strong overall positivity for CML-AGE, IL-1β, IL-2 while the treated roup AAM1 and AAM2 was found to be comparable to that of the untreated control thereby howing AAM are potential anti-glycating agents and are also anti-inflammatory in nature. A ignificant increase in eNOS/NO was observed in the retinal homogenate of the diabetic rat when ompared to the controls *(p<0.01)* whereas in the treated groups namely AAM1 a significant fall n the eNOS / NO levels were observed when compared to diabetic group *(p<0.05)*. Increase in ICAM was observed in the untreated STZ induced diabetic rat when compared to the control gnificantly *(p<0.05)*. The treated groups with AAM1 showed a significant fall in the sICAM vels when compared to diabetic group (50 % decrease), thus indicating that AAM can prevent ascular permeability changes in the retina which is again characteristic of DR. Apoptotic cell eath of retinal neurons is reported in DR. The number of $TUNEL^+$ cells shows significant crease in diabetic retina compared to control *(p<0.01)* and decrease in AAM1 *(p< 0.01;* 70 % ll*)*, AAM2 *(p< 0.05)*. Thus the study indicates that among the two compositions, AAM1 seems be more effective as anti-diabetic (Blood glucose, HbA1c lowering), anti-glycating (decreased GE), anti-oxidant (NO lowering), anti-inflammatory (decreased cytokines), preventing vascular rmeability (decreased ICAM) and anti-apoptotic (reduced TUNEL positivity).

s amino acids are non-toxic and physiological, based on the above results, preclinical studies in uman subjects with T2DM can be done to evaluate the anti-diabetic property of amino acids ixture.

CHAPTER 1: REVIEW OF LITERATURE

1.1 DIABETES MELLITUS

Diabetes Mellitus (DM) is a chronic metabolic disorder that has a significant impact on the health as well as the quality of life of patients. It occurs when the pancreas does not produce enough insulin (insulin insufficiency) or when the body cannot effectively use the insulin (insulin resistance). Uncontrolled hyperglycaemia can lead to serious complications of the macrovascular and the microvascular system resulting in Diabetic retinopathy, neuropathy and nephropathy (American Diabetes Association, 2012). While autoimmune destruction of β-cell is responsible for type 1 DM (T1DM), type 2 DM (T2DM) accounts for more than 90-95 % of the DM and they have relative rather than absolute insulin deficiency (2006)(American diabetes association).

1.1.1 The global burden of diabetes

Diabetes is increasing at an alarming rate. In 1998 prevalence of diabetes in adults worldwide was estimated to be 4.0 % and was predicted to rise to 5.4 % by 2025 (King, Aubert et al. 1998). Later the world prevalence of diabetes among adults was predicted to be 6.4% in 2010 that shall increase to 7.7 %, i.e., 439 million adults by 2030 (Shaw, Sicree et al.). India has the highest number of T2DM cases are reported as 31.7 million in the year 2000 and predicted to be 79.4 million in the year 2030 (Wild, Roglic et al. 2004; Mohan, Mathur et al. 2008) from a nationwide surveillance . The study of T2DM shows that urban areas has a prevalence of 7.3 % of known T2DM while it is 3.2 % in peri-urban/slum areas. This causes a huge economic burden to the society. The study reports that the total amount needed for India to treat T2DM is around 2.2 billion USD (Ramachandran, Ramachandran et al. 2007). Moreover in developing countries like India, the brunt of diabetes and cardiovascular disease occurs among the economically productive age group of 20-45 years (2005)(American diabetes association).

1.1.2 Prevention and treatment

Exercise and diet apart from oral hypoglycemic agents are the effective means of improving glucose homeostasis in T2 DM. A consensus panel felt that metformin should be the only drug considered (Nathan, Davidson et al. 2007). For other drugs, the issues of cost, side effects, and lack of persistent effect led the panel not to recommend their use for diabetes prevention. As an alternative approach, medicinal herbs with antihyperglycemic activities are increasingly promoted by health care professionals and also sought by the Diabetics (American diabetes association 2010). The care of patients with T2DM has been profoundly shaped by the results of the United Kingdom Prospective Diabetes Study (UKPDS) study confirmed the importance of glycemic control in reducing the risk (by 25 %) for microvascular complications.

1.1.3 Pharmacological Treatment and Limitations

1.1.3.1 Oral Glucose-Lowering Drugs

In the United States, five classes of oral agents are approved for the treatment of type 2 diabetes. The drug categories include sulfonylureas, biguanides, alpha-glucosidase inhibitors, thiazolidinediones, and meglitinides. Although initial responses may be good, oral hypoglycemic drugs may lose their effectiveness in a significant percentage of patients (DeFronzo 1999) Kelly,1995; UKPDS 1998) **(Table 1.1)**.

Table 1.1: Limitations of current drug therapies.

Drugs	Effects	Limitations
Sulfonylureas	Enhances insulin secretion	Weight gain due to hyperinsulinemia
Biguanides (Metformin)	➢ Inhibition of hepatic glucose production and increase of muscle glucose uptake. ➢ Reduces plasma triglyceride and LDL-cholesterol levels.	Weakness, fatigue, shortness of breath, nausea, dizziness, lactic acidosis, and kidney toxicity.
Alpha-glucosidase inhibitors (Acarbose)	Decreases postprandial glucose levels by interfering with carbohydrate digestion and delaying gastrointestinal absorption of glucose.	Gas, bloating, and diarrhea.
Thiazolidinediones (Troglitazone, Rosiglitazone and Pioglitazone)	➢ Improving insulin sensitivity in muscle. ➢ Decrease plasma triglyceride levels.	Liver toxicity
Meglitinides (Repaglinide)	Insulin secretion	Weight gain, gastrointestinal disturbances, and hypoglycemia.
Insulin	➢ Increases glucose uptake. ➢ Inhibits breakdown of triglycerides in adipose. ➢ Inhibits gluconeogenesis in liver.	Weight gain and hypoglycemia, atherogenesis.

1.1.3.2 Conventional Approach to Diet Therapy

A typical conventional approach would recommend a diet composed of 60-65 % carbohydrate, 25-35 % fat, and 10-20 % protein, with limited or no alcohol consumption (Schlichtmann, Graber et al. 1997).

1.1.3.3 Alternative Approaches

Alternative treatments for diabetes have become increasingly popular including medicinal herbs, nutritional supplementation, acupuncture, particularly in India. Ideal therapies should have a similar degree of efficacy without side effects.

Medicinal Herbs: Many conventional drugs have been derived from prototypic molecules in medicinal plants. Metformin development was based on the use of *Galega officinalis* (Grover, Yadav et al. 2002) rich in guanidine, the hypoglycemic component. The root of ginseng slows the digestion of food, decreasing the rate of carbohydrate absorption into portal hepatic circulation. Ginseng may affect glucose transport, which is mediated by nitric oxide (NO) (Roy, Perreault et al. 1998) and it may modulate NO-mediated insulin secretion. The most commonly reported side effects of ginseng are nervousness and excitation (Yuan, Wu et al. 1998). Other anti-diabetic beneficial herbs includes *Momordica charantia* (Bitter Melon), *Trigonella foenum graecum* (Fenugreek), *Gymnema sylvestre* (Gurmar), *Allium cepa* (onions), *Allium sativum* (garlic), *Vaccinium myrtillus* (Bilberry) and *Aloe vera*, though there are many more (Dey, Attele et al. 2002).

Nutrients Supplementation: The treatment of diabetes requires nutritional supplementation, as these patients have a greatly increased need for many nutrients. Supplying the diabetic with additional key nutrients has been shown to improve blood sugar control as well as help prevent or ameliorate many major complications of diabetes **(Table 1.2)**. Diet control may result in the restriction of these micronutirients. Therefore supplements can be important. However, currently, there are no large, convincing studies that prove the benefit of specific micronutrients in the management of diabetes (2007) (American Diabetes Association).

1.1.3.4 Protein supplementation

Several studies are supportive of beneficial effects of additional protein intake, along with low carbohydrate/energy diet apart form the oral hypoglycemic agents in T2DM (Gougeon, Styhler et al. 2000). Glucose that is absorbed after the digestion of glucose-containing foods is largely responsible for the rise in the circulating glucose concentration after ingestion of mixed meals. Dietary proteins, fats, and absorbed fructose and galactose resulting from the digestion of sucrose and lactose, respectively, have little effect on blood glucose concentration. Peripheral plasma glucose concentration is reported to be decreased after ingestion of protein due to a modest increase in the rate of glucose disappearance and an increase in circulating insulin was observed (Gannon, Nuttall et al. 2001). A study report on a diet in which the protein content was increased from 15 to 30 % of total food energy, with a corresponding decrease in carbohydrate content showed a moderate but highly statistically significant mean decrease in glycohemoglobin (8.1 - 7.3 %) after 5 weeks on the diet (Gannon and Nuttall 2004). Kinetics of whole-body protein metabolism is elevated. Protein degradation appears to be increased by hyperglycemia and improved by controlling glucose levels. Protein requirements for people with T2DM is now reported to be slightly greater than those for non-diabetic individuals (Gougeon, Styhler et al. 2000).

1.2 INSULIN SIGNALING CASCADE

The pathogenesis of type 2 diabetes is complex, involving progressive development of insulin resistance and a relative deficiency in insulin secretion, leading to overt hyperglycemia.

1.2.1 Insulin

To a large extent, glucose homeostasis and fuel metabolism within the body falls under the regulation of the hormone insulin. Insulin is synthesized and released from the β-cells of the pancreas in response to a rise in plasma glucose concentrations (Cheatham and Kahn 1995). In the plasma, insulin normally circulates at a concentration of 10^{-10} to 10^{-9} M.

Insulin is synthesized as preproinsulin which is inactive molecule composed of 108 amino acids residues and possesses an amino terminus "Signal sequence of 24 amino acids residues. The signal sequence directs translocation of preproinsulin to the Endoplasmic reticulum where it is

packed into secretory vesicles. The proteolytic removal of signal sequence and formation of 3 disulfide bonds results in the formation of smaller molecule (proinsulin, 9000 Da composed of 84 amino acids residues. Proinsulin contains α- and β- chains linked by a connecting sequence (C-peptide) composed of 33 amino acids residues.

When elevated blood sugar triggers insulin secretion, proinsulin is instantly processed in the Golgi complex to remove C-peptide with the help of specific peptidase. Enzymatic removal of C-peptide leads to biologically active insulin with α- β held together by 2 interchain disulphide bond (Ashcroft,1990).

1.2.1.1 Control of insulin secretion

The β-cells of pancreas responds to three classes of physiological stimulants- metabolites, hormones and neurotransmitters. Initiation of secretion by glucose involves the metabolism of the glucose and a rise in (Ca^{2+}) via inhibition of K-ATP channels. Activation of Calmodulin-dependent protein kinase leads to phosphorylation of key components of the release system and exocytotic release of insulin. In addition, in response to hormones and Neurotransmitters, the classical second messenger systems involving inositol phospholipid turn over and adenine cyclase activation generates signals for initiator pathway. Insulin release can be elicited when intra cellular calcium levels are increased by depolarization with extra cellular K^+ **(Fig 1.1)** (Ashcroft, 1990).

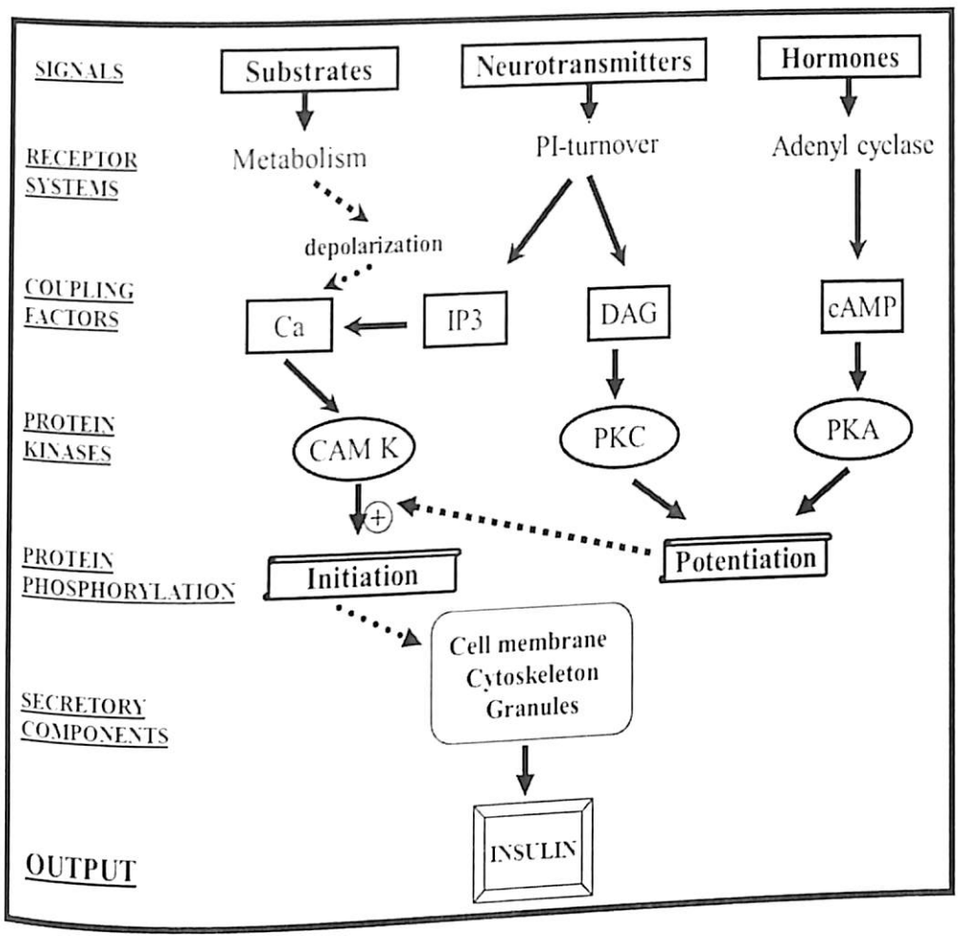


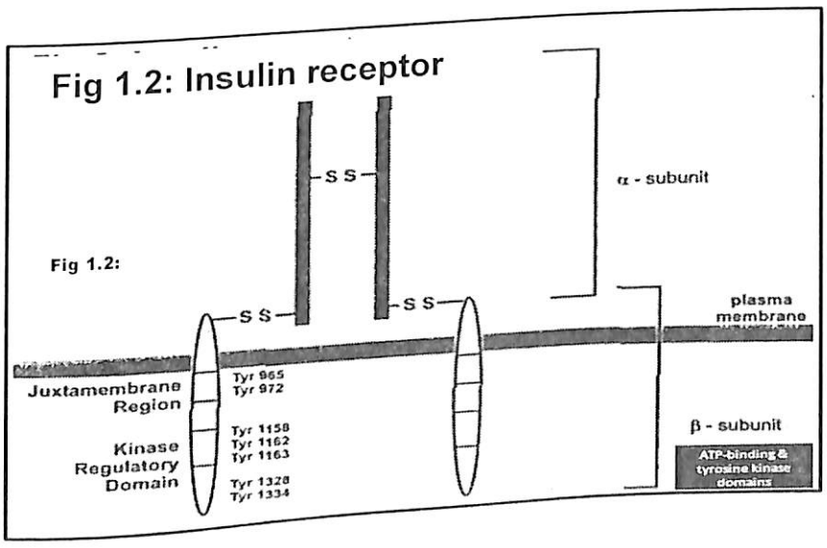
Fig 1.1 : Insulin secretion

1.2.1.2 Biological actions of insulin

As a major function insulin acts upon peripheral insulin sensitive tissues such as muscle, adipose, and liver with diverse biological action (Saltiel and Kahn 2001; Reiter and Gardner 2003). Glucose uptake is achieved at the cellular level in muscle and fat by insulin's ability to cause a translocation of glucose transporters (primarily GLUT4) from an intracellular storage compartment to the plasma membrane (Stephens and Pilch 1995; Holman and Kasuga 1997; Rea and James 1997). Once fused with the plasma membrane, these glucose transporters facilitate the uptake of glucose from the circulation into the cell, thus glucose is free to undergo glycolysis, glycogen synthesis, and other anabolic reactions. This translocation of glucose transporters is achieved through a complex cellular signaling cascade which begins with insulin binding to its specific plasma membrane receptor and then proceeds via several different downstream enzymes and proteins.

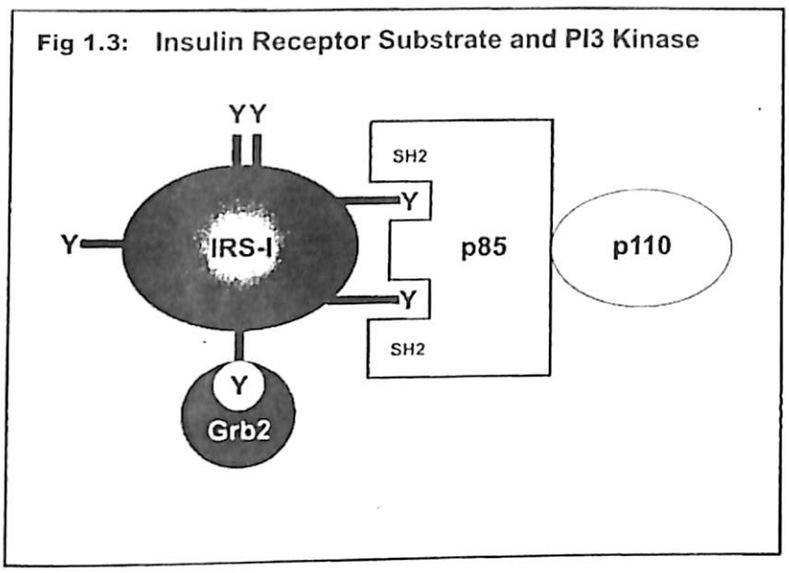
I.2.2 Insulin receptor tyrosine kinase

The insulin receptor is expressed in almost all mammalian tissues although the number of receptors per cell varies from tissue to tissue. They are most abundant on the insulin sensitive tissues adipose, muscle, and liver (Kahn, Baird et al. 1981; Cheatham and Kahn 1995). The insulin receptor consists of two α-subunits and two β-subunits linked through disulfide bonds to form a heterotetramer **(Fig 1.2).** The α-subunits, which are located entirely extracellularly, contain the ligand or insulin binding domain while the β-subunits contain a tyrosine kinase domain. The β-subunit is composed of a short extracellular domain, a 23 amino acid transmembrane domain, and an intracellular domain which has an ATP binding site and tyrosine kinase activity. Insulin binding causes the autophosphorylation of the insulin receptor. The autophosphorylation occurs on seven possible tyrosine residues. Three of these residues, Tyr^{1158}, Tyr^{1162}, and Tyr^{1163}, comprise the kinase regulatory domain which upon phosphorylation, are responsible for further activation of the tyrosine kinase activity of the insulin receptor. There are at least four other possible tyrosines (two in the juxtamembrane region and two at the C-terminal) which may play important roles upon their phosphorylation (Cheatham and Kahn 1995). The importance of the C-terminal phosphotyrosines lies in their ability to act as binding or docking sites for several src homology 2 (SH2) domain containing proteins (Sattar, Berhanu et al. 2007). Tyr^{972} within the juxta membrane region is the phosphotyrosine which plays an important role in insulin stimulated glucose transport (White, Livingston et al. 1988). The insulin receptor is also known to contain many sites of serine and threonine phosphorylation and phosphorylation of these residues may decrease or inhibit the insulin receptor tyrosine kinase (IRTK) activity (Shao, Catalano et al. 2000; Kido, Nakae et al. 2001).



1.2.3 Insulin Receptor Substrate

Immediately downstream of the insulin receptor lies a protein termed the insulin receptor substrate (IRS) (Pederson, Kramer et al. 2001). IRS-1, a protein of approximately 185kDa when fully phosphorylated, associates with the insulin receptor through its protein tyrosine binding (PTB) domain which functions by binding to the phosphorylated tyrosines on the insulin receptor (specifically tyr^{972} in the juxtamembrane region) **(Fig 1.3).** Tyr^{972} appears to play a specific role in binding downstream substrates of the insulin receptor. As mutation of this particular tyrosine residue did not affect insulin binding, autophosphorylation, or kinase activation. but it did prevent phosphorylation of IRS-1 indicating that there was no association between the two molecules (White, Livingston et al. 1988). Another protein domain which has been postulated to play an important role in the association between IRS-1 and the insulin receptor is the pleckstrin homology (PH) domain which is linked to the amino-terminus of the PTB domain (Yenush, Makati et al. 1996). PH domains function by binding to phosphoinositides and are speculated to play a role in targeting molecules to different cell membranes (Lemmon, Ferguson et al. 1996). In addition to these various protein modules, IRS-1 contains greater than 20 potential tyrosine phosphorylation sites which are conserved between IRS-1 homologs. When phosphorylated, these tyrosine residues would serve as a potential binding site for many proteins which contain a src hornology 2 (SH2) domain. SH2 domains are protein modules of approximately 100 amino acids which recognize and bind specific phosphotyrosine residues. Thus, IRS-1 serves as a docking protein which is capable of recruiting several other proteins into its signaling cascade via its phosphotyrosines. IRS-I is also known to contain approximately 40 potential sites of serine and threonine phosphorylation (Pederson, Kramer et al. 2001). Upon insulin stimulation, IRS- 1 undergoes an increase in both tyrosine and serine phosphorylation (Sun, Rothenberg et al. 1991). IRS-2 is similar to IRS-1 both structurally and functionally although there are slight, and possibly, significant differences. Like IRS-1, IRS-2 is also phosphorylated by the activated insulin receptor and is therefore capable of causing the translocation of glucose transporters to the ce11 surface, resulting in a net influx of glucose into the cell (Sesti, Federici et al. 2001)



1.2.4 Phosphoinositide 3-kinase

Phosphoinositide 3-kinase (PI3K) which is downstream of IRS is made of two different subunits, the p85 adaptor or regulatory subunit and the p110 catalytic subunit (Hirsch, Costa et al. 2007). The p85 subunit is known to contain two SH2 domains which allow it to associate with phosphotyrosines on either the IRS-1, IRS-2, or growth factor receptors (Holman and Kasuga 1997). In addition to its primary ability to phosphorylate phosphoinositides, the p110 catalytic subunit is known to have serine kinase activity towards the p85 subunit and IRS-1 which may serve as an autoregulatory feedback mechanism to control the association between these two proteins and possibly to regulate the catalytic activity of the p85/pl10 complex (Dhand, Hiles et al. 1994). Activation of PI3-kinase begins with the tyrosine phosphorylation of the receptor or IRS-1. Phosphorylation of either of these molecules on a tyrosine within the specific YXXM motif facilitates the association between these phosphotyrosines and the two SH2 domains of the p85 subunit (Fu, Aronoff-Spencer et al. 2003). This association or recruitment of the p85 subunit to the phosphotyrosines then leads to activation of cellular PI3-kinase activity through an increase in the catalytic activity of the p110 subunit and by moving the PI3-kinase complex in close proximity to membranes, and therefore to its substrate. Stimulation of cellular P13-kinase activity results in a transient increase in the intracellular levels of PIP3 (Hirsch, Costa et al. 2007).

Four different classes of PI3K are expressed in mammalian cells; depending on their mechanisms of activation, they can be divided into two subtypes: class IA PI3K (PI3K α,β,δ) are

activated mainly by membrane-bound tyrosine kinase receptor. All class of PI3K phosphorylate PIP2 to produce PIP3, a secondary messenger membrane lipid that functions as a docking site to a large number of proteins containing the pleckstrin homology (PH) domain. Class I PI3K exert their large number of biological functions because of their ability to function as docking sites for proteins that contain the pleckstrin homology (PH) domain (Lemmon, Ferguson et al. 1996). Such PH domains are found in Akt, that specifically bind PIP3.

1.2.5 Protein kinase B (Akt)

The protein kinase B (PKB) belongs to AGC kinase (cAMP-dependent, cGMP-dependent and protein kinase C) family and is involved in innumerable cellular signaling circuits. PKB, also known as Akt, regulates cell survival, proliferation and cell growth and is deregulated in many cancers (Fayard, Tintignac et al. 2005). Akt plays a central role in regulation of metabolism, cell survival, motility, transcription and cell-cycle progression. It comprises three mammalian isoforms, PKBα, PKBβ and PKBγ (Akt1, Akt2 and Akt3, respectively). The main function of AKT is to catalyze the phosphorylation of serine and threonine residues at consensus phosphorylation sites in target (Scheid and Woodgett 2001; Hanada, Feng et al. 2004) **(Fig 1.4).**

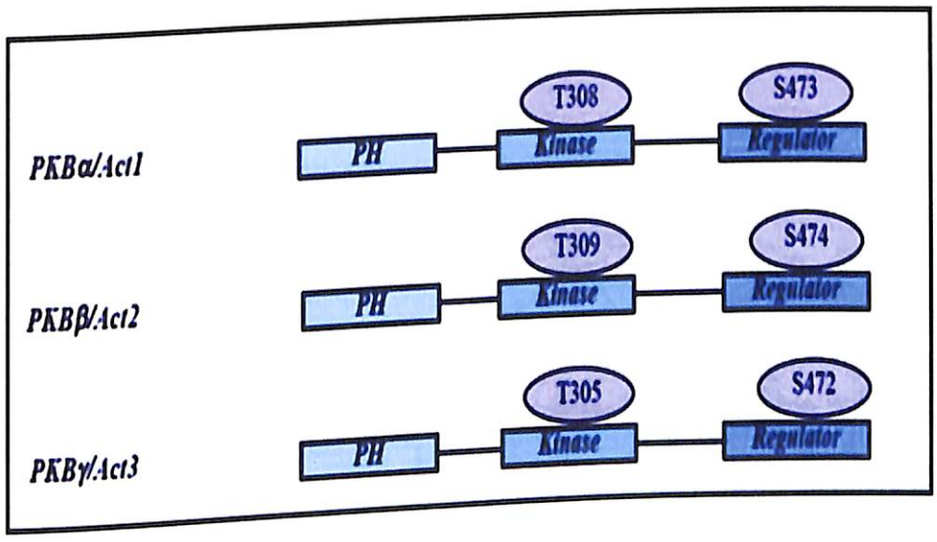


Fig 1.4: Domain structure of human Akt/PKB isoforms

The biologic effects of Akt activation are mediated by the functional consequences of Phosphorylation and activation of Akt/PKB.

Once PI3-Kinase is activated, PIP2 generates the second messenger PIP3. PIP3 levels are tightly regulated by the action of phosphatases such as PTEN, which removes phosphate (Wymann, Zvelebil et al. 2003). PIP3 recruits Akt/PKB to the plasma membrane **(Fig 5)**. Once recruited to the plasma membrane, PKB is activated by phosphorylation (Simpson and Parsons 2001). Although phosphorylation at T308 partially activates Akt/PKB, full activation of Akt/PKB requires phosphorylation on a second site (S473 in PKBα/Akt1) located in the regulatory tail. Phosphorylation of Ser473 is the key step in the activation of PKB because it stabilizes the active conformation state (Alessi, Caudwell et al. 1996). Once activated at the plasma membrane, phosphorylated PKB translocates to the cytosol or the nucleus (Yang, Cron et al. 2002).

1.2.5.1 Downstream effectors of Akt

Activated PKB/Akt, phosphorylate a wide variety of cellular signaling molecules relevant for the regulation of cell growth, cell cycle and cell proliferation, metabolism **(Fig 1.5).**

- **Transcription factors** - NF-kB, forkhead/AFX, CREBand p53.
- **Mediators of apoptosis** - Bad and caspase 9.
- **Cell cycle regulators** - p21, p27, mTOR and cyclin D1.

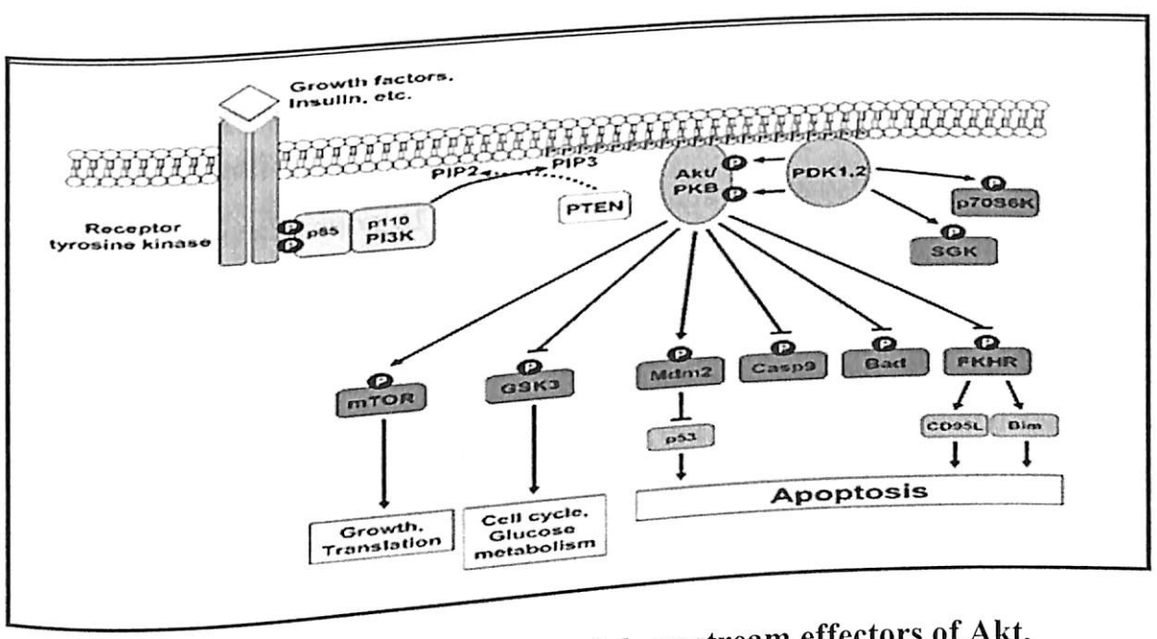


Fig 1.5: PI3K recruiting Akt and downstream effectors of Akt.

1.2.5.2 Akt in glucose metabolism

Akt/PKB regulates several levels of glucose metabolism **(Fig 1.6)**. It enhances glucose uptake in insulin-responsive tissues by affecting the glucose transporters GLUT1, GLUT3, and GLUT4. Akt/PKB induces the expression of GLUT1and GLUT3 and the translocation of GLUT4 to the plasma membrane. It activates glycogen synthesis through the inactivation of GSK3, which in turn leads to activation of glycogen synthase (Kandel and Hay 1999).

Akt2 constitutively active by membrane targeting mimics insulin in eliciting GLUT4 translocation and high levels of glucose transport in the absence of hormone. AS160, a novel Akt Substrate in adipocytes contains phosphorylation sites that are regulated in response to insulin in a PI 3-kinase-dependent mechanism (Sakamoto and Holman 2008).

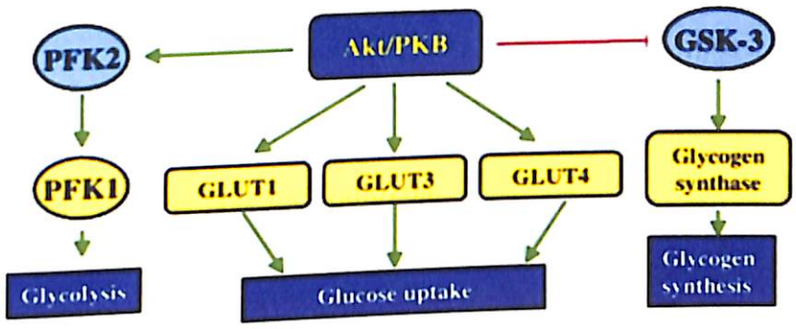


Fig 1.6: Akt/PKB effects glucose metabolism via inhibition of GSK3.

1.2.6 Glucose Transporters

One of the end metabolic effects of insulin is an increased flux of glucose into the cell which is achieved through a translocation of glucose transporters to the cell surface. Insulin stimulates cellular uptake of glucose in its target tissues (Kandror and Pilch 1996; Ceddia, Somwar et al. 2005). There are two types of glucose transporters sodium dependent glucose transports (SGLT) and facilitative glucose transporters (GLUT 1-4). The SGLT which are found in the intestinal tract and kidney are not influenced by insulin. SGLT in the intestinal mucosa of the small intestine and the proximal tubule of the nephron contributes to the renal glucose reabsorption. The Na+/K+ ATPase pump transports sodium from this cell into the peritubular capillary, this creates the sodium gradient inside the cells. SGLT uses the energy from this sodium gradient created by ATPase pump to transport glucose across the membrane against the concentration gradient (Wright, Hirayama et al. 2007).

1.2.6.1 GLUT and its isoforms

The GLUTs are intrinsic membrane proteins which differ in tissue-specific expression and response to metabolic and hormonal regulation. Isoforms of 13 GLUT have been identified. At present the best characterized member of this family are the class-I glucose transporters (GLUT 1-4) differ in their tissue expression, substrate specificity and kinetic characteristics **(Table 1.3)**.

- Glut1 – mediates glucose transport into red cells, endothelial cell of blood barrier tissue, colon, kidney, placenta, adipose tissue, etc.
- Glut2 - provides glucose to liver, kidney, intestine and pancreatic cells, has relatively low affinity for glucose.
- Glut3 – expressed during fetal development, adult neurons, brain, placenta and kidney and has highest affinity for glucose.
- Glut4 – expressed in brown and white fat, skeletal and cardiac muscle and other insulin sensitive cells.
- GLUT5 - small intestine and spermatozoa.

Table 1.3: Various isoforms of GLUT and its tissue distribution.

Name	Tissue location	Km	Role
GLUT1	All mammalian tissues	1 mM	Basal glucose uptake
GLUT2	Liver and pancreatic β cells	15-20 mM	In pancreas – regulation of insulin. In liver – removes excess glucose from blood.
GLUT3	All mammalian tissues	1 mM	Basal glucose uptake
GLUT4	Muscle and fat cells	5 mM	Amount in muscle plasma membrane increases with endurance training.
GLUT5	Small intestine	--	Primarily a fructose transporter

1.2.6.2 Translocation of GLUT4 to plasma membrane

Only GLUT-4 has been found to be regulated by insulin. In the absence of insulin almost all GLUT-4 is found in the cytoplasmic vesicular pool. Insulin induces a rapid increases in translocation of GLUT-4(10 fold) from the intracellular vesicular pool to the plasma membrane which results in a 20-30 fold increase in the rate of glucose uptake (Martin, Lee et al. 2006). In the absence of insulin GLUT4 is rapidly eliminated from the plasma membrane to which it recycles only slowly, sequestering greater than 90% of GLUT4 in the intracellular vesicle membrane.

Attenuated GLUT4 translocation and glucose uptake by muscle and fat cells following insulin stimulation represent a prime defect in insulin resistance. Targeted disruption of GLUT4 selectively in muscle result in insulin resistance and glucose intolerance, demonstrating that GLUT4-mediated glucose transport in muscle is essential to the maintenance of glucose homeostasis (Zisman, Peroni et al. 2000). All of the intracellular GLUT4 of muscle cells recycles to the plasma membrane within 6 hours. GLUT4 molecules from the plasma membrane reach the endosomal recycling compartment (ERC) within 20 min and rapidly exit this compartment 20 min later (Foster,2001). GLUT4 cycles between the intracellular compartments and the plasma membrane. Approximately half of intracellular GLUT4 is located in the 'GLUT4 specialized compartment' (SC) or 'GLUT4 storage vesicle' (GSV), which is segregated from the endocytic recycling compartment (ERC) and the trans-Golgi network (TGN). Insulin-regulated exocytosis pathway of GLUT4 is divided into (1) Glucose transporters "stored" within cell in membrane vesicles. (2) When insulin interacts with its receptor, vesicles move to surface and fuse with the plasma membrane, increasing the number of glucose transporters in the plasma membrane. (3) When insulin levels drops, glucose transporters are removed from plasma membrane by endocytosis, forming small vesicles and (4) The vesicles dock and fuse with the plasma membrane and ready for exocytosis. (5) The smaller vesicles fuse with larger endosome. Patches of the endosome enriched for glucose transporters bud off to become small vesicles, ready to return to the surface when insulin levels rise again. Each step of the GLUT4 traffic may be regulated by insulin-derived signals (Ishikura, Koshkina et al. 2008) **(Fig 1.7).**

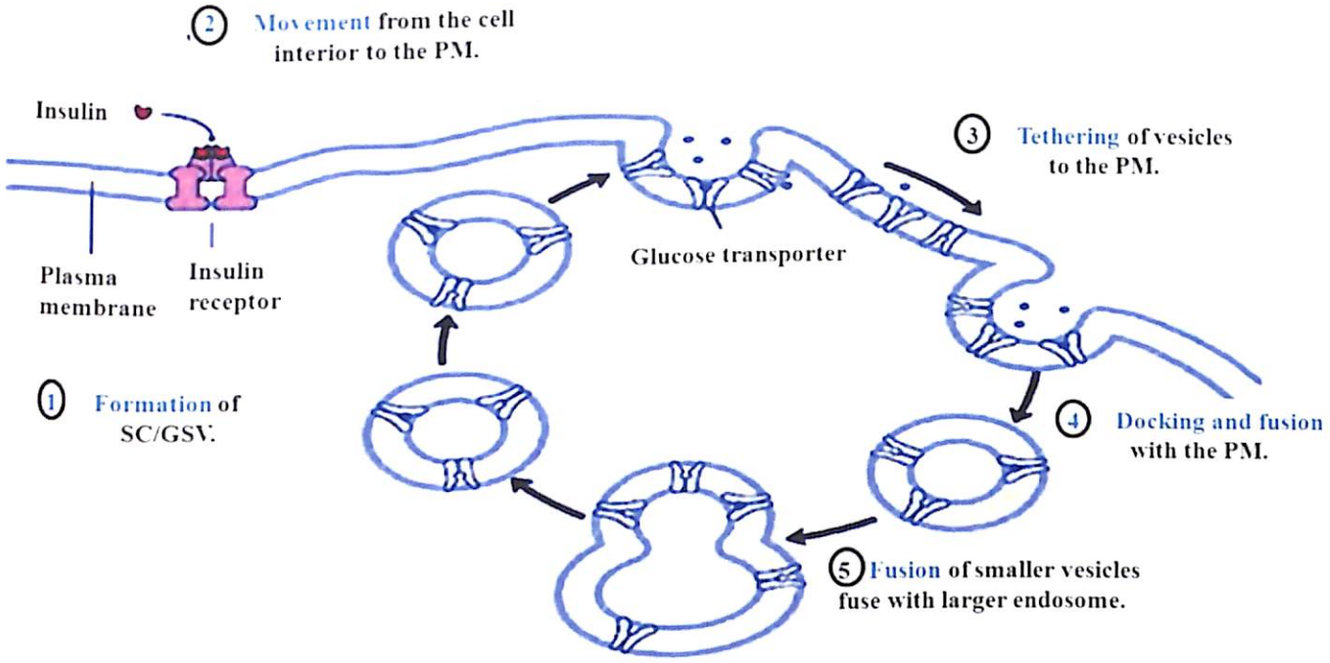


Fig 1.7: Schematic representation of GLUT4 traffic in muscle and fat cells.

1.2.6.3 Various signals for GLUT-4 activation by the insulin

PI3K dependent GLUT4 traffic

Class IA PI 3- kinases play a pivotal role in the translocation of GLUT4-containing vesicles from endosomal compartments to the plasma membrane by activating kinases PDK1, PKB (Akt) and atypical protein kinase C (αPKC) forms PKC-λ and PKC-ζ (Liu, Yang et al. 2007) **(Fig 1.8).**

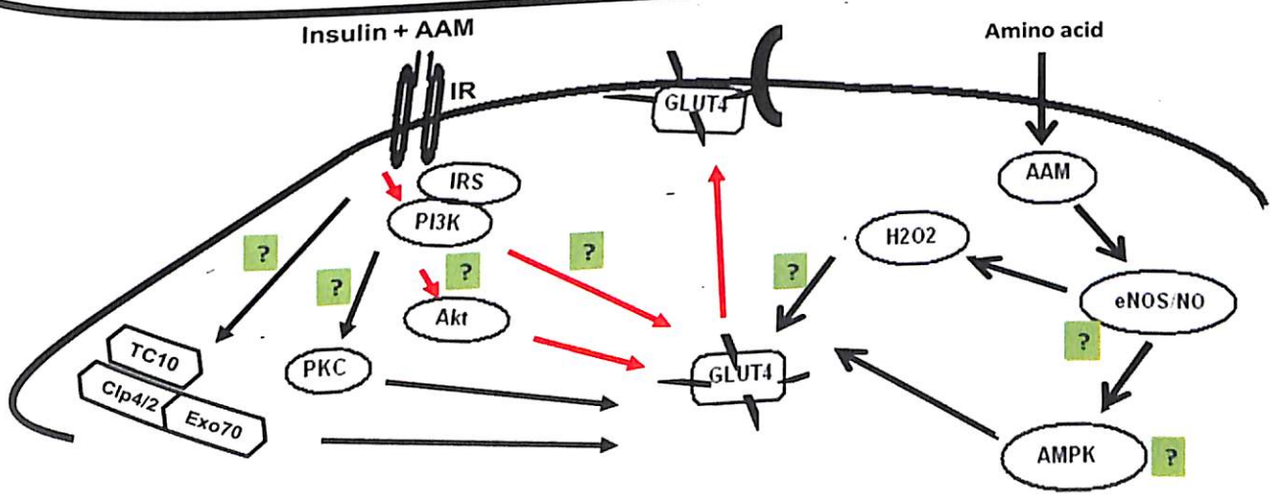


Fig 1.8: Various signals for GLUT4 translocation to plasma membrane.

PI3kinase independent pathway

a) GLUT4 traffic is also regulated via PI3kinase independent pathway. Insulin stimulates tyrosine phosphorylation of c-Cbl in the metabolically responsive cells. C-Cbl is recruited to complex with insulin receptor via the adaptor protein CAP (c-Cbl-associated protein). Upon Cbl phosphorylation, the Cbl/CAP complex is translocated to the plasma membrane domain enriched in lipid rafts or caveolae. In the lipid rafts, CAP associates with caveolar protein flotillin and forms a complex with a number of proteins including TC10, CRKII and other accessory proteins involved in vesicular trafficking and membrane fusion (He, Liu et al. 2007).

b) **Through actin remodelling:** Insulin initiates the translocation of GLUT4 vesicles along microtubules from an intracellular storage compartment to the PM. Actin polymerization specifically regulates this process downstream of vesicle attachment, at a step that may directly drive the incorporation of GLUT4 into the PM. The microtubule network and actin cytoskeleton play a role in Glut4 trafficking, either by linking signaling components or by directing movement of vesicles from the perinuclear region to the plasma membrane in response to insulin (Brozinick, Hawkins et al. 2004; Chang, Chiang et al. 2004).

c) **Microtubules in GLUT4 translocation:** AS160, a substrate of Akt, which mediates insulin effects on the machinery of GLUT4 storage vesicle (GSV) translocation, possesses a GAP domain and regulates the activity of Rab protein(s) involved in GLUT4 trafficking. When phosphorylated by Akt, as in the case of insulin stimulation, the GAP domain of AS160 loses its activity against Rab- GTP and allows Rab(s) to shift from the GDP- to GTP-binding form. Rab in the GTP-binding form recruits various downstream effectors to facilitate transport of GSVs from intracellular localizations to the cell periphery. Intracellular cargo transport occurs through microtubules and GSVs moves along microtubules. Intact microtubules are obligatory for insulin-stimulated GLUT4 translocation (Huang, Imamura et al. 2001; Dugani and Klip 2005).

d) **Role of vesicular H^+-ATPase in GLUT4 recruitment:** GLUT4 is packaged into GSV (GLUT4 vesicle) and stored in cytoplasm until exocytic insertion to the plasma membrane is triggered by insulin. Increased vesicular interior pH would lead to increased H^+ transport via vacuolar type H^+-ATPase activity. This ATPase is electrogenic,

continues to transport H^+ ions until equilibrium has reached at greater vesicular osmolarity owing to accumulation of K^+ The increase in the intra-vesicular osmolarity activity would provide a driving force for influx of water into the GSV, thereby causing it to swell promoting exocytic fusion of GSV with the plasma membrane (Choi, Park et al. 2007).

1.2.7 Glycogen synthase

Insulin stimulates the storage of glucose as glycogen in muscle and adipose tissue through the coordinate increase in glucose uptake and modulation of glycogen metabolizing enzymes. Insulin stimulates glycogen synthase activity by promoting its net dephosphorylation, through phosphatase activation and kinase inhibition. This action of insulin is facilitated by the stimulation of glucose uptake, because the resulting increase in glucose 6 phosphate leads to the allosteric activation of glycogen synthase and increased susceptibility to dephosphorylation and activation of glycogen synthase, which incorporates UDP-glucose into glycogen chains (Brady 2003; Dixon, Nolan et al. 2003; Frojdo, Vidal et al. 2009) **(Fig 1.9)**.

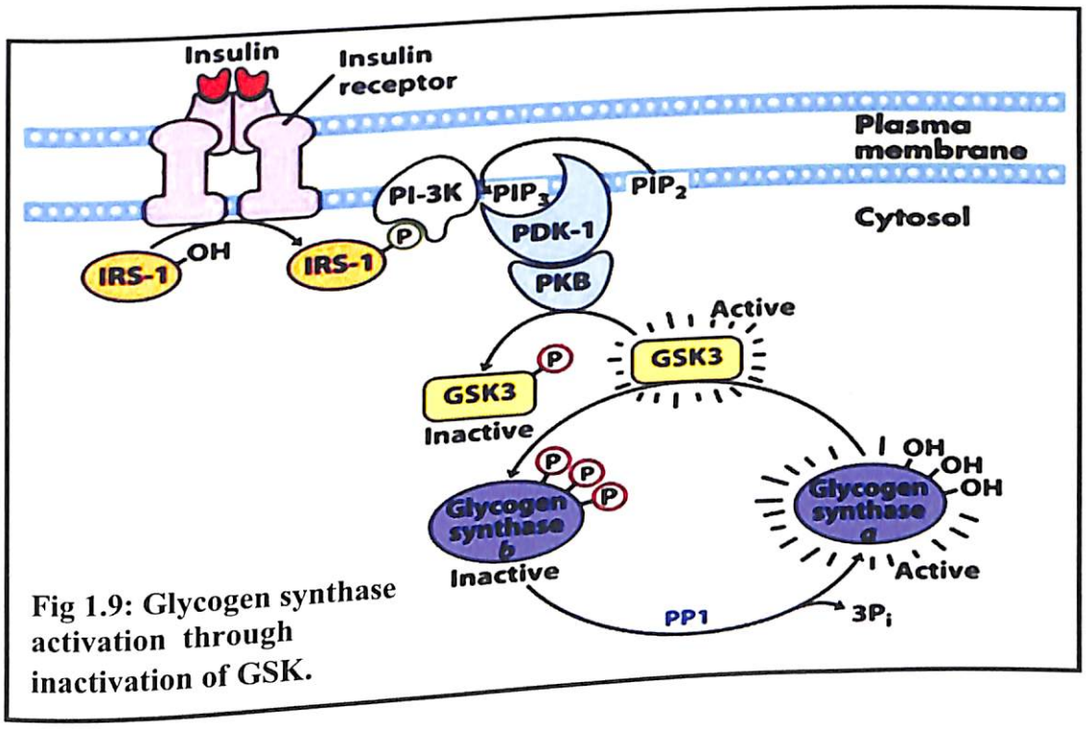


Fig 1.9: Glycogen synthase activation through inactivation of GSK.

1.3 AMINO ACIDS

Amino acids are the basic structural building units of proteins containing both amine and carboxyl functional groups. Twenty amino acids are encoded by the standard genetic code and are called proteinogenic or standard amino acids. Of the 20 standard proteinogenic amino acids, the essential amino acids and non-essential amino acids are listed in **table 1.4**. Post-translational modifications are often essential for the function or regulation of a protein. The twenty standard amino acids are either used to synthesize proteins and other biomolecules, or oxidized to urea and carbon dioxide. The oxidation pathway starts with the removal of the amino group by a transaminase, the amino group is then fed into the urea cycle. The other product of transamidation is a keto acid that enters the citric acid cycle (Brosnan 2000). Glucogenic amino acids namely alanine, aspartic acid, arginine, etc., can also be converted into glucose, through gluconeogenesis (Young and Ajami 2001).

Table 1.4: Essential and non-essential amino acids which is proteinogenic.

Essential	Non-essential
Isoleucine	Alanine
Leucine	Asparagine
Lysine	Aspartate
Methionine	Cysteine
Phenylalanine	Glutamate
Threonine	Glutamine
Tryptophan	Glycine
Valine	Proline
Arginine*	Serine
Histidine*	Tyrosine

1.3.1 Amino acids in metabolism

All tissues have capability for synthesis of the non-essential amino acids, amino acid remodeling, and conversion of non-amino acid carbon skeletons into amino acids and other derivatives that contain nitrogen. The potentially toxic nitrogen of amino acids is eliminated via transaminations, deamination, and urea formation; the carbon skeletons are generally conserved as carbohydrate, via gluconeogenesis, or as fatty acid via fatty acid synthesis pathways. In this respect amino acids fall into three categories: glucogenic, ketogenic, or glucogenic and ketogenic.

Glucogenic amino acids are those that give rise to a net production of pyruvate or TCA cycle intermediates - ***All amino acids except lysine and leucine*** are at least partly glucogenic.

Ketogenic amino acids - ***Lysine and leucine*** are the only amino acids that are solely ketogenic, giving rise only to acetylCoA or acetoacetylCoA, neither of which can bring about net glucose production.

Glucogenic and ketogenic amino acids - ***Isoleucine, phenylalanine, threonine, tryptophan, and tyrosine.***

Under starvation - The reduced carbon skeleton is used for energy production, with the result that it is oxidized to CO_2 and H_2O.

Amino acids are reported to be beneficial in T2DM by three mechanisms as follows:

a) Stimulating the pancreas for insulin secretion (Dixon, Nolan et al. 2003)

b) Scavenging the excess glucose in the blood by acting as preferred substrates for glycation either at the primary amino group or at the side chain as in lysine (Ramakrishnan and Sulochana 1993; Ramakrishnan, Sulochana et al. 1996; Ramakrishnan, Sulochana et al. 1997).

c) Up-regulation of the insulin receptor tyrosine kinase (Sulochana, Rajesh et al. 2001).

d) Amino acids are reported to be beneficial as anti-cataract, anti-glycating, anti-diabetic **(Table 1.5).**

Table 1.5: Studies indicating amino acids to be beneficial in diabetes both *in vitro* and *in vivo*.

Amino acids	Effect	Mechanism	Cell type	Reference
Lysine	Anti-cataract	Anti-glycating	*In Vitro*	(Ramakrishnan and Sulochana 1993)
alanine, aspartic acid, or glutamic acid	Anti-glycating	reduce the glycation	Human lens, *in vitro*	(Ramakrishnan, Sulochana et al. 1996)
Glycine, Aspartic acid, Alanine and Gluctamic acid	Anti-glycating	reduce the glycation by mitigating galactose	Human lens, *in vitro*	(Ramakrishnan, Sulochana et al. 1997)
Lysine, amino acid mixture	Anti-cataract	Anti-glycating	Rat lens	(Sulochana, Punitham et al. 1998)
Free amino acids	Anti-diabetic	Lowering blood sugar	T2DM	(Sulochana, Lakshmi et al. 2002)
Amino acids mixture	Insulin signal molecule	IRTK, PI3K	Human monocytes	(Srinivasan, Rajesh et al. 2001)
Amino acids mixture	Anti-glycation	Cytoskeleton actin	Human mononuclear cells	(Sulochana, Indra et al. 2001)
Amino acid mixture	↑ Glucose uptake	GLUT4	Adipocyte & CHO-K1 cells	(Bogan, McKee et al. 2001)
Alanine	Insulin secretion	Metabolic stimulus secretion coupling factors such as L-glutamate.	Rodent islet	(Dixon, Nolan et al. 2003)
Amino acid mixture	Insulin secretion	Insulin response	T2DM	(van Loon, Kruijshoop et al. 2003)
Amino acid mixture + carbohydrate	Glucose disposal	Insulin secretion	T2DM	(Manders, Wagenmakers et al. 2005)
Amino acid mixture	Insulin signaling molecule	AS160, Glycogen synthase	Rat	(Bernard, Liao et al.)
Amino acids - Free/ mixture	↑ Glucose uptake	↑ GLUT4	CHO-K1 cells	(Selvi, Angayarkanni et al.)

1.3.2 Amino acids as antiglycating agent

Structural alterations in proteins lead to decreased functional activity of these biomolecules. Glucose can slowly condense with protein amino groups non-enzymatically forming, initially, a Schiff's base, which will rearrange to form the Amadori adduct, and this process is termed glycation (Bagner,1999). These compounds can react with proteins to form cross-links as well as chromo/fluorophoric adducts called Maillard adducts or Advanced Glycation End Products (AGE). It has been proved earlier that free lysine decreases non-enzymatic glycation of lens proteins in an in-vitro model and thereby act as antiglycating agent (Ramakrishnan and Sulochana 1993). Lysine decreased the blood glucose levels in diabetic rats in addition to mitigating cataractogenesis in these animal models (Sulochana, Punitham et al. 1998). Apart from lysine other amino acids like glycine, aspartic acid, glutamic acid and alanine were also found to have antiglycating effect (Ramakrishnan, Sulochana et al. 1997). Thus amino acids are reported to have antiglycating and antidiabetic property apart from their secretogogue effect on the beta cells of the pancreas, which increases the plasma insulin level.

1.3.3 Amino acids as secretogogue

Amino acids under appropriate conditions can enhance insulin secretion from primary isletcells and β-cell lines (Smith, Sakura et al. 1997; Brennan, Shine et al. 2002; Dixon, Nolan et al. 2003; Newsholme, Brennan et al. 2005). Amino acids mixture along with carbohydrates is much effective in provoking insulin secretion (van Loon, Kruijshoop et al. 2003; Manders, Wagenmakers et al. 2005). *In vivo*, L-glutamine and L-alanine are quantitatively the most abundant amino acids in the blood and extracellular fluids followed closely by the branched chain amino acids (Sener and Malaisse 1981). However, unlike glucose, individual amino acids do not provoke insulin secretion in vitro when added at physiological concentrations. The mechanisms by which amino acids enhance insulin secretion are varied that are listed below **(Fig 1.10)**.

1. Combinations of amino acids at physiological concentrations or high concentrations of individual amino acids are much more effective (Kimball, Farrell et al. 2002). .
2. Specific amino acid mixtures at physiological concentrations, with *in vitro* mouse islet incubations showed insulin secretion that was robustly stimulated (Bolea, Pertusa et al. 1997).

3. Four amino acids were found to be particularly important for stimulating β-cell electrical activity, essential for insulin secretion namely leucine, isoleucine, alanine, and arginine (Thams and Capito 1999; Newsholme, Brennan et al. 2005).
4. Mixture of free leucine, phenylalanine, and arginine can produce a large insulinotropic effect when ingested in combination with carbohydrates (van Loon, Kruijshoop et al. 2003) .
5. The combined intravenous administration of arginine-leucine and arginine-phenylalanine, together with glucose, resulted in the largest increase in plasma insulin concentrations (Floyd, Fajans et al. 1966).
6. L-glutamine and L-alanine may modulate glucagon release from pancreatic α-cells, which subsequently may influence insulin secretion from β-cells (Newsholme, Brennan et al. 2005).
7. The cationically charged L-arginine, by direct depolarization of the plasma membrane at neutral pH leads to insulin secretion, but only in the presence of glucose, whereas other amino acids, co-transported with Na^+, can also depolarize the cell membrane as a consequence of Na^+ transport and thus induce insulin secretion by activating voltage-dependent calcium channels (Newsholme, Brennan et al. 2005).
8. Metabolism, resulting in partial oxidation, e.g., L-alanine, may initially increase the cellular content of ATP, leading to closure of the ATP-sensitive K^+ (K_{ATP}) channel, depolarization of the plasma membrane, activation of the voltage-activated Ca^{2+} channel, Ca^{2+} influx, and insulin exocytosis (Brennan, Shine et al. 2002) .
9. Additional mitochondrial signals may be generated that affect insulin secretion. Mitochondria generate ATP, which is the main coupling messenger in insulin secretion, and other coupling factors, which serve as sensors for the control of the exocytotic process (Newsholme, Brennan et al. 2005).
10. Amino acids were shown to release insulin from pancreas as oral anti-diabetic tablet does. Glycine was found to increase insulin secretion in healthy first-degree relatives of T2DM patients. Amino acids are suggested to be nutrient secretogogues to release insulin secretion (Gonzalez-Ortiz, Medina-Santillan et al. 2001).

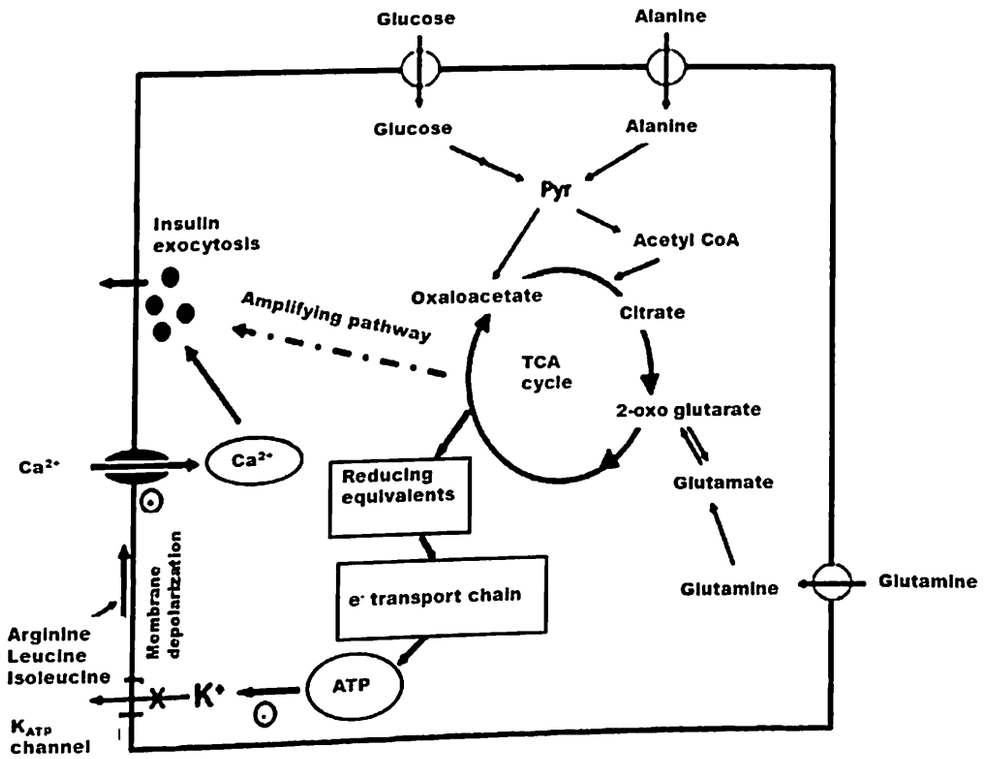


Fig 1.10: A summary of potential regulatory mechanisms of insulin secretion.

1.3.4 Amino acids in glucose uptake

Leucine and isoleucine is reported to stimulate glucose uptake in an insulin-independent manner via PI3K and PKC, and the effect of isoleucine was greater than that of leucine as studied in C2C12 myotubes (Doi, Yamaoka et al. 2003). Isoleucine administration increased glucose uptake in the rat muscle. Ile known to increase the glucose uptake as a result of decreased AMP concentration, leading to a decrease in the AMP:ATP ratio and AMPK activity (Doi, Yamaoka et al. 2005).

1.3.5 Amino acids in up-regulating insulin signaling molecules

Free amino acids such as lysine, arginine, isoleucine has been reported to influence glucose uptake by various mechanism including insulin signaling. However more studies are warranted to understand the complete cascade of events involved.

1.3.5.1 Lysine

The amino acid lysine, when given as oral tablets to diabetic individuals showed increased insulin sensitivity in monocytes by an increase in the insulin receptor tyrosine kinase activity. Further, free amino acids were shown to have a positive role in improving leukocyte

deformability by mitigating cytoskeletal actin glycation and improving F-actin content (Sulochana, Indra et al. 2001).

1.3.5.2 Branched chain amino acids (BCAA)

Leucine increases glucose uptake, reduces blood glucose and increases the use of intracellular glucose with activation of glycogen synthase in the rat skeletal muscles (Nishitani, Matsumura et al. 2002; Nishitani, Takehana et al. 2005). Leucine in combination with Glutamine and other amino acids increases insulin secretion from diabetic pancreas. L-glutamine however, in the absence of L-leucine does not influence insulin release. Leucine also activates the translational regulators mTOR (Mammalian target of rapamycin) that are associated with increased protein synthesis. The structural isomer of leucine, isoleucine differs in the activity, and the difference might be due to stearic hindrance or any other enzyme retarding activity. A small structural variation in the amino acid can cause large difference in their activity of enhancing the enzyme (Xu, Kwon et al. 2001).

1.3.5.3 Arginine

Iwasaki et al have shown that insulin rapidly translocates protein N-arginine methyltransferase (PRMT1) and evokes its methyl transferase activity in the membrane fraction, and that the processes are essential for sustained activation of IR/IRS-1/PI3-K cascade followed by glucose uptake in skeletal L6 myotubes. Nitric oxide (NO), the metabolic mediator for L-arginine, potentiates insulin-mediated glucose uptake through the increase in blood flow (Linden, Wadley et al.; Paolisso, Tagliamonte et al. 1997).

1.3.5.4 Amino acid mixture

A novel link between glucose and amino acid metabolism is given by Bogan et al 2001. Mixture of amino acids rapidly mobilized GLUT4 from the intracellular compartments to the plasma membrane as studied in 3T3-L1 and CHO cell lines. *Bogan et al* study states that amino acids regulate distribution of GLUT4 through a rapamycin-sensitive pathway (Bogan, McKee et al. 2001).

1.3.6 Amino acids and insulin resistance

Flati et al have reported that oral supplements of AAs stimulated both GLUT4 and protein synthesis through independent insulin signals in rat skeletal muscles. This hypothesis suggests the presence of an metabolic pathway in eukaryotic cells that is not active when insulin intracellular signaling is efficient but can be activated by alternative stimuli, such as AAs, when insulin signaling is impaired and use of anabolic nutrients such as AAs, together with standard therapies, can overcome insulin resistance syndrome (Flati, Pasini et al. 2008).

Prizant et al showed that individual supplementation of lysine, histidine and threonine maintained a low level of IRS-1 phosphorylation, which was dose-dependently increased by their combined addition (Prizant and Barash 2008).

The recent study on the early markers of diabetes indicated isoleucine, leucine, valine, tyrosine and phenylalanine (5 amino acids) as an indication of the metabolic derangement in T2DM shows that amino acids metabolism may play a major role in DM (Wang, Larson et al.). However, more studies are required to delineate the cause from the effect.

1.3.7 Interrelationship between Insulin and Nutrients

Nutrient substances that influence insulin gene levels are mannose, amino acids, fatty acids/keto acids (Taylor 1999) **(Fig 1.11)**. Insulin secretion is governed by a feedback relationship with the exogenous nutrient supply. When nutrient supply is high or abundant, insulin is secreted in response and the hormone in turn stimulates the utilization of these nutrients while inhibiting the mobilization of endogenous substrates. When nutrient supply is low, insulin secretion is dampened and there is an enhanced mobilization of endogenous substrates.

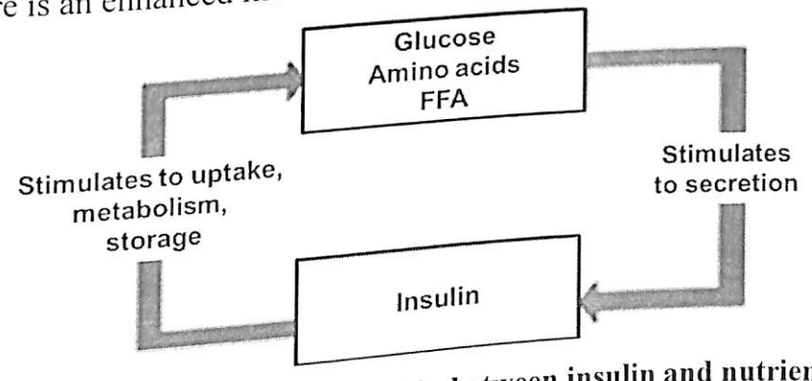


Fig 1.11: Feedback relationship between insulin and nutrients.

For carbohydrates, insulin stimulates the transport of glucose from the plasma, across the cell membrane, and into the cytoplasm in muscle and fat (adipose) tissue. For fats, insulin stimulates

transfer of fatty acids into the adipose cells for fat synthesis. For protein, insulin stimulates the transport of certain amino acids from the plasma across the cell membrane for protein synthesis (Taylor 1992).

Concurrently as studied in adipocytes , amino acids inhibit early steps in insulin action critical for glucose transport and inhibition of gluconeogenesis, including decreased insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2, and a marked inhibition of insulin stimulated phosphatidylinositol 3–kinase. Amino acids act as specific positive signals for maintenance of protein stores, while inhibiting other actions of insulin at multiple levels. This bidirectional modulation of insulin action indicates crosstalk between hormonal and nutritional signals and demonstrates a novel mechanism by which nutritional factors contribute to insulin resistance (Caro, Ittoop et al. 1986).

1.4 REACTIVE OXYGEN SPECIES

Oxidative stress has long been associated with the complications of diabetes (nephropathy, retinopathy, and macro vascular complications) via the chronic hyperglycemia. The prolonged hyperglycemia is known to deplete cellular antioxidants and cause an increase in free radicals (Ceriello 2000; Maritim, Sanders et al. 2003). Mechanisms by which increased oxidative stress is involved are activation of transcription factors, protein kinase C. and accumulation of AGEs.

1.4.1 Free radicals and diabetes complications

Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids, and eventually cell death. Various mechanisms have been suggested to contribute to the formation of these reactive oxygen-free radicals. Glucose oxidation is believed to be the main source of free radicals. In its enediol form, glucose is oxidized in a transition-metal dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to super-oxide anion radicals **(Fig 1.12)**. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by catalase or glutathione peroxidase, and in the presence of transition metals, can lead to production of extremely reactive hydroxyl radicals (Jiang, Woollard et al. 1990). Superoxide anion radicals can also react with nitric oxide to form reactive peroxynitrite radicals (Hogg, Kalyanaraman et al. 1993) **(Fig 1.13)**.

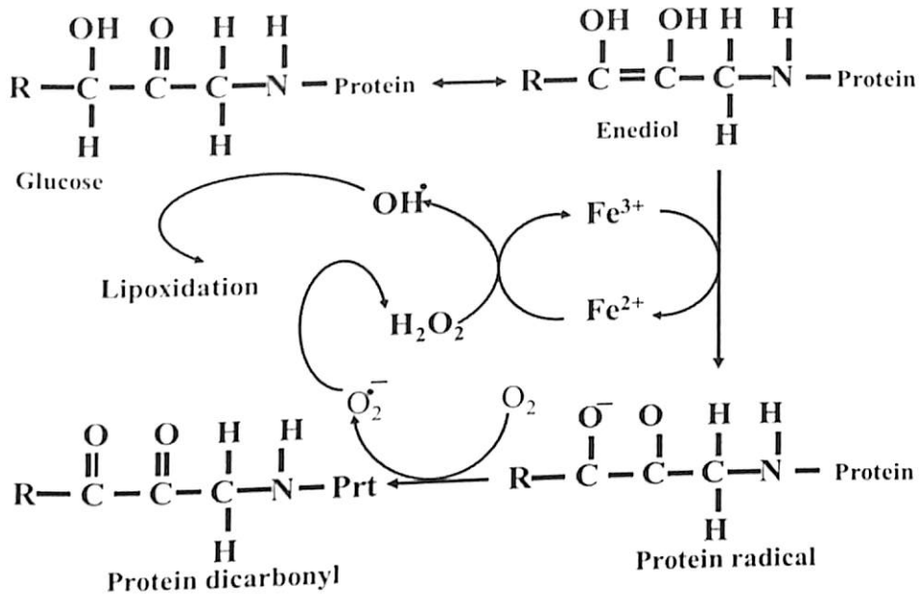


Fig 1.12: Mechanism of glycoxidation and lipoxidation

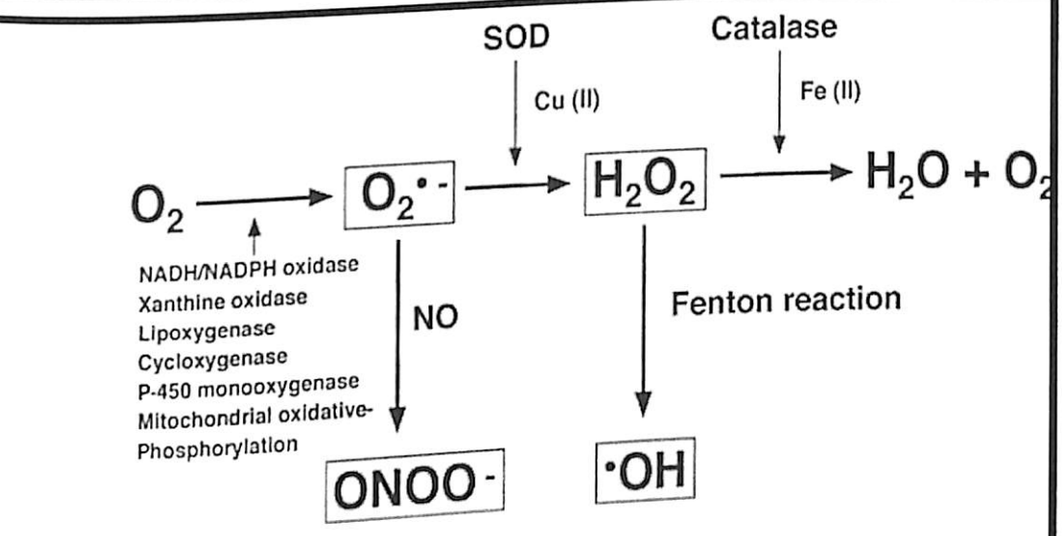


Fig 1.13: Formation of free radicals and overcoming by antioxidants.

The rate constant for the reaction of superoxide anion with NO is 10-fold faster than with SOD, leading to the diffusion-controlled formation of peroxynitrite. Peroxynitrite is a powerful oxidant that causes protein modification via oxidation of protein-associated thiol groups or nitration of tyrosine residues. VEGF's angiogenic signaling in vascular endothelial cells is that superoxide anion and hydrogen peroxide may serve as intracellular messengers involved in autophosphorylation of VEGFR2 (El-Remessy, Al-Shabrawey et al. 2007) **(Fig 1.14)**.

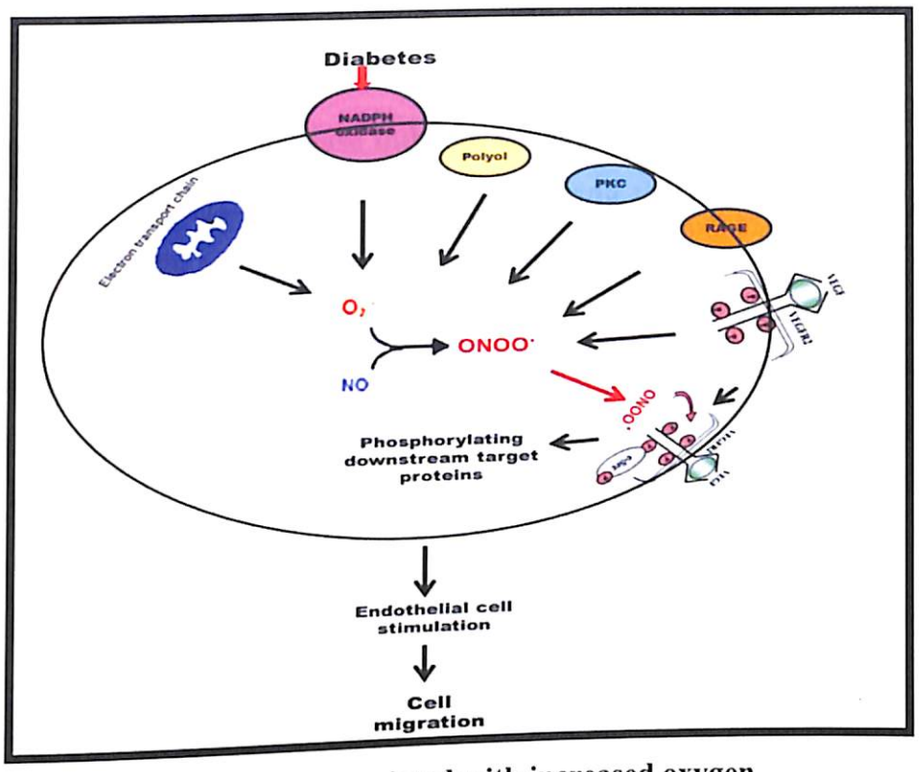


Fig 1.14: NADPH oxidase associated with increased oxygen species and decreased NO bioavailability.

1.4.2 ROS increasing Vascular endothelial growth factor via NFkB pathway

Hyperglycemia, ROS, and oxidative stress activates the transcription factor NF-κB (Mohamed, Bierhaus et al. 1999; Bierhaus, Schiekofer et al. 2001). NF-κB plays a critical role in mediating immune and inflammatory responses and apoptosis. NF-κB regulates the expression of a large number of genes, including several of those linked to the complications of diabetes including VEGF and the receptor for AGE namely RAGE (Ho, Liu et al. 2000) **(Fig 1.15)**.

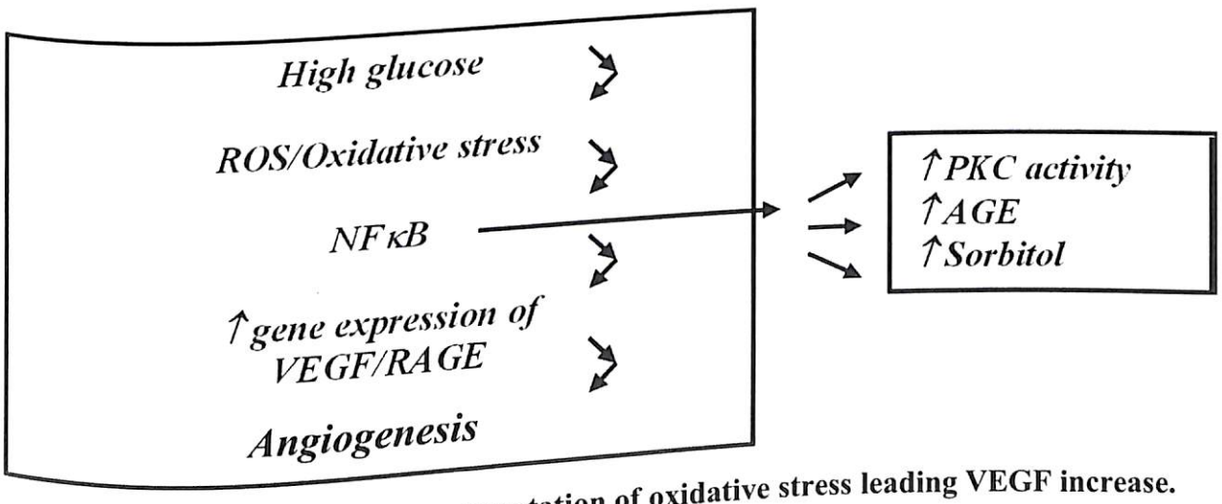


Fig 1.15: Schematic representation of oxidative stress leading VEGF increase.

1.4.3 Cascade of events in high glucose induced angiogenesis

New blood vessel growth, or angiogenesis, occurs during the development and maturation as well as during critical physiological processes, including wound healing and reproduction. However, angiogenic processes also contributes to pathologies including cancer, inflammation and autoimmune disease. Angiogenesis is the principal factor responsible for disease pathology, such as is the case in diabetic retinopathy and macular degeneration.

Under hyperglycemia and retinal hypoxia, a number of vasoactive factors may interact to promote pathology in a variety of cell types including the microvasculature, neurons and glia. In addition to hyperglycemia, overproduction of reactive O_2 species by mitochondria, AGEs, hexosamines and increased polyol metabolism of glucose leads to altered signaling of pathways involving protein kinase C, nuclear factor kappa-B (NF_K-B) and MAP kinase. These changes damage retinal endothelial cells, pericytes, neurons, glia and pigment epithelial cells and recruit inflammatory cells which produce vasoactive compounds, growth factors (VEGF), coagulation factors and adhesion molecules that eventually leading to angiogenesis and tissue remodeling (Pelikanova 2007; Qazi, Maddula et al. 2009) **(Fig 1.16)**.

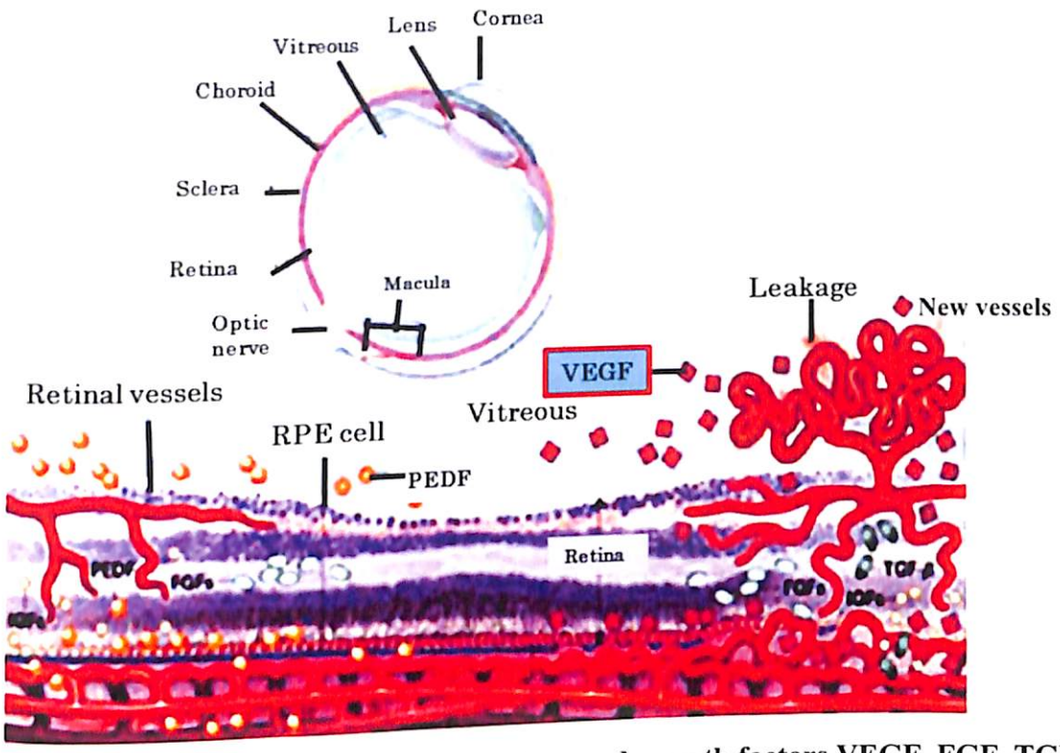


Fig 1.16: Retinal angiogenesis showing increased growth factors VEGF, FGF, TGF, IGF.

The major factors promoting angiogenesis are high glucose, hypoxia, reactive O_2 species, AGEs, hexosamines, protein kinase C, Nuclear factor kappa-B (NF_K-B), MAP kinase, VEGF.

1.4.4 Vascular endothelial growth factor

VEGF, a 40-kDa glycoprotein acts as a primary regulator of blood vessels throughout the body, including the retina. It is a proinflammatory molecule that plays a well recognized role in neovascularizaton and in increased permeability. Elevated levels of VEGF are associated with angiogenesis in proliferative diabetic retinopathy (PDR) (Penn, Madan et al. 2008). It is produced in response to hypoxic and inflammatory stimuli as secreted by macrophages, T cells, retinal pigment epithelial cells, astrocytes, pericytes and smooth muscle cells and by multiple cell types in the retina in diabetes, including ganglion cells, muller cells, and pericytes.

Alternative splicing of the VEGF gene yields five isoforms, VEGF115, VEGF121, VEGF165, VEGF189 and VEGF206 (Shibuya and Claesson-Welsh 2006). VEGF binds to tyrosine-kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (Flk-1), and also the neuropilins which lack tyrosine-kinase activity.

Hyperglycemia leads to functionally and anatomically incompetent capillaries as they become acellular which leads to capillary nonperfusion, hypoxia and consequently induction of VEGF-A leading to angiogenesis (Mahabeleshwar, Feng et al. 2006; Crawford, Alfaro et al. 2009). The rate constant for the reaction of superoxide anion with NO is 10-fold faster than with SOD, leading to the diffusion-controlled formation of peroxynitrite (El-Remessy, Al-Shabrawey et al. 2007). Peroxynitrite is a powerful oxidant that causes protein modification via oxidation of protein-associated thiol groups or nitration of tyrosine residues. VEGF's angiogenic signaling in vascular endothelial cells is that superoxide anion and hydrogen peroxide may serve as intracellular messengers involved in autophosphorylation of VEGFR2 (Colavitti, Pani et al. 2002).

The levels of VEGF in vitreous was increased (Patel, Tombran-Tink et al. 2006; Angayarkanni, Selvi et al. 2009) and correlates with the activity of neovascularisation in retinopathy, breakdown of the blood-retinal barrier, increased microvascular permeability (Aiello, Avery et al. 1994; Murata, Ishibashi et al. 1995). VEGF plays a key role in the development of both PDR and diabetic macular oedema (DMO). Intravitreal injection is an effective means of delivering anti-VEGF drugs to the retina. However, this is an invasive procedure associated with potentially serious complications, such as endophthalmitis or retinal detachment. In addition, although delivered within the vitreous, anti-VEGF drugs could pass into the systemic circulation, which could potentially result in hypertension, proteinuria, increased cardiovascular events and impaired wound healing. Pegaptanib, ranibizumab and bevacizumab are the currently available anti-VEGF agents. Ranibizumab and bevacizumab block all VEGF isoforms, thus impairing both physiological and pathological neovascularisation. Pegaptanib only blocks the VEGF(165) isoform (Simo and Hernandez 2008). After intravitreal Bevacizumab treatment for vascularised pigment epithelial detachment secondary to AMD RPE rip was observed (Bakri and Kitzmann 2007). RPE tears may occur after intravitreal injection of ranibizumab (Gamulescu, Framme et al. 2007). A safe mode of treatment for decreasing the VEGF is not yet arrived.

1.5 DIABETIC RETINOPATHY

1.5.1 Complications involving retinal changes

Diabetic retinopathy (DR) is a significant cause of severe vision loss and blindness in adults. The diabetic complications include primarily retinopathy, nephropathy and neuropathy. The incidence of retinopathy worldwide is reported as 28.5 %, nephropathy 44 %, and 60-70 % with nerve damages. In South India the retinopathy incidence is 18 % (Saumya Pal, 2011). The retinopathy of prematurity which is seen among premature babies is yet another form of vasoproliferative disorder of retina has an incidence of 20-51 % (Chawla, 2010). The pathogenesis of DR is mediated by inflammatory processes, including leukocyte adhesion and the cytokine network. Retinal vasculature in diabetes is accompanied by inflammatory cell adhesion, which triggers vascular hyperpermeability due to tight junction disassembly and pathologic retinal neovascularization. DR is the most common cause of blindness and characterized by (1) leaking blood vessels, (2) retinal swelling, such as macular edema, (3) pale, fatty deposits on the retina (exudates) with leaking blood vessels, (4) damaged nerve tissue (neuropathy), and (5) vascular occlusion, angiogenesis, microaneurysms, haemorrhages and infarction any changes in the blood vessels. DR patients are classified into non proliferative and proliferative based on the angiogenesis (Bloomgarden 2001).

Prolonged hyperglycemia-induced retinopathy changes include, pericyte death and thickening of the basement membrane of the vascular walls. These damages change the formation of the blood-retinal barrier leading to more permeable. In the initial stage, it is called as non-proliferative diabetic retinopathy (NPDR). When the damaged blood vessels leak fluid and lipids onto the macula develops a condition called macular edema. The fluid makes the macula swell, which blurs vision. As the disease progresses, severe NPDR enters an advanced, or proliferative, stage. The lack of oxygen in the retina causes fragile, new, blood vessels to grow along the retina and in the clear, gel-like vitreous humour. These new blood vessels can bleed, cloud vision, and destroy the retina. Fibrovascular proliferation cause tractional retinal detachment. The new blood vessels can also grow into the angle of the anterior chamber of the eye and cause neovascular Glaucoma. NPDR shows up as cotton wool spots, or microvascular abnormalities or as superficial retinal hemorrhages advancing to PDR (Bloomgarden 2001; Gardner, Antonetti et al. 2002).

1.5.2 Cellular changes in the retinal capillaries in DR

The normal retina is composed of four major classes of cells:

a) Blood vessels - Endothelial cells & Pericytes.

b) The second category is Glial cells comprise Macroglia (Muller cells & Astrocytes).

c) Third class of cells includes neurons.

d) Fourth class of cells are Microglia.

The interactions and functional integration of all of these cell types are required for normal vision so disruption of any of them may impair vision (Gardner, Antonetti et al. 2002) **(Table 1.6)**.

Table 1.6: The cell type that undergoes pathological changes in DR.

Cell type	Normal retina	DR
Pericytes	Regulates retinal vascular flow by dilating and contracting.	Loss of pericytes
Endothelial cells	Regulates hemostatic functions and constitute the blood–retinal barrier.	Endothelial dysfunction
Macroglial cells	Support cells that regulate retinal metabolism and modulate the function of neurons and blood vessels.	Produces cytokines such as VEGF which increase vessel permeability.
Muller cells	i) Span the thickness of the retina from the pigment epithelium to the internal limiting membrane ii) Have their cell bodies in the inner nuclear layer. iii) Major regulators of glutamate metabolism, extracellular ionic balance, and neuronal function.	Proliferates in the formation of epiretinal membranes in proliferative retinopathy.
Astrocytes	By contrast, are limited to the nerve fiber layer where their processes wrap around blood vessels and ganglion cells.	Astrocytes dramatically decreases the expression of the intermediate filament, glial fibrillary acidic protein (GFAP) there by increased vascular permeability.
Neurons (photoreceptors, bipolar cells, amacrine cells, and ganglion cells)	Mediate photo transduction, and modulate and convey nerve impulses that are ultimately transmitted to the brain through the axons of the ganglion cells that comprise the nerve fiber layer and optic nerve.	Produces cytokines such as VEGF which increase vessel permeability.
Microglia	Normally quiescent	Becomes activated and produces cytokines such as VEGF & tumor necrosis factor which increase vessel permeability.

1.5.3 Biochemical changes in DR

DR is mediated by inflammatory processes, including leukocyte adhesion and the cytokine network. Retinal vasculature in diabetes is accompanied by inflammatory cell adhesion, which triggers vascular permeability (ICAM-1, VCAM-1) and pathologic retinal neovascularization by growth factors such as VEGF (Joussen, Poulaki et al. 2002; Ishida, Usui et al. 2003; Joussen, Poulaki et al. 2004), IGF (Poulaki, Joussen et al. 2004), enzymes such as CD45 (leukocyte common antigen) known as transmembrane protein-tyrosine phosphatase (PTPase), polymerase (ADP-ribose) (Zheng, Szabo et al. 2004), small molecules - Angiopoietin-1, Chemokines - Monocyte chemotactic protein (MCP) (Joussen, Poulaki et al. 2002).

1.5.4 Early changes in DR animal models

1.5.4.1 Advanced glycation end products (AGE)

AGEs have been detected in retinal blood vessel walls and are believed to contribute towards vascular occlusion and increased permeability of retinal endothelial cells causing vascular leakage (Stitt 2003). AGEs are toxic to pericytes, which possess AGE receptors (RAGE) and damage to pericytes is seen in DR (Chibber, Molinatti et al. 1997).

1.5.4.2 Apoptosis induced by AGE

Apoptosis of pericytes usually precedes vascular changes and is a characteristic of early retinopathy. Vascular apoptosis was explored using terminal dUTP nick-end labeling (TUNEL) in human and rat retinas treated by trypsin digestion. The increase in vascular cell apoptosis also suggested a potential mechanism for the appearance of acellular capillaries as the endothelial cell disappear while leaving their intact basement membranes behind (Engerman and Kern 1995; Mizutani, Kern et al. 1996). Apoptosis may also account for the appearance of pericyte "ghosts" (pockets within the basement membrane that appear to have once contained a pericyte) frequently noted in trypsin digest samples (Kern, Tang et al. 2000).

1.5.4.3 Vascular Cell Loss - Pericytes

Loss of retinal pericytes is an early and characteristic hallmark sign of DR. Microvascular endothelial functions disrupted, includes perturbations in endothelial cell survival, microvascular stabilization, capillary tone, and tissue perfusion (Benjamin, Hemo et al. 1998). Decrease of

pericyte survival and formation of acellular capillaries during DR have been linked to hyperglycemic activation of PKC and to inhibition of platelet-derived growth factor β signaling via a novel PKC target phosphatase, SHP-1 (Geraldes, Hiraoka-Yamamoto et al. 2009), by the Ang/Tie signaling cascade has been proposed as an alternative mechanism in pericyte dropout (Pfister, Feng et al. 2008). Diabetes-associated changes impairs normal pericyte-endothelial communication, altering direct cell-cell contacts. Notably, gap junctions are particularly vulnerable as glucose-induced downregulation of connexin 43 results in gap junction disassembly and vascular apoptosis (Bobbie, Roy et al.; Sato, Haimovici et al. 2002). Pericyte loss leads to endothelial growth arrest as impaired pericyte recruitment leads to endothelial hyperplasia in vivo (Hellstrom, Gerhardt et al. 2001). Loss or decrease of pericyte abundance may lead to end-stage microvascular damage preceding the proliferative angiogenic response (Fig 1.17).

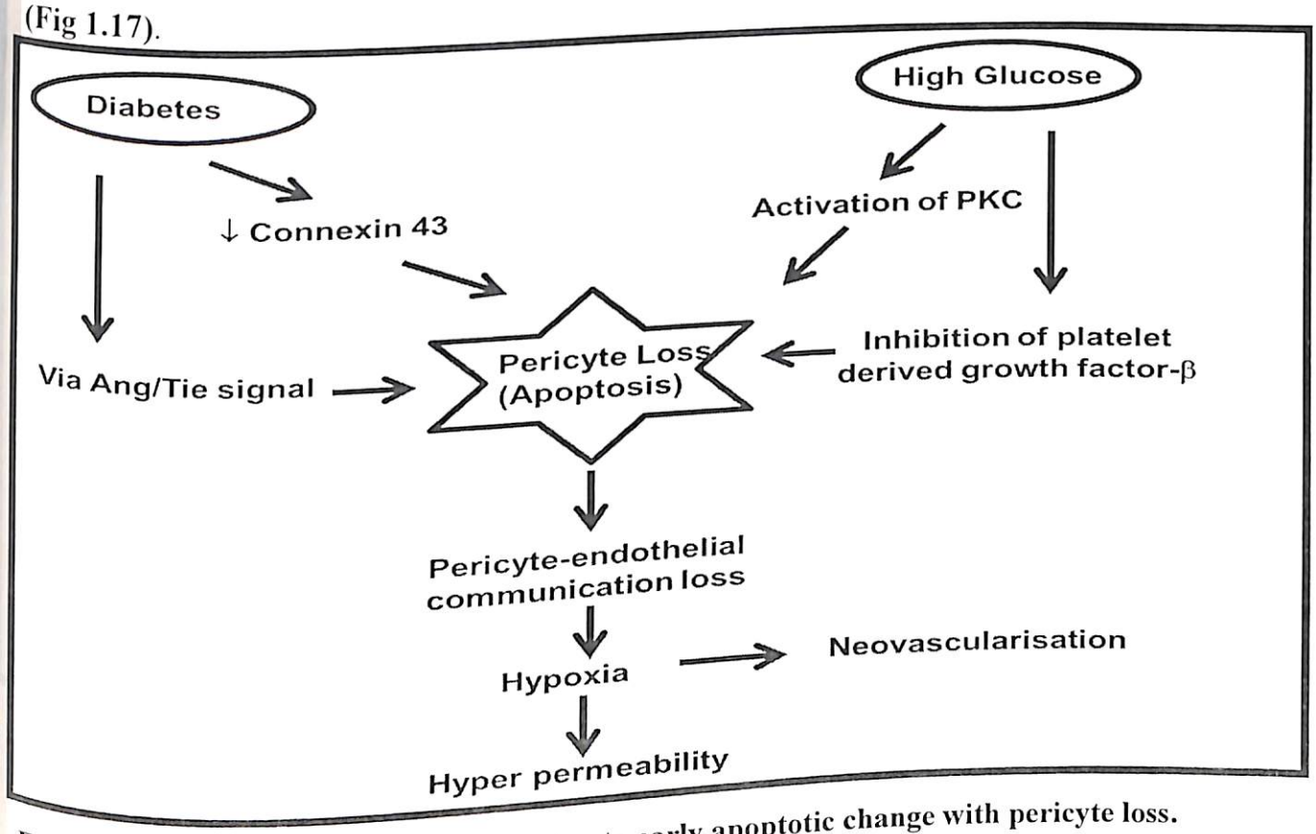


Fig 1.17: High glucose environment leading to early apoptotic change with pericyte loss.

1.5.4.4 Vascular Cell Loss - Endothelial Cells

Microvascular insult is further accelerated by the degeneration of capillary endothelial cells, accumulation of acellular capillaries, increase in endothelial apoptosis, decrease in recruitment of endothelial progenitor cells to damaged vessels (Brunner, Hoellerl et al.). Various factors leading

endothelial degeneration includes shear stress in the physiologic range is by prosurvival signal for endothelial cells (PI3K/Akt pathway), under pathologic strain induces endothelial apoptosis (Dardik, Chen et al. 2005).

1.5.4.5 Pericyte-endothelial cell interactions

Additionally, pericyte-endothelial cell interactions are critical in maintaining vascular stability. Upon contacting endothelial cells, mural cells (pericytes) upregulate several endothelial survival factors, including VEGF (Darland, Massingham et al. 2003) and through multiple means including gap junctions, soluble mediators, and direct cell-cell contact **(Fig 1.18)**.

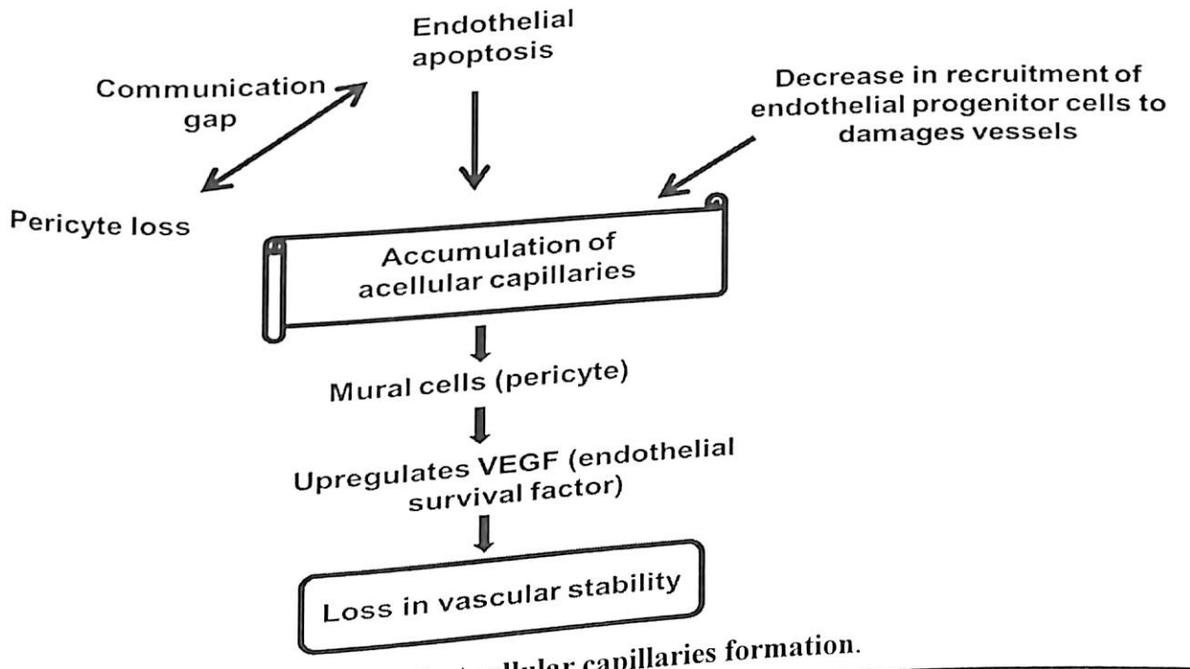


Fig 1.18: Acellular capillaries formation.

Thus AGE, oxidative stress pave ways to the DR complications by increasing the growth factors and cytokines. Based on these data, inhibition of formation of glycated proteins can be a target for reduction of diabetic vascular complications by using suitable antiglycating agents. As our previous study have shown antidiabetic effect of amino acids mixture in Type 2 diabetic patients and mitigation of cataract in diabetic rats, the potential antiglycating effect of this novel mixture of amino acids may prevent the retinal changes attributed to prolonged hyperglycemia.

1.5.4.6 Early apoptotic changes in diabetic retina

Neural apoptosis is an earlier event than vascular apoptosis and that the rate of neural apoptosis remains constant throughout the duration of diabetes. Neurons are unable to proliferate, apoptosis of these cells will result in a cumulative loss leading to chronic neuro degeneration (Barber, Lieth et al. 1998; El-Remessy, Al-Shabrawey et al. 2006). The total number of cell bodies in the retinal ganglion cell layer was reduced by 10% after 7.5 months of diabetes. This was accompanied by a 22% reduction in the thickness of the inner plexiform layer and a 14% loss in the thickness of the inner nuclear layer with no change in the thickness of the outer nuclear layer (Zeng, Ng et al. 2000). Along with loss of retinal ganglion cells, there was a cumulative loss of amacrine cells in diabetic rats (Gastinger, Singh et al. 2006; Martin, Lee et al. 2006).

1.5.5 Genetics and diabetic retinopathy

The pathogenetic mechanism of retinal disease caused by DM is still largely unclear and there is no satisfactory therapy for diabetes so far. The immediate goals of therapy are to stabilize blood sugar and eliminate its symptoms. Expression profile analysis of genes and proteins in diabetic retina tissue shows complex interaction among multiple susceptibility genes and between genetic and environmental factors. A host of genes involved in these pathways/processes include angiotensin-I converting enzyme (ACE), angiotensin II type 1 receptor (AGTR1), angiotensinogen (AGT), vascular endothelial growth factor (VEGF), aldose reductase (AR2), receptor for advanced glycation end products (RAGE), glucose transporter1(GLUT1), inducible and constitutive nitric oxide synthases (NOS2A, NOS3), transforming growth factor beta (TGFβ), endothelin isoforms, and its cellular receptors (Ng) (2010).

1.5.6 Animal models of diabetes

The widely used animal model for DR are Male wistar non-diabetic rats, Sprague-Dawley rat, spontaneously Diabetic Torii (SDT) rats (Srinivasan and Ramarao 2007). Intraperitoneal injection of streptozotocin, 55 mg/kg, at day 2 of age is given. Streptozotocin (STZ) and alloxan (ALX) are two commonly used drugs to induce diabetes in animal models. A single dose of STZ has direct toxic effect on β-cell by alkylation, while ALX toxicates the β-cell by the generation

of reactive oxygen species. In addition, there are nutritionally induced type 2 models such as the high-fat diet (HFD) C57Blk/6J mouse, galactose-enriched feed diet (Shaikh and Somani).

1.5.6.1 Limitations in animal models

In most models (including rodents), diabetes appears as a consequence of the inability to increase β-cells mass in response to obesity-induced insulin resistance. Furthermore, animals (except monkeys and cats) usually develop diabetes without displaying the same islet pathology as in humans (islet amyloidosis), change in physiological or pathophysiological mechanisms such as the fact that the human brain needs about 20 % of the energy of the resting metabolic rate compared with 3 % in rodents. Additionally, cost, availability, time is also a limitation when it comes to T2DM, due to the fact that (as in humans) disease progression or the development of its complications in animals (especially in primates) need frequently long periods to appear (Chatzigeorgiou, Halapas et al. 2009). One of the limitations of research into DR is the lack of suitable animal models. Rodent models of diabetes, including streptozotocin-induced rats and mice, such as the Ins2Akita mice, show some of the features of retinal and neural alterations. However, the disease in these animals, never progresses from the background phase to the proliferative stage associated with neovascularisation.

1.6 ADVANCED GLYCATION END PRODUCT

High glucose leads to a deleterious effect of formation of AGE such as $^{\varepsilon}$N-carboxymethyl-lysine, pentosidine, or methylglyoxal derivatives. Glucose and other reducing sugars can react nonenzymatically with the amino groups of proteins to form reversible Schiff bases and, then, Amadori products. These early glycation products undergo further complex reactions such as rearrangement, dehydration, and condensation to become irreversibly cross- linked, heterogeneous fluorescent derivatives termed AGE **(Fig 1.19)**. The formation and accumulation of AGE in various tissues progress during normal aging and at an extremely accelerated rate in diabetes mellitus resulting in the development of diabetic micro- and macro-vascular complications. AGE induces NFkB signaling pathway causing an increase in inflammatory response, increases reactive oxygen species, cytokines (Yamagishi, Nakamura et al. 2006).

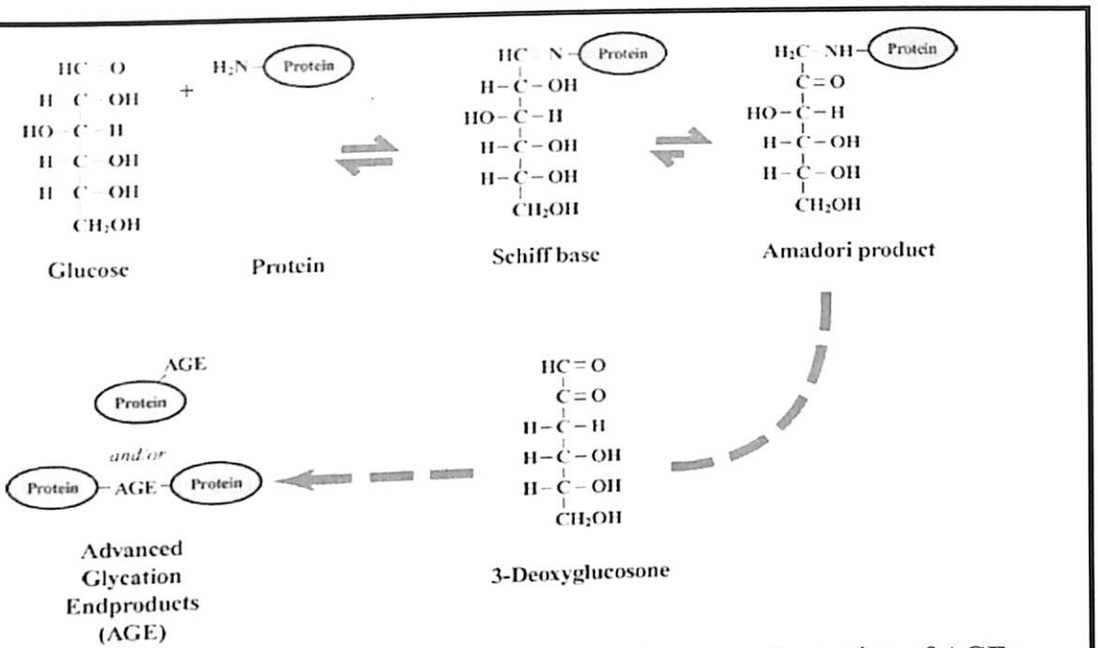


Fig 1.19: Glycation of proteins by glucose and the subsequent formation of AGEs.

AGEs are complex, heterogenous molecules that cause protein cross-linking, exhibit browning and generate fluorescence. Over a dozen AGEs have been detected in tissues and can be divided into three categories.

1. Fluorescent cross-linking AGEs such as pentosidine and crossline.
2. Non-fluorescent cross-linking AGEs such as N-carboxymethyllysine, imidazolium dilysine cross-links, alkyl formyl glycosyl pyrrole (AFGP) cross-links and arginine–lysine imidazole (ALI) cross-links.
3. Non-cross-linking AGEs such as pyrraline and N-carboxymethyllysine (CML).

1.6.1 Serum AGE levels marker of DR

Serum levels of non-CML AGEs are significantly associated with the severity of diabetic nephropathy and retinopathy, suggesting a role of non-CML AGE in the progression of microvascular complications. Recent studies indicate that non-CML AGEs may play a role in accelerating micro- and macrovascular complications of diabetes (Sampathkumar, Balasubramanyam et al. 2005).

1.6.2 AGE to Oxidative stress

AGE induces free radicals exaggerates oxidative stress due to an excess free oxygen species production, secondary to increased oxidation of substrates (sugars, non-saturated fats, and

glycated proteins. Hyperproduction of free radicals is responsible for endothelial dysfunction, *via* a decrease in nitric oxide (NO) production. Oxidized LDL decreases NO production, by a reduction in NO synthase, AGEs quenches the NO, and thus contribute to defective vasodilation. AGEs also increase susceptibility of LDL to oxidation (Wautier and Guillausseau 2001). Covalent modification of intracellular proteins by dicarbonyl AGE precursors alters several cellular functions. Modification of extracellular matrix proteins causes abnormal interactions with other matrix proteins and with integrins. Modification of plasma proteins by AGE precursors creates ligands that bind to AGE receptors, inducing changes in gene expression in endothelial cells, mesangial cells and macrophages (Brownlee 2001), up regulating the expressions of vascular endothelial growth factor (VEGF), vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), Tumor necrosis factor-α, etc,. (Bai, Tang et al. 2003; Ishida, Usui et al. 2003; Nyengaard, Ido et al. 2004; Mahabeleshwar, Feng et al. 2006).

1.6.3 AGE - adhesion molecules changes and permeability

Increasing evidence suggests that the pathogenesis of diabetic retinopathy is mediated by inflammatory processes, including leukocyte adhesion and the cytokine network (Bai, Tang et al. 2003). Retinal vasculature in diabetes is accompanied by inflammatory cell adhesion, which triggers vascular hyperpermeability due to tight junction disassembly and pathologic retinal neovascularization (Davis 1992). Stress would activate microglia to produce proinflammatory cytokines and acquire an activated morphology. Activated microglia produce chemokines such as monocyte chemoattractant protein-1, inducing expression of adhesion molecules, which can promote the leukostasis of neutrophils, on endothelium and potentially inducing the extravasation of inflammatory macrophages.

Interaction of AGEs with RAGE on macrophages causes oxidative stress and activation of nuclear factor-kB (NF-kB) via activation of the p21ras and the mitogen-activated protein (MAP) kinase signaling pathway (Yan, Schmidt et al. 1994). NF-kB modulates gene transcription for endothelin-1, tissue factor and thrombomodulin and generation of pro-inflammatory cytokines such as interleukin-1α (IL-1α), interleukin- 6 (IL-6) and tumour necrosis factor- a (TNF-α) (Wang, Vom Hagen et al. 2008). There is also enhanced expression of adhesion molecules

including vascular cell adhesion molecule- 1 (VCAM-1) and intercellular adhesion molecule- 1 (ICAM-1), in addition to other effects such as increased vascular permeability.

1.7 CURRENT FOCUS OF ATTENTION

The serious complications of diabetes are cardiovascular disease, nephropathy, retinopathy and neuropathy. These microvascular and macrovascular complications can have devastating consequences on the health of people with diabetes (Saydah, Fradkin et al. 2004). To reduce the risk or slow the progression of retinopathy, glycemic control is required. Lifestyle changes (diet and exercise) are typically the first-line treatment for type 2 diabetes and can be very effective in glycemic control. When this treatment fails to maintain adequate glucose control, oral antihyperglycemic agents are usually the next step (Cooppan 2003). Over time, due to the progressive nature of type 2 diabetes, a combination of oral agents is frequently necessary to maintain glucose control (White,2003). In addition to glycemic control, it is also indicated that nutritional interventions can be beneficial to improve endogenous insulin secretion (Manders, Wagenmakers et al. 2005). Individuals with prediabetes or diabetes are recommended by medical nutrition therapy (MNT) (American diabetes association,2011). MNT procedures defines the level, content and frequency of nutrition services that are appropriate for optimal care and nutrition outcomes with the optimal mix of macro and micro nutrients for diabetic subjects are recommended (Franz, Bantle et al. 2002). Since amino acids used are nontoxic, physiological, Ubiquitous, water-soluble substances. Although this is a preliminary study, the exact pharmacological and therapeutic dosage, the mode of administration of amino acids, their long term effects in delaying or preventing diabetic complications, and the molecular mechanisms by which they provide these beneficial effects are issues that need to be addressed.

CHAPTER 2: OUT LINE OF THE WORK

Diabetes Mellitus (DM) is a chronic metabolic disorder that has a significant impact on the human subjects. There are limitation in the current drugs used for treating diabetes. A typical conventional approach in controlling diabetes would be a recommended diet composed of 60-65 % carbohydrate, 25-35 % fat, and 10-20 % protein, with limited or no alcohol consumption and life style. The treatment of diabetes too requires nutritional supplementation such as amino acids. The proposed work is done in knowing the various properties of amino acids in high glucose condition. At high glucose environment , the glucose uptake and GLUT4 translocation was studied in CHO-K1 and human adipocyte cells. Amino acids studies are controversial, few studies state it to have beneficiary and few prove them to be a causative for insulin resistance. This study is proposed to look in the role of amino acids in insulin signaling cascade molecules namely by looking in the IRTK, PI3K, Akt, GLUT4. Insulin resistance is state of where insulin is unutilized and glucose been prevented in entering the cells. We further explored the glucose utilization by studying the glycogen synthase activity, an enzyme when activated helps in glycogen synthesis.

Diabetic retinopathy remains one of the leading causes of blindness world wide. One of the clinical presentation is angiogenesis. The major causative factor for angiogenesis is VEGF and oxidative stress is a triggerer for this potent factor. Intravitreal injection is an effective means of delivering anti-VEGF drugs to the retina. However, this is an invasive procedure associated with potentially serious complications, such as endophthalmitis or retinal detachment. Amino acids are proved to be anti-glycating by our previous studies. Therefore in the present study the focus is on amino acids effect of VEGF as studied in CHO-K1 cells.

Animal studies have demonstrated that accumulation of AGE is associated with formation of microaneurysm, neovascularization and pericyte loss. AGE could increase the oxidative stress, cytokines and other growth factors. Amino acids are proved to be anti-glycating in our previous study, this study was further extended to animal study by inducing diabetes, retinal changes and early marker changes were studied in SD rats.

2.1 OBJECTIVES

1. To study the effect of amino acids on cell morphology, cell viability, cell proliferation, cell toxicity, cell metabolism in CHO-K1 cells under normal glucose and high glucose in addition to amino acids in mixture and free form.

2. To study the beneficial effect of amino acids on insulin signaling cascade proteins in CHO-K1 cells under normal glucose and high glucose in addition to amino acids mixture.

3. To study the effect of amino acids on oxidative stress pathway and vascular endothelial growth factor in CHO-K1 cells under normal glucose and high glucose in addition to amino acids mixture and free form.

4. To study the beneficial effect of amino acids on glucose uptake and glucose transporter 4 in human 3T3 adipocyte cell line under normal glucose and high glucose in addition to amino acids mixture and free form.

5. To study the effect of amino acids on the retinal changes in the diabetes induced Sprague Dawley rats.

CHAPTER 3: METHODOLOGY

3.1 PREPARATION OF F_{12}-K_1 KAIGHN'S MEDIUM

A bottle of commercially available F_{12}-K_1 nutrient mixture (Sigma) was reconstituted in 900 ml of autoclave sterilized Milli-Q water. The glass bottles used for the cell culture are autoclaved at 121° C for 15 minutes in 15 lbs pressure. The medium was filter sterilized and 90 ml was distributed onto sterilized glass bottles.

3.2 PREPARATION OF GROWTH MEDIUM

- F_{12}-k_1 kaighn's nutrient mixture - 90 ml
- Fetal bovine serum - 10 ml
- L-Glutamine - 1 ml
- Sodium bicarbonate - till the pH is alkaline
- Ciprofloxacin - 0.05 ml (10 mg / 10 ml PBS)
- After reconstitute the medium in solution the mixture is kept at room temperature for 2 days for sterility checkup. Likewise the final medium prepared after adding all the constituents is checked for sterility

3.3 RETRIVEL OF CHO-K1 CELLS FROM LIQUID NITROGEN

- Chinese Hamster Ovarian-K1 (CHO-K1) cells were purchased from National Centre for Cell Science, Pune. As these cells were preserved in cryovials in Liquid Nitrogen, they were retrieved for experimental purpose.
- All experiments were done between passage number 20 – 24.

- The cryovials were immediately thawed in 37°C water bath or incubator after removed from Liquid N2. This step was carried out to wash DMSO (sigma) which has inhibiting effect on the growth of the cells.
- The thawed suspension was added to 8ml of the growth medium and mixed thoroughly and centrifuged at 2000 rpm for 5-10min.
- The supernatant was discarded and fresh growth medium was added to the cell pellet to create a cell suspension and Trypan Blue Dye Exclusion test was done to find the viability of cells.

3.4 MAINTENANCE OF CHO-K1

CHO cells were obtained from National Center For Cell Science (NCCS), Pune. The cells were cultured onto F_{12} - K_1 kaighn's medium. The cells were observed under phase contrast microscope for growth. If the cells were grown to confluence they were trypsinized using 0.1% trypsin and were sub cultured into a new flask. (25 CM^2 Falcon Culture flasks-50 ml Canted neck)

3.5 TRYPSINIZATION

Principle

For experimental purpose, the cells are grown in 25-cm^2 tissue culture flasks. In such flasks, cells are provided with growth medium comprised of the essential nutrients required for proliferation, and the cells adhere to the container and each other as they grow. This process of Cell culture or tissue culture requires a method to dissociate the cells from the container and each other. Trypsin, an enzyme commonly found in the digestive tract, is used to "digest" the proteins that facilitate adhesion to the container and inter-cellularly. The process of trypsinization is often done to permit passaging the cells to a new container.

Procedure

- The medium was removed from 25-cm^2 tissue culture flask.
- 0.1 % Trypsin-EDTA was added to the cells uniformly, and were just made to come in contact with the cell monolayer and aspirated.

- Immediately the cells were incubated for 1-2 minutes at 37°C CO_2 incubator in sterile environment.
- Then approximately 3 ml of growth medium was added to the cells, aspirated and collected in a separate container.
- According to the need of cell no. the cells from the mother flask was subcultured to daughter flask/ wells.
- 5 ml of medium was added to both the mother and daughter flask.
- Trypan blue dye exclusion test was done to find the viability.
- Viable cell count = 2860 cells / 10 µl
- CHO-K1 cell count was found to be 2860 cells / 10µl
- The cells were then incubated at 37°C in a 5 % CO_2 atmosphere. The growth of cells was observed every 24 hours under phase contrast microscope.

3.6 SEEDING OF CHO-K1 CELLS IN VARIOUS TISSUE CULTURE FLASKS

The CHO-K_1 cells were seeded at the bottom surface of 25-cm^2 tissue culture flasks (Greiner) and grown at 37°C in 5 % CO_2 incubator. Change of medium was given every 24 hours. CHO-K_1 cells between 20^{th} and 24^{th} passages were taken for experiments. After confluence of growth obtained, the medium was removed from the flask, the cells were trypsinized and seeded to 25 cm^2, 75 cm^2, 6 well, 24 well, 96 well based on the experiment requirements. The table below gives the amount of cells added **(Table 3.1).**

Table 3.1: Details on number of cells added in each well/ flask for the experiment purpose.

S. No.	Culture dishes	No. of cells per well
1	75 cm^2	8.5×10^4
2	25 cm^2	2.8×10^4
3	6 well	1.5×10^4
4	24 well	1×10^4
5	96 well	3×10^3

3.7 CONDITIONS EXPOSED

Following are the various concentrations of glucose and amino acids used for the experiment parts.

- **Control:** Basal glucose in the medium 7 mM is considered as normal exposing condition.
- **High Glucose:** 12, 17 & 27 mM
- **Amino acids (Free, Mixture):** 2.5, 5.0, 10 & 20 mM.
- **Insulin:** 100 nM
- The different concentration of G and AA maintained in cell exposing are listed in the **Table 3.2.**

Table 3.2: Maintenance of cells in high glucose concentration and incubation of cells with amino acids.

- CHO cells were incubated with glucose alone and glucose with different concentration of amino acids as follows:

Glucose	Glucose + X mM following free amino acids	Glucose + X mM amino acids mixture
Cells with glucose alone	L-Glutamic acid	---
	L-Alanine	---
	L-Leucine	---
	L-Isoleucine	---
	L-Lysine	---
	L-Arginine	---
	L-Aspartic acid	---
	---	Mixture of the above amino acids

- The cells were incubated at 37°C in CO_2 for 24 hours and observed the morphology under phase contrast microscope.

Table 3.3: Experimental conditions exposure times.

EXPERIMENT	TIME OF EXPOSURE
Morphology	24 hours
MTT	24 hours
Lactate	24 hours
Glucose uptake	8 min
IRTK	2 min
PI3K	2 min
Akt	2 min
GLUT4	5 min
Glycogen synthase	20 min

3.8 TRYPAN BLUE DYE EXCLUSION TEST

Principle

Trypan Blue is a vital stain used to selectively colour dead cells as blue. It is a diazo dye. Live cells or tissues with intact cell membrane do not take up the stain. Since cells are very selective to the compounds that pass through the membrane, in a vital cell Trypan blue is not absorbed; however, it traverse the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under microscope. Since live cells are excluded from staining, this staining method is described as a Dye Exclusion Method.

The cell suspension is mixed in a volume of buffer or PBS containing a water-soluble dye (Trypan blue). The dye leaks into the cells that have damaged plasma membranes. By counting total cells and stained cells the percentage of viability can be studied.

Procedure

20 µl of 0.4 % trypan blue stain was added to 20 µl of cell suspension and were mixed thoroughly (1:2 ratio)

↓

Mixture is kept at room temperature for 3 minutes

↓

The hemocytometer was taken and the cover slip was placed in position.

↓

The chambers of hemocytometer were added with 10µl of the stained cell suspension.

↓

Cells were counted in all the four 4 corner primary squares of each grid.in the phase contrast microscope

↓

Both the dead cells and live cells were counted separately. Only the Dead cells were stained blue

Calculations

1. Live cell count = (Number of live cells × Dilution factor) / (Area counted × Depth factor) = (Number of cells /10 µl)

Where dilution factor is 2, depth factor = 0.1

2. Live cell count (cells per ml) = Average cell count per square x dilution factor x 10^4
3. % of cell viability = (number of viable cells / total number of cells) × 100

3.9 MTT ASSAY (3-(4,5-dimethylthiazol-2-yl) 2,5diphenyl tetrazoliumbromide) (Wu, Lei et al. 2009)

Principle

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced by metabolically active cells,

ıpart by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH nd NADPH. The resulting intracellular purple formazan can be solubilized and quantified by ɔectrophotometer at 540 nm.

rocedure

)nce the cells have reached confluence growth serum free medium was added and incubated for 4 hours

↓

The cells were exposed to various glucose and amino acid concentration for 24 hours

↓

fter the incubation period is over the medium was removed and fresh serum free medium along with MTT was added (1 mg/ml), such that the concentration should be 100 µg in each well

↓

The cells were incubated at 37^0C for 4 hours in the incubator

↓

The medium was removed and DMSO was added and incubated for 10 minutes at 37°C to dissolve the formazan crystals

↓

The color developed was read at 540 nm spectrophotometrically

↓

The results were expressed in % taking the untreated cells absorbance value as 100 %

10 LACTATE ESTIMATION

inciple

e lactic acid reacts with sulphuric acid and releases acetalaldehyde, the amount of which is asured by means of the purple color given by p-hydroxydiphenyl in the presence of copper.

agents required

1. 10% TCA: 10 gm in 100 ml
2. 4% $CuSO_45H_2O$: 4 gm in 100 ml
3. Concentrated Sulphuric Acid.

4. P-hydroxy diphenyl reagents: Dissolve 1.5 g of p-hydroxy diphenyl in 95 % ethanol.
5. Stock standard: Lithium lactate - 21.3 mg of Lithium lactate in 100 ml.
6. Working standard. Dilute the stock standard with water to get a solution containing 10 µg/ml.
7. Instrument: DU 640 spectrophotometer.

Table 3.4: Standardization protocol for lactate estimation.

Reagents	Blank	S_1	S_2	S_3	S_4	S_5	T
Working standard (ml)	-	0.1	0.2	0.3	0.4	0.5	-
Concentration (µg)	-	1	2	3	4	5	-
Distilled water (ml)	0.5	0.4	0.3	0.2	0.1	-	0.45
Cell lysate (1:5 diln)	-	-	-	-	-	-	0.05
Sulphuric acid (ml)	3.0	3.0	3.0	3.0	3.0	3.0	3.0
	Boiling water- 10 minutes and cool						
Copper sulphate (ml)	0.05	0.05	0.05	0.05	0.05	0.05	0.05
p-hydroxy diphenyl (ml)	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	Room temperature –30 minutes						
O.D at 570nm							

Calculation: Test O.D/Std O.D x concentration of Std. final value is expressed in mM (Barker & Summerson, 1941).

3.11 LABELED $U^{14}C$ AMINO ACID UPTAKE

3.11.1 Exposure conditions

After 24 hours serum free medium starvation, SFM was removed from the 24 well plates and the cells were exposed to varying glucose (7, 12 & 17 mM) and amino acids (Labeled -0.5 µCi & unlabeled- 5 mM) along with the presence (100 nM) and absence of Insulin with the following combinations:

Glucose and free amino acids

Glucose + Insulin

Glucose + Insulin + $U^{14}C$ Alanine + unlabeled alanine

Glucose + Insulin + $U^{14}C$ Arginine + unlabeled arginine

Glucose + Insulin + $U^{14}C$ Leucine + unlabeled leucine

Glucose + Insulin + $U^{14}C$ Isoleucine + unlabeled isoleucine

Glucose + Insulin + $U^{14}C$ aspartic acid + unlabeled aspartic acid

Glucose + Insulin + $U^{14}C$ Glutamic acid + unlabeled glutamic acid

Glucose + Insulin + $U^{14}C$ Lysine + unlabeled lysine

Glucose and mixture of amino acids

Glucose + Insulin

Glucose + Insulin + $U^{14}C$ Alanine + unlabeled Ala, Arg, Leu, Ile, Asp, Glu, Lys

Glucose + Insulin + $U^{14}C$ Arginine + unlabeled Ala, Arg, Leu, Ile, Asp, Glu, Lys

Glucose + Insulin + $U^{14}C$ Leucine + unlabeled Ala, Arg, Leu, Ile, Asp, Glu, Lys

Glucose + Insulin + $U^{14}C$ Isoleucine + unlabeled Ala, Arg, Leu, Ile, Asp, Glu, Lys

Glucose + Insulin + $U^{14}C$ aspartic acid + unlabeled Ala, Arg, Leu, Ile, Asp, Glu, Lys

Glucose + Insulin + $U^{14}C$ Glutamic acid + unlabeled Ala, Arg, Leu, Ile, Asp, Glu, Lys

Glucose + Insulin + $U^{14}C$ Lysine + unlabeled Ala, Arg, Leu, Ile, Asp, Glu, Lys

- The same experimental condition was done in the absence of insulin.
- The medium was collected at the end of 8 minutes and stored in eppendorfs, immediately the metabolic reactions of the cells were arrested using ice cold PBS and repeatedly washed.
- The cells were lysed using 0.1 N NaOH and the 24 well plates were stored at 4°C.
- The uptake of individual labeled amino acid was measured using Liquid Scintillation System.

3.12 LIQUID SCINTILLATION SYSTEM

Principle

A small number of organic solvents fluoresce when bombarded with radioactivity. The light emitted is of very short wavelength and is not efficiently detected most photomultipliers. However, if a compound is dissolved that can accept the energy from the solvent and itself fluoresce at a longer wavelength, and then the light can be more efficiently detected. Such a

compound is known as primarily fluor and the most frequently used example is 2,5-diphenyl oxazole (PPO). Unfortunately the light emitted by PPO is not always detected with very high efficiency but this can be overcome by including a secondary fluor or wavelength shifter such as 1,4-bis(5-phenyloxazol-2yl) benzene (POPOP). This method is particularly useful in quantifying weak β-emitters such as ^{3}H, ^{14}C and ^{35}S, which are frequently used in biological work. The Liquid Scintillation counting is carried out in Liquid Scintillation system (LSS 6500, Beckman).

Preparation of scintillant

Toluene	- 666 ml
Triton X 100	- 322 ml
POP	- 5 gm
POPOP	- 0.15 gm

Procedure

2 ml of scintillant and 200 µl of NaOH lysed sample was added in each vials.

↓

The vials were closed properly and mixed thoroughly by shaking.

↓

Using tissue papers, the outer surface of the vials were wiped gently.

↓

The vials were arranged in the racks.

↓

Command card with Calibration instruction was introduced

↓

The racks containing the vials were placed into the sample changer of the instrument.

↓

The LSS recognized the sample as ^{14}C radioisotope.

↓

Finally the rack containing halt was kept in order to stop the counting process.

↓

The system always takes one minute to count one sample at a time.

3.13 Cell lysate preparation

3.13.1 Radio Immuno Precipitation assay buffer (RIPA buffer)

To 10 ml of PBS (pH 7.4), the following were added.

PMSF – 100 µl

Sodium orthovandate – 100 µl

Aprotinin – 100 µl

Triton X 100 – 50 µl.

3.13.2 Procedure

At the end of incubation with exposing conditions, the medium was removed from the flask

↓

Reaction was stopped by adding ice cold PBS

↓

Washed thrice in PBS

↓

Incubated with 1 ml of ice cold RIPA buffer, at 4°C for 15 minutes

↓

Cells were scraped off with cell scraper and transfered to the eppendorfs

↓

Disrupted the cells by sonication for 10 sec (Sonicator - Misonix Ultrasonic Liquid ProcessorsXL-2000 series)

↓

Centrifuged at 10,000 rpm for 15 minutes at 4°C

↓

The supernatant was transfered to a pre cooled centrifuge tube

↓

Cell lysate was stored at -80°C until processed after protein estimation Lowry method

3.14 PROTEIN ESTIMATION (LOWRY METHOD)

Principle

The phenolic group of tyrosine and tryptophan residues (amino acid) in a protein will produce a blue purple color complex, with maximum absorption in the region of 660 nm wavelength, with Folin-Ciocalteau reagent which consists of sodium tungstate molybdate and phosphate. Thus the intensity of color depends on the amount of these aromatic amino acids present and will thus vary for different proteins. Most proteins estimation techniques use Bovine Serum Albumin (BSA) universally as a standard protein, because of its low cost, high purity and ready availability. The method is sensitive to around 10 µg/ml and is probably the most widely used protein assay despite it being only a relative method, subject to interference from Tris buffer, EDTA, nonionic and cationic detergents, carbohydrate, lipids and some salts. The incubation time is very critical for a reproducible assay. The reaction is also dependent on pH and a working range of pH 9 to 10.5 is essential (Lowry, Rosebrough et al. 1951).

Procedure

Table 3.5: Standard and test protocol of protein estimation.

Reagents	Blank	Standard	Sample
Std: BSA (µl)	---	50	---
Sample (µl)	---	---	50
Alkaline copper reagent (ml)	5.0	5.0	5.0
Room temperature: 10 minutes.			
Folin's reagent (ml)	0.5	0.5	0.5
Room temperature: 20 minutes; read at 660 nm.			

3.15 SDS PAGE / WESTERN BLOT

Principle

Polyacrylamide gels are prepared by the free radical polymerization of Acrylamide and cross-linking agent N N' - methyl bis Acrylamide. Chemical polymerization is controlled by initiator catalyst system - APS and TEMED.

Reagents

1. 30 % Acrylamide
   Acrylamide 29.2 g
   Bis acrylamide 0.8 g
   D.H_2O water 100 ml

2. TRIS-HCl (pH 8.8)
   18 gm of TRIS dissolved in 50 ml of MQ water and made upto 100 ml (adjusted pH with conc. HCl)

3. TRIS-HCl (pH 6.8)
   6 gm of TRIS dissolved in 100 ml of MQ water.

4. Electrophoretic buffer (pH 8.6)
   0.6 gm of TRIS and 2.88 gm of Glycine in 1000 ml of MQ water with 1 gm of SDS

5. 10 % APS in D.H_2O

6. 10 % SDS in D.H_20

7. TEMED

8. 10 x TBS (tris buffered saline)
   24.2 gm TRIS base
   80 gm NaCl
   1 liter of D. H_2O

9. SDS Sample buffer
   0.5 M Tris - HCl (pH- 6.8) – 1.0 ml, 10 % SDS - 1.6 ml, Glycerol - 0.8 ml, β - Mercaptoethanol - 0.4 ml, Bromophenol blue - 0.2 ml, MQ.H_2O - 4.0 ml Sample is diluted to 1:4.

10. 0.1% Agarose.

11. Transfer Buffer

Tris	3.3 g (25 mM)
Glycine	14.3 g (0.2 M)
SDS	0.5 g
DH_2O	800 ml

Methanol 200 ml
pH to 8.5 before adding Methanol. Stored at -20°C.

12. Phosphate Buffered Saline Tween (pH 7.4)

NaCl	8.0 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15 g
KCl	0.2 g
$D.H_2O$	1000 ml
Tween 20	1.0 ml

13. Blocking buffer:
1X TBS , 0.1 % tween 20 with 5 % skimmed milk (non-fat dry milk)

14. Washing buffer-TBST: 0.1 % tween-20 in 1X TBS

15. Ponceau stain- 1 gm in 1 % acetic acid

16. Primary Antibody: ***Ref Table 7***

17. Secondary antibody: HRP conjugate: ***Ref Table 7***

18. Antibody diluent buffer
2.5 % BSA in 1X TBS with 0.1 % tween-20

19. Luminescent Mixture-Millipore Immunobilon Western
0.5 ml of HRP substrate peroxide solution
0.5 ml of HRP substrate Luminol reagent

Materials

- PAGE apparatus
- Electroblotting cassette
- Whatmann No: 3 MM Paper
- Magnetic stirrer
- Nitrocellulose paper
- Mini transfer western blot- BioRad

Protocol

Preparation of separating gel and separating gel

Table 3.6: Percentage of stacking and separating gel.

Reagents	10 % Separating gel (10 ml)	12 % Separating gel (10 ml)	4 % Stacking gel (10 ml)
30 % Acrylamide (ml)	3.33	4.0	1.33
Tris HCl (pH 8.8) (ml)	2.5	2.5	--
Tris HCl (pH 6.8) (ml)	--	--	2.5
D. H_2O (ml)	4.17	3.5	6.1
SDS (µl)	100	100	100
APS (µl)	50	50	50
TEMED (µl)	5	5	10

Casting the gel

The slab is assembled, sealed at the bottom with agarose and separating gel is carefully poured. After allowing about 45 minutes to polymerize with a layer of saturated butanol, the stacking gel was poured above the separating gel and combs were placed. Samples were loaded along with molecular weight marker. The gel was run at 150 V for approximately 2-3 hours till the samples ran the whole length of the gel.

Clean glass plates with 1.5 mm spacer clamped

↓

Sealed with molten agarose gel

↓

10 % Separating gel was poured up to the ¾ th level of the plate and allowed to solidify

↓

Then stacking gel was poured and comb was placed and allowed to solidify

↓

After solidification comb was removed and washed twice with buffer

↓

The sample containing 50 µg of protein 3 parts + 1part of 5X SDS sample buffer was added

↓

Electrophoretic movement with 150 V current till tracking dye reach agarose layer(seal)

↓

After, gel was removed from the electric field

Transfer

Cut the gel and incubated in the transfer buffer for 20 min

↓

Cut the nitrocellulose membrane for required size and incubated in the transfer buffer for 20 min

↓

Arranged the gel and nitrocellulose membrane in the mini transfer western blot without any air bubble

↓

Kept for transfer under 100 V electric field for 1 hour at 4°C with coolent for the transfer to occur

↓

Then the membrane was removed and washed thrice in TBST each for 5 min

↓

Stained the gel with Ponceau stain to confirm complete transfer

↓

Ponceau stained membrane was washed in water

↓

Incubated the membrane in blocking buffer for 1 hour at RT under mild rocking

↓

Washed in TBST - 3 times

↓

Overnight incubation in primary antibody (Dilution ref table 7) at 4°C with mild rocking

↓

Washed in TBST - 5 times

↓

Incubated in secondary antibody (1:2000) for 1hour at RT

↓

Washed in TBST – 3 times

Detection (Dark Room)

Membrane placed in the even surface on the food wrapper

↓

Luminescence mixture was added over the membrane and incubated for 2-10 min at RT

↓

After that membrane was exposed to X-ray sheet, inside the hypercasette (timing dependent on the strength of the signal)

↓

Then membrane was developed in developer (1in 10 dilution) for 1 min

↓

Washed in water

↓

Soaked in fixer (1 in 5 dilution) for 1 min

↓

Finally washed in water

↓

Then the intensity of the bands were analysed by GS 800- Calibrated densitometer (Biorad)

Table 3.7: Antibody details used for the western blot analysis of GLUT4, Akt, VEGF.

Particulars	Primary antibody	Secondary antibody
GLUT4	Goat polyclonal IgG GLUT4 (Santa Cruz) – 1:1000	Anti-goat raised in rabbit IgG HRP conjugated (Santa Cruz) – 1:2000
Akt	Rabbit phospho Ser 473 Akt (Cell signaling) - 1:1000	Anti-rabbit raised in mouse IgG HRP conjugated (Santa Cruz) - 1:2000
VEGF	Rabbit polyclonal IgG VEGF (Santa Cruz) - 1:1000	Anti-rabbit raised in mouse IgG HRP conjugated (Santa Cruz) - 1:5000

3.16 IMMUNOPRECIPITATION

Principle

This technique is based on the antigen- antibody reaction. The specific antigen is precipitated using monoclonal antibodies using monoclonal hybridoma technique, which are directed against the antigen of interest. Immunoprecipitation is a procedure by which peptides or proteins that react specifically with an antibody are removed from the solution and examined for quantity or physical characteristics (molecular weight, isoelectric point, etc). Antibody-antigen complexes are removed from solution by addition of an insoluble form of an antibody binding protein such as Protein A, Protein G or secondary antibody.

Procedure

500 µg of cell lysate supernatant was taken in a microfuge tube

↓

To this added 10 µl of agarose beads and incubated for which is a pre-clearing step of non-specific

↓

Centrifuged

↓

To the supernatant added 7.5 µl (1.5 µg) of primary antibody and kept at 4° C for over night rocking

↓

Then to this added 20 µl of agarose protein conjugate and incubated at 4° C on rocker platform for 2 hours

↓

Immunoprecipitate was collected by centrifuging at 3000 rpm for 10 minutes at 4°C

↓

Immunoprecipitate was washed with 400 µl of RIPA Buffer twice

↓

Supernatant was discarded and the pellet was washed with wash buffer 2 times

↓

Third wash was done with assay buffer for twice

↓

Resuspended the pellet in 200 µl of RIPA Buffer and stored at -20°C

↓

Protein estimation was done with the above immunoprecipitate

3.17 GLUCOSE UPTAKE BY INDIRECT MEASUREMENT OF GLUCOSE ENTRY BY GLUCOSE OXIDASE / PEROXIDASE TEST METHOD

Experiment

- The glucose entry into the cell is measured by the disappearance of glucose from the medium which is analyzed by clinical analyzer. The experiments were done in duplicates.
- The 90 % confluent cells were starved for 24 hours.
- Serum free medium (SFM) from CHO-K1 cells were removed and a fresh SFM was added with the varying concentration of glucose 7 mM, 12 mM and 17 mM in the presence and absence of insulin (100 nM) along with 5, 10 & 20 mM AAM.
- The cells were then incubated for 8 minutes and immediately the medium was collected in eppendorfs and the cell metabolism was arrested using ice cold PBS.
- The medium was stored in -20°C. These medium was later used to calculate the glucose remaining by subjecting to Glucose oxidase and peroxidase test.
- The difference between the glucose present in the medium before and after exposed to the cells showed the uptake of glucose by the starved CHO-K1 cells.

Principle

The reaction sequence employed in the assay of Glucose is as follows:

$$\text{Glucose} + 1/2O_2 + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconate} +$$

$$2H_2O_2 + \text{4-aminoantipyridine} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

Glucose is oxidized by Glucose oxidase and produces Gluconate and Hydrogen peroxide. The Hydrogen peroxide is then oxidatively coupled with 4-aminoantipyrine and phenol. The amount of coloured complex (Quinoneimine) is proportional to glucose concentration in sample, and can be measured photometrically.

Procedure

- The reagent and the Glucose analyzer was pre-warmed to room temperature
- To 1 ml of reagent, 10 µl of sample was added and incubated for 5 min at 37°C.
- After incubation, the colour was read in Glucose analyzer.

3.18 GLUCOSE UPTAKE STUDY BY $U^{14}C$ LABELED GLUCOSE

CHO-k1 Cells were grown in 24 well plates and incubated
in 5 % CO_2 incubator at 37°C for 24 hours

↓

The medium was discarded
Serum free medium was added and incubated for 24 hours
in 5 % CO_2 incubator at 37°C

↓

SFM was removed and conditions media were added and incubated for
incubated for 2, 8, 30 and 60 minutes in 5 % CO_2 incubator at 37°C
for time standardization of glucose uptake study

↓

The CHO-K1 cells were treated with different glucose concentration (5 mM-
30 mM) along with this was added 0.2 µCi of radiolabelled $U^{14}C$ glucose/deoxy glucose,
incubated for 8 minutes
to see effect of amino acids on glucose

↓

Medium was discarded and the cells were washed thrice with ice cold PBS

↓

1.0 ml of 0.2 N NaOH is added and cells are scrapped with the help of vigorous syringing

↓

To 1 ml of this cell lysate, 4 ml of scintillant is added and amount of radioactivity is read
using liquid scintillation system *(Section 3.12)*

3.19 AUTORADIOGRAPHY OF INSULIN RECEPTOR TYROSINE KINASE

Reagents

1. PBS (Phosphate Buffer Saline) (pH – 7.4): 8 gm of NaCl, 1.15 gm of Na_2HPO_4, 0.2 gm of KCl was dissolved in 1000 ml of distilled water.
2. RIPA buffer ***(Ref Section 3.13.1)*** (Makoto *et al.*, 2001).
3. Primary antibody ($P85_\alpha$ Rabbit Polyclonal IgG)
4. A/G agarose conjugate
5. ***IRTK buffer:*** TRIS-HCl - 50 mM (pH –7.4), NaCl - 100 mM, DTT - 1 mM, EDTA - 1 mM, Triton X - 0.1 %, LiCl- 500 mM, PMSF - 1 mM, Protease Inhibitors (1 mg/ml - 300 µl for 10 ml).
6. ***Protein kinase assay buffer*** in PBS: HEPES buffer - 50 mM, EDTA - 0.1 mM, Brij25 - 2.5mg, NaCl - 0.15 M, filter sterilized and stored at 4°C.
7. ATP - 50 mM
8. $MgCl_2$ - 2 M
9. Artificial substrate – (poly GLU, TYR) (Sigma) - 4:1,
10. Radio labeled γ ^{32}P ATP (BRIT, Hyderabad) - 40µCi.
11. ***Reaction mixture:*** 10 µl Assay buffer , 10 µl of Cold ATP , 1.5 µl $MgCl_2$, 20 µl of Substrate, 2 µCi (0.2µl) of Radio labeled γ ^{32}P was mixed together and 41.7 µl of Cock tail was taken for the experiment for one reaction.

Protocol

Cells were grown, serum starved (24 hours), pre-incubated with 100 nM insulin and exposed to experimental conditions (glucose - 7, 17 &27 mM and amino acid mixtures - 2.5, 5 & 20 mM) for 2 min. Then the reaction was stopped by ice cold PBS, washed thrice with PBS, added 1 ml RIPA buffer, kept for 10 min at 4°C, cells were scrapped off and transferred to the eppendorfs, sonicated for 10 sec and centrifuged at 10,000 rpm for 10 mins, 4°C. Protein was estimated in supernatant and stored at -20 °C until processed for immunoprecipitation.

3.19.1 Immunoprecipitation of IRTK

To 250 µg of protein added 5 µl (1 µg conc.) of primary antibody

↓

Incubated with rocking for 1 hour, 4°C

↓

Agarose conjugate (10 µl) was added & incubated overnight rocking, 4°C

↓

Centrifuged at 3500 rpm, 10 min, 4°C

↓

200 µl for buffer was added

↓

Wash step was repeated to 2-3 times

↓

The lower organic layer was collected

↓

Pellet was solubilized with buffer

↓

Protein was estimated (Lowry's method) and was stored at -20° C
(Ferrara, Reynolds et al. 1998)

3.19.2 IRTK activity by autoradiography

30 µg of protein and cocktail 41.7µl was added

↓

It was incubated for 30 minutes

↓

5 X sample buffer (3 µl for 42 µl) was added

↓

It was boiled for 3 minutes to break if any sub units were present

↓

7 % SDS PAGE was run and the wells were loaded with samples at 160 V for 2 hours

↓

After 2 hours the gel was dried along with whatman 1 filter paper and food wrapper with the help of a vacuum dryer at 80°C for 2 hours

↓

The dried gel paper was placed in the hyper cassette

↓

The gel was exposed to an x-ray film for varying hours such as 1, 12, 24, 48 h respectively

↓

The x-ray film was developed after the exposure time.

↓

Quantified by LSS

↓

After quantification, cpm values for the amount of radioactive material present in the samples for IRTK expressed as specific activity in

nmole ATP incorporated/min/mg protein

(Sulochana, Rajesh et al. 2001; Tremblay and Marette 2001)

3.20 AUTORADIOGRAPHY OF PI3-K

Reagents

1. Primary antibody ($P85_{\alpha}$ Rabbit Polyclonal IgG)
2. Agarose conjugate
3. **PI3-K buffer1**: 50 mM HEPES (pH -7.5), 150 mM NaCl, 10 % Glycerol (5 ml) , 1 % Triton X 100 (100 µl), 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 1 mM Sodium orthovanadate , 1 mM DTT, 30µl from10 X stock of Protease Inhibitor.
4. **PI3-K Buffer2**: 20 mM Tris HCl, 10 mM NaCl, 1 mM Sodium orthovanadate, 0.5 mM EDTA was dissolved in 50 ml of PBS and was filter sterilized and stored at 4°C.
5. **Reaction Mixture:** 50 µl of Buffer, 1 µl of $MgCl_2$, 5 µl of ATP, 5 µCi (0.5 µl) of $\gamma\ ^{32}P$ ATP , 0.3 mg/ml of 2 µl PI substrate. 58.5 µl of Cock tail was taken for experiment for one reaction.
6. Solvents ($CHCl_3$: CH_3OH: 28%NH_4OH) in the ratio (129: 114 : 15)

3.20.1 Immunoprecipitation of PI3-K

Refer section **3.19.1**

3.20.2 PI3-K activity by autoradiography

30 µg of proteins and cocktail were added

↓

Incubation for 20 minutes

↓

The reaction was terminated by chloroform

↓

Centrifugation at 5000 rpm for 10 minutes was done and the lower organic layer was taken

↓

The sample was spot on Thin Layer Chromatographic (TLC) plate

↓

The TLC plate was dried

↓

The samples were made to run by keeping them in solvents for 1 hour

↓

The TLC plates were dried and was placed in the hyper cassette

↓

The TLC plates were exposed to an x-ray film for varying hours such as 1, 12, 24, 48 hours respectively

↓

Overnight exposure means the hyper cassette should be kept at -80° C

↓

It is kept at room temperature for 1 hour before the x-ray was developed

↓

The x-ray film was developed after the exposure time

↓

For quantifying the TLC plates too were stained with iodine vapours

↓

The spots were marked, scraped and dissolved in scintillant and kept for 10 mins

↓

Quantified by LSS

↓

After quantification, cpm values for the amount of radioactive material present in the samples for PI3K expressed as specific activity in

pmole ATP incorporated/min/mg protein

(Tremblay and Marette 2001) (Srinivasan, Rajesh et al. 2005)

3.21 IMMUNOFLUORESCENCE OF AKT / GLUT4

Principle

Immunofluorescence staining is known as the fluorescent antibody technique. In indirect immunofluorescence assay secondary antibody is conjugated with the fluorchrome and it recognizes the primary antibody in target cells and function as a reporter for the presence of target cell.

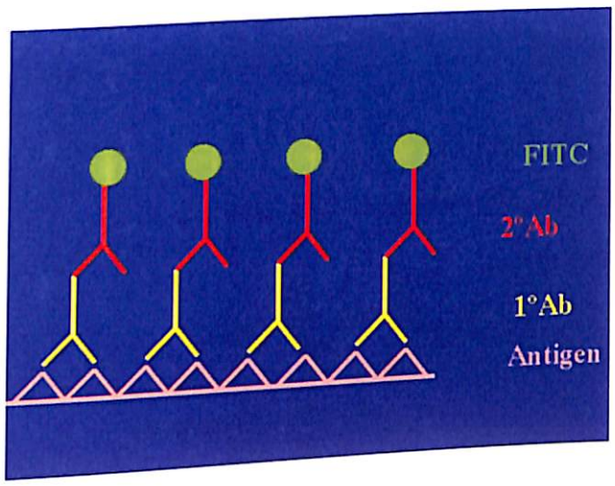


Fig 3.1: Indirect immunofluorescence - principle

Reagents

1. Glucose stock (Sigma) : 90 mg in 5 ml, conc.100 mM
2. Insulin : Recombinant human insulin (Sigma)
3. 4 % Para formaldehyde in PBS (pH 7.0) dissolved heating at 100°C and small amount of 1N NaOH was added.
4. 0.1 % Triton X100 in PBS.
5. 2 % BSA in PBS (Himedia)
6. Primary antibody 1 % BSA/ PBS
7. FITC conjugated secondary antibody in 2 % BSA /PBS
8. DAPI (4,6 Diamino 2 phenylindol, Sigma)

   Stock: 5 mg/ml in PBS (pH7.0)

   Working: 1:50,000 dilution in PBS
9. Antifade: Mowiol (sigma)

6 ml of Glycerol was taken; 2.4 g of Mowiol and 6 ml of D H2O was added and stirred for overnight at RT. Then 12 ml of 0.2 M Tris (pH 8.5) and 0.02 % of sodium azide was added and incubated in hot water (50-60°C) for 10 min to dissolve the Mowiol. Then centrifuged at 5000 RPM for 15 min to remove any undissolved solids. Stored in -20°C

Table 3.8: Antibody details used for the immunofluorescence assay.

Particulars	Akt	GLUT4
Primary antibody	phospho Ser 473 Akt raised in Rabbit (Cell signaling) 1:50	Human Anti Glut raised in rabbit (Abcam) 1:100
Secondary antibody	Anti rabbit raised in Goat conjugated to FITC (Bangalore Genei) 1:100	Anti rabbit raised in Goat conjugated to FITC (Bangalore Genei) 1:200

Experiment Procedure

Cells were plated in 24 wells plate with 22 mm cover slip

↓

When cells reached 80-90 % confluence

↓

Serum starved for overnight with serum free media

↓

Next day pre-incubated in 100 nM insulin / 1 hour at 37°C

↓

Exposed to experimental condition 2 min (Akt), GLUT4 (5 min) at 37°C

↓

Washed thrice in ice cold PBS

↓

Fixed with 4 % Para formaldehyde for 20 min at RT

↓

Permebilised with 0.1 % Triton for 10 min at RT

↓

Blocked by 2 % BSA in PBS for 10 min at RT

↓

Primary antibody over night at 4°C

↓

Washed in PBS thrice

↓

Secondary antibody FITC conjugated for 45 min at RT in Dark

↓

Washed in PBS thrice

↓

Counter stain DAPI (1:50,000) for 15 min at RT in dark

↓

Washed several times in PBS, final in Tris-HCl (pH 8.5)

↓

Allowed to dry

↓

Mounted with Anti-fade and coversliped

↓

Kept in dark for 2 hrs in dark

↓

If needed stored in 4°C

↓

Viewed under fluorescence microscope-carl zeiss inverted fluorescent microscope
(Shigematsu, Miller et al. 2001)

3.22 PROTEIN ESTIMATION- BRADFORD ASSAY (Muretta, Romenskaia et al. 2008)

Principle

Coomassie dye binds to the protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant color change from brown to blue.

Reagents

1. Coomassie plus- Bradford assay kit (Pierce, Rockford)
2. Standard – Bovine serum albumin – 1 mg/1ml in normal saline
3. Normal saline – 0.9 % NaCl

Table 3.9: Protocol for protein estimation by bradford assay.

PARTICULARS	BLANK	STANDARD	TEST
Normal saline (ml)	1.0	1.0	1.0
Reagent (ml)	1.0	1.0	1.0
Standard (µl)	-	5	-
Test (µl)	-	-	2
Incubated at RT for 10 min, Read OD at 595nm.			

3.23 GLUT4 ANALYSIS BY FLOW CYTOMETRY

Principle

The flow cytometry is hydrodynamic focusing for presenting cells to a laser. The sample is injected into the center of a sheath flow. The combined flow is reduced in diameter, forcing the cell into the center of the stream. As cells intercept the light source they scatter light and fluorochromes are excited to a higher energy state. This energy is released as a photon of light with specific spectral properties unique to different fluorochromes. Scattered and emitted light from cells and particles are converted to electrical pulses by optical detectors. Collimated (parallel light waveforms) light is picked up by confocal lenses focused at the intersection point of cells and the light source and light is send to detector photomultiplier tube (PMT). The

electrical pulses originating from light detected by the PMTs are then processed by a series of linear and log amplifiers. Logarithmic amplification is most often used to measure fluorescence in cells. This type of amplification expands the scale for weak signals and compresses the scale for "strong" or specific fluorescence signals.

Protocol

The CHO-K1 cells were exposed to varying glucose concentration (7 - 27 mM) in the presence and absence of amino acids mixture (5 & 10 mM) with or without insulin (50,100 nM)

↓

At the end of two minutes of exposure at 37 °C, the reaction was arrested by adding ice cold PBS immediately, followed by trypsinisation (0.1 %)

↓

The cells were transferred and stained in polystyrene tubes

↓

The cells were spun down such that the supernatant was removed with little loss of cells. The viability of the cells was not less than 90 %

↓

The cells were resuspended to approximately 1-2x10^6 cells/ml in ice cold PBS, 10 % FCS, 1 % sodium azide

↓

100 µl of cell suspension was added to each tube, along with an additional 2 µg of the primary antibody in 3% BSA in PBS, and incubated for 30 min at 4°C in the dark

↓

The cells were washed three times by centrifugation at 400 g for 5 min and were resuspended in ice cold PBS

↓

The FITC-labeled secondary antibody was diluted in 3 % BSA in PBS at 1:100 dilution

↓

The cells were resuspended in this solution and incubated at 4°C for 30 minutes in dark

The cells were washed thrice by centrifugation at 400 g for 5 min and resuspended in ice cold PBS, 3 % BSA, 1 % sodium azide.

(Muretta, Romenskaia et al. 2008)

Flow cytometry instrumentation

One-color flow cytometric analysis was performed on a BD FACS Calibur four-color flow cytometer (BD Biosciences) equipped with two air-cooled lasers: a 15 mW Argon laser (488 nm) and a Red Diode laser (632 nm). The emission of fluorochromes was recorded through specific band pass filters: 525 nm for fluorescein isothiocyanate. The flow cytometer was regularly checked by using standard BD Calibrite 3 beads (BD Biosciences) for photomultiplier tube (PMT) voltage adjustment, color compensation setup, and sensitivity test. Cells were gated on a forward scatter (FS) versus side scatter (SS) plot based on their morphological features and on FL2 versus side scatter (SS) dot plot where FITC-conjugated GLUT4 was used to trace the cells of interest. Green fluorescence (anti-GLUT4) was displayed on FL1 single histogram gated on the previous two plots. An appropriate discriminator was set on the FS parameter to exclude small debris and other cell fragments. Data acquisition and analysis were performed using the BD CELL Quest Pro software (BD Biosciences, USA). The data are expressed as % FITC positive cells of the total 10,000 cells gated which is over and above the fluorescence seen in the control cells that are not exposed to experimental condition.

3.24 GLYCOGEN SYNTHASE ACTIVITY FOLLOWED BY RP-HPLC

Principle

The regulatory enzyme glycogen synthase catalyses the essentially irreversible reaction -

$$\text{Glycogen (n)} + \text{UDP-Glc} \xrightarrow{\text{Glycogen synthase}} \text{Glycogen (n+ 1)} + \text{UDP}$$

The activity of glycogen synthase in the cell lysate is estimated by decrease in the concentration of UDPG (one of the reactant) after the reaction. The UDPG was assayed by reverse phase HPLC.

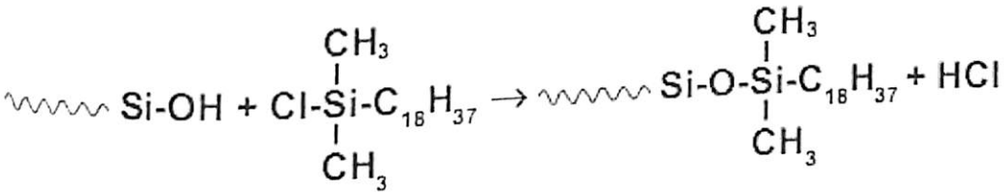
3.24.1 Solid phase extraction

- C 18 column, Solid Phase Extraction Cartridges (Cayman chemical company)
- It is an Octadecyl bonded end capped silica.
- Particle size – 40 µm.
- Pore size – 60 A°.

- Used for reversed phase extraction of nonpolar to moderately polar compounds.

Principle

Reversed phase SPE separations involve a polar sample matrix (mobile phase) and a nonpolar stationary phase. The analyte of interest is typically mid- to nonpolar. The hydrophilic silanol groups at the surface of the raw silica packing (typically 60Å pore size, 40µm particle size) have been chemically modified with hydrophobic alkyl or aryl functional groups by reaction with the corresponding silanes.



Retention of organic analytes from polar solutions (e.g. water) onto these SPE materials is due primarily to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. These nonpolar-nonpolar attractive forces are commonly called van der Waals forces, or dispersion forces. To elute an adsorbed compound from a reversed phase SPE tube or disk, a nonpolar solvent to disrupt the forces are used.

Requirements

1. UDPG (Sigma)
2. UDP (Sigma)
3. Glycogen
4. Glucose 6 phosphate
5. 500 mM Tris HCl pH 8.2
6. 300 mM $MgCl_2$
7. 100 mM EDTA

Reagents

1. **Reaction mixture**
   Tris HCl (pH 8.2) - 0.2 ml
   $MgCl_2$ - 0.085 ml
   EDTA - 0.02 ml

β mercaptoethanol - 0.05 ml
MQ water - 1.6 ml

2. **Reaction cocktail**
Reaction mixture - 2 ml
Glycogen - 5 mg
G6P - 1.4 mg

3. **Stock standard**
UDPG – 1 mg /1 ml in 10 % Acetonitrile

Table 3.10: Working standard of UDGP

PARTICULARS	S1	S2	S3	S4
Vol. of stock (µl)	122	244	366	488
Made up to 1ml using 10 % Acetonitrile				
Final conc.(µM)	200	400	600	800

4. **Stock standard**
UDP– 1 mg /1 ml in 10 % Acetonitrile

Table 3.11: Working standard of UDP

PARTICULARS	S1	S2	S3	S4
Vol. of stock (µl)	80	160	240	320
Made up to 1ml using 10 % Acetonitrile				
Final conc.(µM)	200	400	600	800

3.24.2 Protocol – Glycogen Synthase activity

0.5 ml of the cocktail of the reaction mixture was taken

↓

122 µl of the stock UDPG was added

↓

100 µl of the cell lysate was added mixed well (enzyme source)

↓

Incubated for 5 min at 30°C

↓

Then immediately transferred and kept in 100°C for 5 min to stop the reaction

↓

Cooled and used for further analysis

↓

Purification was performed by SPE (solid phase extraction) cartridge.

3.24.3 Equilibration of the cartridge for solid phase extraction *(fig 3.2)*

1.0 ml of MQ water was added to the cartridge and centrifuged at 2500 RPM for 5 min

↓

Then 1 ml of 10 % Acetonitrile was added and centrifuged at 2500 RPM for 5 min

↓

Then this cartridge was used for the extraction purpose

3.24.4 Sample preparation for HPLC

To the equilibrated cartridge the 1 ml of standard/samples were added and centrifuged for 5 min at 2500 RPM

↓

Then the elute was filtered through the 22 µm syringe filter

↓

Then filtrate was kept at 60°C for 1 hour to enable Acetonitrile evaporations

↓

Then concentrated by speed vac concentrator (Savant SPDlll V, Thermo scientific)

↓

Reconstituted with 100 µl of 10 % Acetonitrile

↓

From this 50 µl was used for HPLC injection

(Danforth 1965)

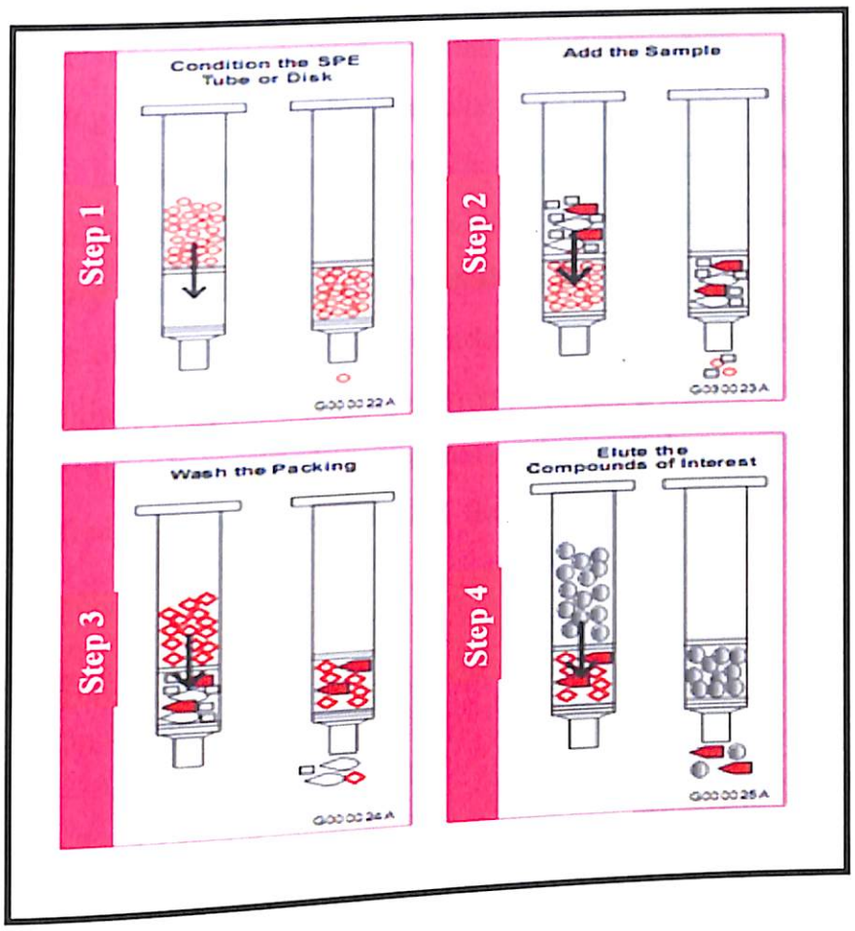
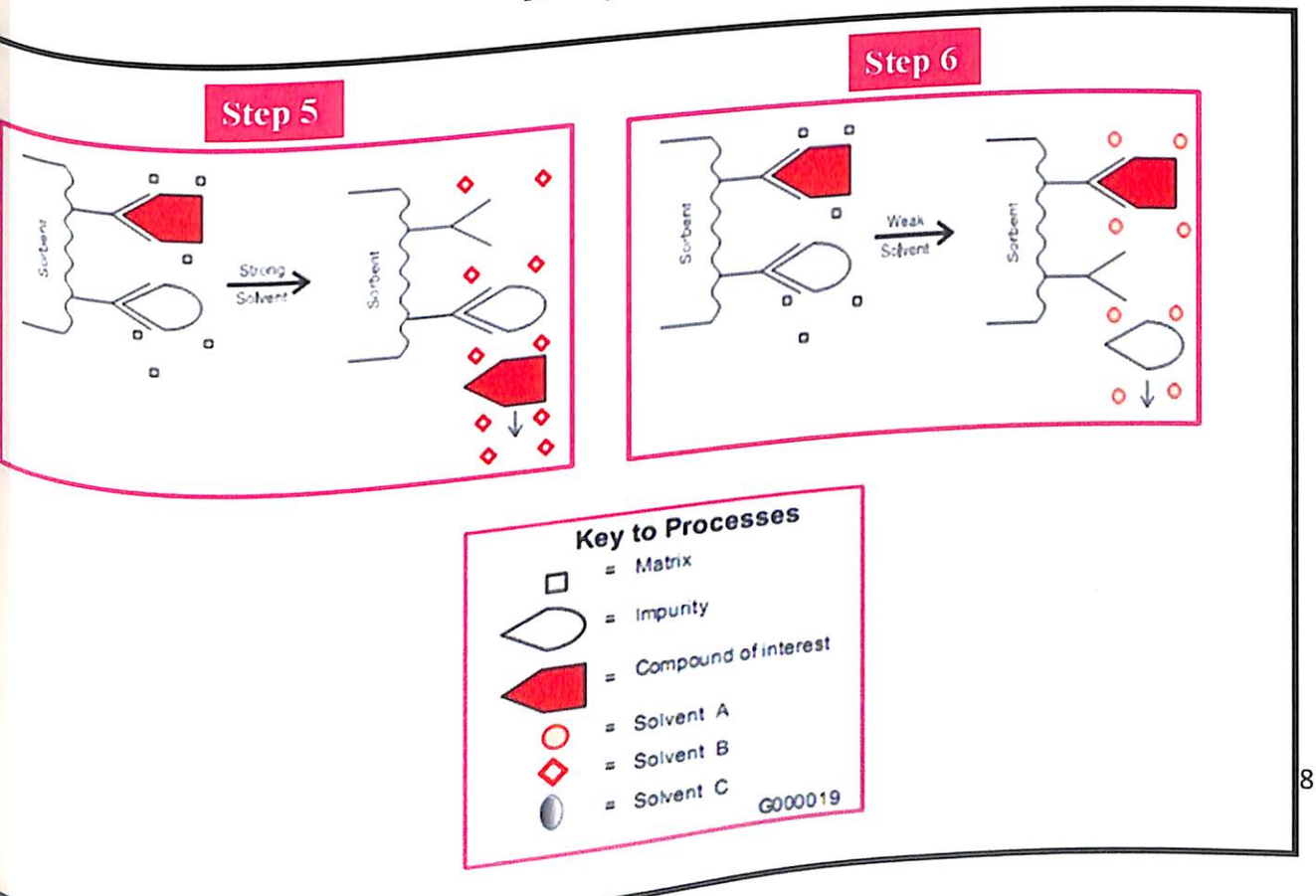


Fig 3.2: Steps involved in the sample preparation before the HPLC run by SPE cartridge.



Reverse phase HPLC separation depends on the reversible adsorption/desorption of solute molecules with varying degrees of hydrophobicity to a hydrophobic stationary phase. It is an adsorptive process by experimental design, which relies on a partitioning mechanism to effect separation. The solute molecules partition (i.e. an equilibrium is established) between the mobile phase and the stationary phase. The distribution of the solute between the two phases depends on the binding properties of the medium, the hydrophobicity of the solute and the composition of the mobile phase. Initially, experimental conditions are designed to favour adsorption of the solute from the mobile phase to the stationary phase. Subsequently, the mobile phase composition is modified to favour desorption of the solute from the stationary phase back into the mobile phase.

Column parameters

Reversed phase column - C18

Column length - 25 cm

Internal diameter - 4.6 mm

Particle size - 5 micron

Liquid Chromatography parameters

Pressure : 400 max pressure

Temperature : 37°C

Flow rate : 1.5 ml/min

Detector: Variable wavelength detector

Injection volume: 50 µl

Mode : Gradient

Solvent A : 0.005 M Tetra butyl ammonium hydrogen sulphate
0.01 M Disodium hydrogen phosphate in MQ water.

Solvent B : 100 % Acetonitrile

Time : 22 min

Filter : 254 nm

Table 3.12: Gradient program for HPLC

Time (min)	% B (Acetonitrile)	Flow Rate (ml/min)	Max Pressure
0	0	1.5	400
3	5	1.5	400
12	5.1	1.5	400
13.5	25	1.5	400
15	25	1.5	400
16.5	0	1.5	400
22	0	1.5	400

3.25 REACTIVE OXYGEN SPECIES ASSAY BY (2'7')- DICHLORODIHYDRO FLUORESCENCE DIACETATE (DCF) METHOD

Principle

The acetate ester form of 2', 7' Dichlorodihydrofluorescein Diacetate (H_2DCFDA-AM) is a membrane permeable molecule that passes through the cell membrane. Once inside the cell, cellular esterases act on the molecule to form the non-fluorescent moiety H_2DCFDA, which is ionic in nature and therefore trapped inside the cell. Oxidation of H_2DCFDA by ROS converts the molecule to 2' 7'dichlorodihydrofluorescein (DCF), which is highly fluorescent. Upon stimulation, the resultant production of ROS causes an increase in fluorescence signal over time **(Fig 3)**.

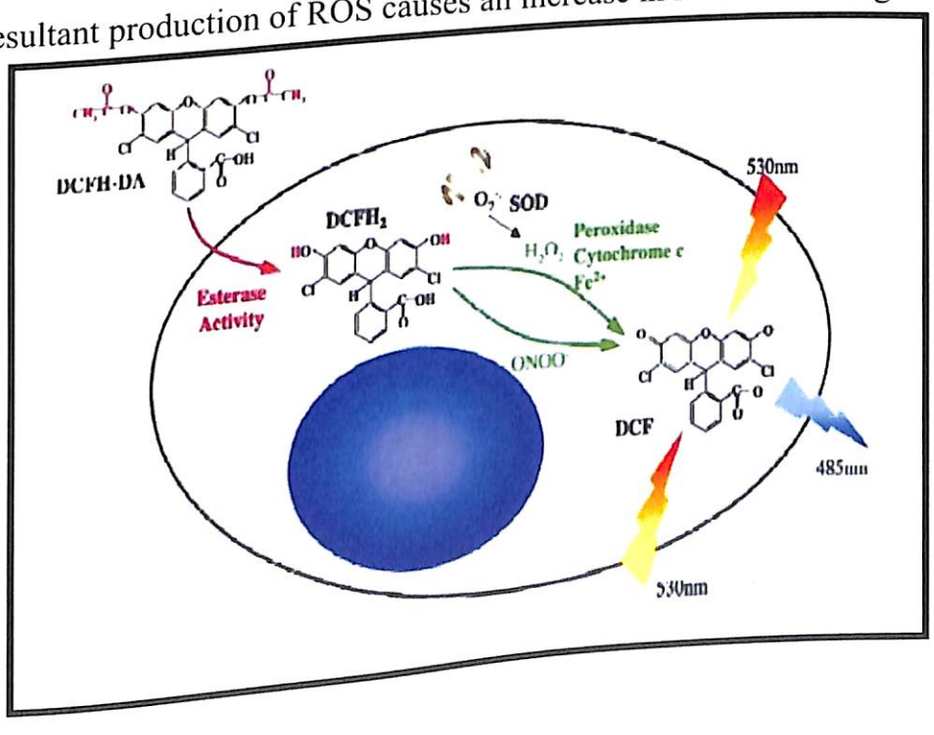


Fig 3.3: Principle of DCF dye used in ROS measurement.

Reagents

2', 7' Dichlorodihydrofluorescein Diacetate (Sigma) - 1 mg/ ml Dimethyl sulfoxide (DMSO).

Protocol

Cells were plated in 96 wells plate

↓

When cells reached 80-90 % confluence

↓

Serum starved for overnight with serum free media

↓

Exposed to experimental condition with 100 nM insulin for 24 hours and incubated at 37°C

↓

After incubation 5 µM of stock DCF-DA added to each well

↓

Then incubated in dark for half an hour

↓

Kinetic readings were taken in Dynex Triad Elisa reader for 1 hour

↓

Excitation Filter 485 nm; Emission Filter 535 nm (Xu, Li et al. 2009)

3.26 SUPER OXIDE DISMUTASE

Principle

Super oxide, $O_2^{-\bullet}$, is an intermediate in the autoxidation of epinephrine. The ability of super oxide dismutase to inhibit the autoxidation of epinephrine at pH 9.8 provides the basis of the assay for the enzyme.

SOD catalyses the following reaction,

$$O_2^{-\bullet} + O_2^{-\bullet} + 2\,H^+ \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

Reagents

1. Carbonate - bicarbonate buffer - 50 mM, pH 9.8
   Na_2CO_3-529 mg; $NaHCO_3$- 420 mg; EDTA-1 mg made up to 100 ml with distilled water.
2. Epinephrine: 1.8 mM solution, prepared freshly - 1 mg in 1 ml, dissolved by adding dilute HCl.
3. Instrument: DU 640 spectrophotometer (Kinetic assay mode)

Procedure

Table 3.13: Assay protocol for the estimation of SOD

	Blank	Control	Test
Water (ml)	500	100	--
Carbonate Buffer (µl)	900	900	900
Cell lysate (µl)	--	--	100
Epinephrine (µl)	--	400	400

As soon as the epinephrine is added, immediately the increase in absorbance at 480 nm is measured in a DU 640 spectrophotometer for 240 sec.

Autoxidation of epinephrine to adrenochrome is performed in a control tube without the enzyme. One unit of enzyme activity is defined as the quantity of enzyme required to produce 50 % inhibition in epinephrine autoxidation (Misra and Fridovich 1972).

Calculation

The delta OD values (absorbance at 165 sec - absorbance at 105 sec) for epinephrine control and test were obtained.

$$\text{Specific activity of SOD} = \frac{C\text{-}T}{C/2 \text{ X protein per100 } \mu l \text{ lysate}} = \text{Units / min / mg protein}$$

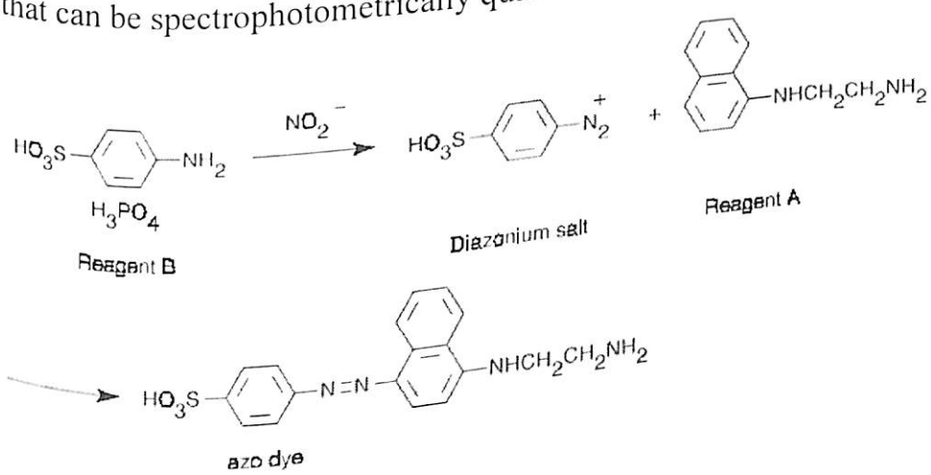
C = ΔOD for epinephrine control

T = ΔOD for test

3.27 NITRIC OXIDE BY GRIESS METHOD

Principle

Sulfanilic acid is quantitatively converted to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to N-(1-naphthyl) ethylenediamine, forming an azo dye that can be spectrophotometrically quantitated based on its absorbance at 548 nm.



Reagents

1. N-(1-naphthyl) ethylenediamine dihydrochloride - 25 mL of a 0.1 % (1 mg/mL) solution, sealed under argon.
2. Sulfanilic acid - 25 mL of a 1 % (10 mg/mL) solution in 5 % phosphoric acid
3. Nitrite standard solution - 1.0 mL of 1.0 mM sodium nitrite in deionized water

3.14: Microplate Assay for NO (Biovision, USA)

	Blank	Std	Test
Medium (µl)	--	--	85
Buffer (µl)	85	83	--
Std (µl)	--	2	100
Buffer (µl)	115	115	115
Nitrate reductase	--	5	--
Co-factor	--	5	--
	Room temperature	1 hour	
Enhancer	--	5	5
Griess R1	50	50	50
Griess R2	50	50	50
	Room temperature	10 min	
	OD at 540 nm		

3.28 CELL CYCLE ANALYSIS BY PROPIDIUM IODIDE

Principle

Apoptotic cell is typically characterized by DNA fragmentation and consequently, loss of nuclear content. Propidium iodide (PI) binding and labeling to DNA gives a rapid and precise evaluation of cellular DNA content by flow cytometric analysis and subsequent identification of hypodiploid cell.

Materials

1. PI - 1 mg/ml
2. Cells in appropriate phase of cell cycle suspended in PBS
3. Ice cold ethanol (70 %)
4. 0.1 % Trypsin (Himedia - cell culture grade)
5. DNase free RNase A - 10 mg/ml (Biogene India)
6. Taurine - 1 mg/ml
7. N-aceytl cysteine (NAC) - 1 mg/ml

8. Di-ethly dithiocarbamate (DDC) - 1 mg/ml
9. Insulin - 100 nM
10. Instrument - Flow cytometry (BD Bioscience)

Procedure

Isolate cells:

Cells with passage 23 were retrieved

↓

1 X 10^4 cells were seeded in two 6 well plates

↓

After 80 % confluence (48 hours)

↓

The cells were serum starved for 24 hours

↓

Cells exposed to experimental conditions and kept for 24 hours

↓

The cells were trypsinized and removed with PBS

↓

Centrifuged at 12,000 rpm for 5 mins at 4°C, decanted the supernatant and gently resuspend the cells in PBS

↓

Cell are counted by hemocytometer

↓

Washed the cells with PBS (1X10^6 cells per tube), and centrifuge at 12,000 rpm at 4°C

↓

Resuspend the pellet in 0.3 ml of PBS

Fixing the cell:

Cells were fixed by gently adding *0.7 ml ice cold ethanol (70%) dropwise to the tube containing 0.3 ml of cell suspension* in PBS while vortexing gently

↓

The cells kept in ice at 4°C for a day

↓

Centrifuged the cells as above, washed one time with ice cold PBS and re-centrifuge

↓

Re-suspended the pellet in 0.25 ml of PBS, added 5 µl of 10 mg/ml Rnase A
Incubate at 37°C for 1 hour

↓

Added 10 µl of 1 mg/ml PI. Kept in dark and at 4°C until analysis

↓

Analyzed on FACS by reading on cytometer at 488 nm
(Nunez 2001)

3.29 APOPTOSIS ASSAY by ANNEXIN-V

Principle

Annexin-V-Alexa fluor 488 severs as a fluorescent probe with a high affinity to phosphatidylserine (PS). Cell-surface PS detection with annexin-V severs as a marker for apoptotic cells. Necrotic cells being leaky enable annexin-V-alexa fluor 488 to bind to the inner membrane PS. To differentiate apoptotic cell from the necrotic cell, simultaneous staining with both annexin-V-alexa fluor 488 and DNA stain PI is done. Exclusion of PI coupled with annexin-V-alexa fluor 488 indicates the apoptotic cells.

Materials

1. Annexin binding buffer: 10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$ pH 7.4
2. Ice cold PBS
3. PI - 1 mg/ml
4. 0.1 % Trypsin
5. Annexin-V-Alexa fluor 488 (Invitrogen)
6. Insulin - 100 nM
7. Instrument - Flow cytometry (BD Bioscience)

Procedure

Cell were serum starved for 24 hours and exposed to experimental conditions

↓

After incubation period, cells were trypsinized and washed with ice cold PBS

↓

Re-centrifuged the washed cells, discard the supernatant and resuspended the cells ($1X10^6$) in annexin-binding buffer (100 µl)

↓

Added 5 µl of annexin-V conjugate to each 100 µl of cells along with 5 µl of PI

↓

Incubated the cells at room temperature for 15 mins

↓

After incubation period, 400ul of annexin-binding buffer was added, mixed gently and kept in ice

↓

The stained cells were analyzed by flow cytometry.
(Morais, Westhuyzen et al. 2005)

3.30 ADIPOCYTE CELL CULTURE

Source

Poietics™ Human Visceral Preadipocytes are isolated from visceral (kidney and bladder) adipose tissue by enzymatic digestion and selective culturing techniques. Unpassaged cryopreserved in a solution containing EGM™-2 MV, FBS and DMSO are from LONZA.

Preparation of Media

- Pre-warmed (37°C), supplemented medium for culturing preadipocytes
- Decontaminated the external surfaces of a 500 ml bottle of Preadipocyte Basal Medium-2 with 70 % v/v ethanol or isopropanol.
- Made up Preadipocyte Growth Medium-2 by adding the entire contents of the FBS (10 %), L-glutamine (2 mM), GA-1000 SingleQuots™ to the bottle of Preadipocyte Basal Medium-2 (50 µg/ml). Reserved 100 ml of Preadipocyte Growth Medium-2 for subsequent preparation of differentiation medium.

Thawing of Cells / Initiation of Culture Process

- Removed the cryovial of cells from liquid nitrogen storage and thaw rapidly in a 37°C water bath, no more than 2 minutes. Decontaminate the external surfaces of the cryovial of cells with 70% v/v ethanol or isopropanol.
- Using a micropipet, gently added the thawed cell suspension to 50 ml of pre-warmed Preadipocyte Growth Medium-2.
- Rinsed the cryovial with medium and added the rinse to the cell suspension.
- Centrifuged at 300 x g for 10 minutes at room temperature.
- When washing the cells, leave a minimum of 1 ml of wash at the bottom of the tube.
- Added 2 or 3 ml of Preadipocyte Growth Medium-2 to the 1 ml of wash and resuspended the pellet of cells. Diluted 20 µl of the cell suspension in 20 µl of 0.4 % Trypan Blue, and a cell count was done to determine % viability. Recovery should be approximately 90 %.

If the cells are to be expanded prior to use for assays

- Plated the preadipocytes at approximately 9,000 cells/cm2 in 0.2 ml medium/cm2 growth area in tissue culture flasks and gently rock to dispersed the cell suspension over the growth surface.
- Human primary preadipocytes will appear round when first plated. Within four hours, greater than 90 % of the cells will be attached and begin to flatten and elongate. Within 24-36 hours the cells are adherent, elongated and spindle shaped. As preadipocytes begin to divide, they will round up. During mitosis the cells stay loosely attached and once division is completed, gets flatten and elongate.
- Preadipocyte cultures are fed every 3-4 days after plating.
- Preadipocyte cultures are cultured to 70 % confluency as they undergo growth arrest, a precursor to differentiation. At this point the cells are harvested and used for subculturing.
- Primary human preadipocytes should not be passaged more than once.

Differentiation to preadipocyte to adipocyte

- Added the entire contents of the SingleQuots™ of insulin, dexamethasone, indomethacin and isobutyl-methylxanthine to 100 ml of Preadipocyte Growth Medium-2 prewarmed to 37°C.
- NOTE: The 100 ml of Differentiation Medium will be "2X" – the concentrations of the differentiation agents will be diluted 2-fold when added to the pre-plated cells.
- Induced the preadipocytes to begin differentiating into adipocytes with the addition of 0.1 ml of Adipocyte Differentiation Medium to each well.
- No further additions or medium changes are required. Differentiated adipocytes are delicate and care should be used to avoid disrupting the numerous lipid vacuoles in the cells.
- The extent of adipocyte differentiation may be noted by microscopic observation of lipid vacuoles in the induced cells. The intracellular lipid vacuoles will begin to appear 4 to 5 days after induction and will continue to increase in number and size for 7 to 10 days. Non-induced cells will have few, if any, lipid vacuoles.
- To document adipocyte differentiation, cultures may be carefully rinsed with PBS, fixed with 10% buffered formalin and stained with Oil Red O.

3.31 ADIPOCYTE STAINING WITH OIL RED O

Reagents

1. Oil Red O stock

   0.7 g Oil Red O in 200 ml Isopropanol

   Stirred overnight, then filter with 0.2 µm and store at 4°C
2. Oil Red O working solution

   6 parts Oil Red O stock & 4 parts d.H2O.

   Mixed and made to sit at room temp for 20 min, filtered.
3. 10 % Formalin in PBS
4. Isopropanol 60 %

Method

- After 10 days of 90 % differentiation of preadipocytes to adipocytes, removed most of the medium
- Add 10 % formalin and incubated 5 min, RT
- Discarded formalin and added the same volume of fresh formalin. Incubate at least 1 hour, or longer. Note: Cells can be kept in formalin for a couple of days before staining.
- Removed all the formalin and washed wells with 60 % isopropanol.
- The wells were dried completely.
- Added Oil Red O working solution for 10 min.
- Removed all Oil Red O and immediately added d.H2O, washed with water thrice.
- Removed all water and viewed under bright field microscope (Sheng, Zhang et al. 2008).

3.32 IMMUNOFLUORESCENCE FOR GLUT4 in adipocytes cell

Ref section 3.21 & Table 3.8 for procedure and antibody dilution

3.33 GLUCOSE UPTAKE IN ADIPOCYTE CELLS

Reagents

1. Amino acids-each 5 mM of alanine, arginine, aspartic acid, glutamic acid, lysine, leucine, isoleucine, cysteine.
2. Cold 2-Deoxy Glucose.(M.W=164mg/L=1mM)

   Stock=1.6mg/ml

   0.1mM= 10uL in 0.5ml
3. Hot 2-Deoxy Glucose

   Stock=1ml/50μci

   20ul=1μci/ 24 well of 0.5ml

NOTE: 8 mM extra glucose added to 25 mM high glucose to have a total of 33 mM as high glucose in adipocyte cells exposure condition.

Protocol

1. After 80 % confluence, cells were serum starved for 2 hours.
2. Then insulin 100 nM pretreatment were given for 30 min at 37°C.
3. Washed with PBS.
4. Cells were incubated with PBS containing 0.1 mM 2-deoxyglucose and 0.2 μCi/ml $U^{14}C$ deoxyglucose for 5 min along with amino acids mixture].
5. Removed the medium.
6. Washed with ice cold PBS.
7. Solubilized with 0.4 ml of 1 % SDS.
8. Added 4 ml of scintillant.
9. Read in Beckman LS6500 scintillation counter (Harmon, Paul et al. 2004).

3.34. GLUT4 mRNA EXPRESSION

3.34.1 RNA extraction by trizol method (Chomczynski and Sacchi 1987)

Principle

TRI Reagent has phenol and guanidine thiocyanate in a monophase solution to facilitate the immediate of RNAse activity. Biological samples are homogenized or lysed in TRI Reagent; the Subsequent addition of bromochloropropane or chloroform results in the separation of the

homogenate into aqueous and organic phase. RNA partitions to the aqueous phase, DNA to the interphase, and protein to the organic phase. The RNA can then be precipitated from the aqueous phase with the addition of isopropanol. The isolated RNA is suitable for any downstream application, including RT-PCR.

Materials required

1. Phosphate buffered saline
2. 0.1% Trypsin
3. Trizol Reagent
4. Chloroform
5. Isopropanol
6. 70 % Ethanol
7. DEPC
8. Agarose
9. 1X TBE (Diluted form 10X TBE)

Procedure

1. The confluent cells were trypsinised using 0.1% Trypsin
2. It was then centrifuged at 14,000 RPM for 3 mins.
3. 1 ml of cold Trizol reagent was added to the pellet.
4. It was incubated at room temperature for 5 mins.
5. 200µl of chloroform was added and it was mixed well for 15sec.
6. It was incubated at room temperature for 3 mins.
7. It was the centrifuged at 12,000 RPM for 15 mins.
8. The aqueous layer was transferred to a new vial.
9. 500 µl of isopropanol was added and it was incubated at room temperature for 10 mins.
10. It was centrifuged at 12,000 RPM for 10 mins.
11. The supernatant was discarded and 1 ml of 70% ethanol was added to pellet and it was incubated on ice for 3 mins.
12. It was centrifuged at 12,000 RPM for 5 mins.
13. The supernatant was discarded and it was dried for 3 mins.

14. 30 µl of DEPC treated water was added.
15. 2 µl of extracted RNA was quantified by Nanodrop (Spectrophotometer).
16. 2 µl of extracted RNA was electrophoresed on 0.8 % agarose prepared using 1X TBE Buffer.

3.34.2 cDNA synthesis

RNA was converted to cDNA using iScript RT-PCR Kit (Biorad) following the manufacturer's protocol using the reagents provided in the kit.

Procedure

1. The following reaction mix was prepared

Reagents	Volume
RNA Template	2 µl
Nuclease free water	13 µl
5x Iscript Reaction Mix	4 µl
Reverse Transcriptase	2 µl
	20 µl

2. The following protocol was followed:

25°C		42°C		85°C	4°C
5:00		30:00		5:00	∞

3. The cDNA was quantified by using Nano drop (spectrophotometer).

3.34.3 PCR for GLUT4 / housekeeping gene- glyceraldehyde 3-phosphate dehydrogenase

GLUT4 Forward primer:

5' – ATG ACT GTG GCT CTG CTC CT -3'

Tm – 59.4°C

GLUT4 Reverse primer:

5' – TGA TGA AGT TGC TCG TCC AG -3'

Tm – 57.3^{0}C

GAPDH Forward primer:

5' – TGT TCC AGT ATG ATT CCA CCC -3'

Tm - 62^{0}C

GAPDH Reverse primer:

5' – GTC TTC TGG GTG GCA GTG AT -3'

Tm - 62^{0}C

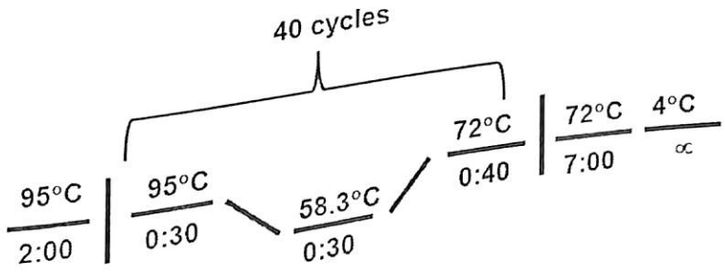
Procedure

1. The following reaction mixture was prepared.

DNTP'S	4 µL
Buffer (10X)	2.5 µl
FP	1.4 µL
RP	1.5 µL
Taq Polymerase	0.3 µl
MQ water	13.61 µl
DNA	2.0 µg
	25 µl

2. A Negative control was also set.(The above reaction mix was prepared where instead of cDNA autoclaved Milli-Q water was added)

3. The following PCR profile was followed.



4. The amplified PCR product was electrophoresed on 2 % agarose using 1X TBE.

3.35 ANIMAL STUDY

3.35.1 Animal procurement and ethics

Male Sprague Dawley (SD) was procured and housed at Madras in Tamil Nadu veterinary animal and science university (TANUVAS), Chennai. All animal was acquired lawfully as per the animal ethics guidelines.

3.35.2 Diet

Animals were fed with standard laboratory Hindustan chow and drinking water ad libitum. Every day sufficient amount of feed and drinking water was given.

Body weight and blood glucose level of each rat was taken at the base line after acclimatization of the animal to the environment.

3.35.3 Streptozotocin induced Diabetes mellitus in SD rats

Dosage of 55 mg/kg STZ was prepared in fresh 0.01M citrate buffer pH= 4.0 (Sithole,2009; Vaishya,2009).

Preparation of ***0.01 M citrate buffer*** pH= 4.0

Solution A: Trisodium citrate : 29.4 mg (10 ml)

Solution B: Citric acid : 38.4 mg (20 ml)

Mixed the solution A + B in the ratio of 2:3

Adjusted the pH to 4.0 using 1N NaOH

3.35.4 Grouping of animals

Animals were separated into groups. The control group (C) is the normal untreated healthy rats. Animals that showed blood glucose levels greater than 250 mg/dL were considered diabetic and were distributed as Diabetic group (D), Aminoguanidine (AG) treated group 50 mg/Kg body weight , SNAAM1 (Sankara Nethralaya amino acid mixture 1) and SNAAM2 supplemented groups respectively.

The groups are as follows:

Group 1: C (Healthy control) (n= 8)

Group 2: SNAAM1 (Composition I) (n=8)

Group 3: SNAAM2 (Composition II) (n=8)

Group 4: D (Diabetic) (n=8)

Group 5: AG (Amino guanidine) (n= 8)

AAM1 & AAM2 composition: Application to be filed for patent.

3.35.5 Blood glucose test

To quantitatively measure the concentration of glucose in blood by glucometer by tail puncturing. Before STZ injection and after 72 hours the blood sugar level were tested.

Principle

The glucose dehydrogenase enzyme in the presence of the coenzyme, on the test strip converts the glucose in the blood sample to gluconolactone.

Procedure

- Inserted the test strip into the meter and the meter turned on.
- Code number on the display matched the code number on the test strip container was verified.
- Touched the drop of blood to the edge of the strip.
- Waited for the results in mg% in Accu-check.

3.35.6 HbA1c

To measure the glycated hemoglobin in rat whole blood, initial i.e. before STZ was induced rats were analyzed for glycated hemoglobin levels. The same done after 8 weeks.

Materials

- The kit contains test devices with a porous membrane filter, test tubes prefilled with reagent washing solution.
- The reagents contains agents lyse the red blood cells and precipitate the haemoglobin particularly.
- A blue boronic acid conjugate that binds cis-dots of glycated hemoglobin.

Procedure

- HbA1c is a boronate affinity assay.
- When blood is added to the reagent, the red blood cells immediately lyse.
- All hemoglobins precipitates.
- The boronic acid conjugate binds to the cis-diol configuration of glycated haemoglobin.
- An aliquot of the reaction mixture is added to the test device and all the precipitate haemoglobin, conjugate-bound and unbound, remains on top of the filter.
- Excess of colored conjugate is removed with the washing solutions.
- The precipitate is evaluated by measuring the blue (glycated haemoglobin) and the red (total haemoglobin) color intensity with the NYCO CARD reader II, the ratio between them being proportional to the percentage of HbA1c in the sample.

3.35.7 Specimens collected and usage

- Sprague dawley rats was sacrificed after two months
- Lens, eyeball, retina and serum was stored at -80°C until processed.
- From the phase 1 the sample were taken and used for the following assay.
- Retinas were removed from rat eyes which were enucleated after 2 months, were processed, embedded and sectioned and used for TUNEL stain and Immuno histochemistry.
- Lens, retinal lysate and serum were used for measuring AGE levels.
- Retinal lysate for ELISA of eNOS, NO and ICAM.

3.36 TISSUE PROCESSING

Stored retinas were processed, embedded and sectioned for further assay (Ramakrishnan, Sulochana 2012).

3.36.1 Steps in Processing

- 100 % alcohol - 30minutes
- 100 % alcohol - 30 minutes
- 100 % alcohol - 30 minutes
- Xylene - 4 minutes
- Xylene - 4 minutes
- Paraffin wax - 30 minutes.

- Paraffin wax - 60 minutes
- Wax in vacuum - 30minutes

3.36.2 Steps in Embedding

- Orientation of tissues was decided.
- Molten paraffin wax 2° or 3°C above the melting point was dispensed into the mould.
- It is the process of placing the tissue in a support medium (paraffin wax)
- Thin film of solid has formed on surface.
- Gently pressed the tissue into the wax in the oriented plane.

3.36.3 Vacuum impregnation

- Transferred the cleared tissues to a heated, sealed container of molten wax.
- Degree of vacuum should not exceed 400-500 mm of Hg.

3.36.4 Sectioning the tissues

The sections of thin microtome 5 µm was taken on the charged slides (fisher scientific) and slides were kept for drying and next day deparffinisation was done

3.36.5 Deparafinisation

- xylene 1 - 4 minutes
- xylene 2 - 4 minutes
- xylene 3 - 4minutes
- 100 % Isopropyl alcohol - 4 minutes
- 100 % Isopropyl alcohol - 4 minutes
- 95 % Isopropyl alcohol - 4 minutes
- 80 % Isopropyl alcohol - 4 minutes
- 70 %Isopropyl alcohol - 4 minutes
- 60 %Isopropyl alcohol - 4 minutes
- Distilled water - 4 minutes

3.37 Immuno histochemistry for CML-AGE, IL-1β, IL-2 in rat retina

Principle

The qualitative identification by light microscopy of antigens in sections of formalin-fixed, paraffin-embedded tissue are studied by immune histochemistry (IHC). The sections are subjected to epitope retrieval prior to staining. Endogenous peroxidase activity is neutralized using the NovocastraTM peroxidase block. This is followed by application of the protein block to reduce non-specific binding of primary and polymer. The sections are subsequently incubated with optimally diluted primary antibody. Post primary block is used to enhance penetration of the subsequent polymer reagent. The polymer recognizes mouse and rabbit immunoglobulins, it detects any tissue-bound primary antibody. Sections are further incubated with the substrate/chromogen, 3,3' - diaminobenzidine (DAB). Reaction with the peroxidase produces a visible brown precipitate at the antigen site.

Reagents

1. Peroxidase Block - 3 % Hydrogen peroxide.
2. Protein Block - 0.4 % Casein in phosphate-buffered saline, with stabilizers, surfactant, and 0.2 % Bronidox L as a preservative.
3. Post Primary Block - Polymer penetration enhancer containing 10 % animal serum in tris-buffered saline/0.09 % ProClin™ 950.
4. NovoLink TM Polymer. Anti-mouse/rabbit IgG -Poly-HRP (each at 8 µg/ml) containing 10 % animal serum in tris-buffered saline/0.09 % ProClin™ 950.
5. DAB Chromogen. 1.74 % 3,3' - diaminobenzidine, in a stabilizer solution.
6. NovoLinkTM DAB Substrate Buffer (Polymer) - Buffered solution containing 0.05 % hydrogen peroxide and preservative.
7. Hematoxylin - 0.02 % Hematoxylin.

Protocol (Leica biosystems, UK)

- Enucleated rat eye balls were fixed in 10 % neutral buffered formalin, processed, embedded and 5 µm sections were done as per **section 3.37**.
- The deparafinized sections were treated with trypsin for 10 min for antigen retrieval.

- Washed slides in de-ionised water.
- Neutralized the endogenous peroxidase using Peroxidase Block for 5 minutes.
- Washed in TBS for 2 x 5 minutes.
- Incubated with Protein Block for 5 minutes.
- Washed in TBS for 2 x 5 minutes.
- Incubated with optimally diluted primary antibody (CML-AGE 1:200, IL-1β & IL-2 1:50).
- Washed in TBS for 2 x 5 minutes.
- Incubated with Post Primary Block for 30 minutes.
- Washed in TBS for 2 x 5 minutes.
- Incubated with NovoLink TM Polymer for 30 minutes.
- Washed in TBS for 2 x 5 minutes with gentle rocking.
- Developed peroxidase activity with DAB working solution for 5 minutes.
- Rinsed the slides in water.
- Counterstained with Hematoxylin.
- Rinsed the slides in water for 5 minutes.
- Dehydrated, cleared and mounted the sections.

3.38 TUNEL ASSAY

Principle

Cleavage of genomic DNA during apoptosis may yield double stranded, low molecular weight DNA fragments (mono and oligonucleosomes) as well as single stranded break (nicks) in high molecular DNA. These DNA strand breaks are identified by labeling with terminal deoxynucleotidyl transferase (TdT) which catalyzes polymerization of labeled nucleotide to free 3' –OH DNA ends in a template independent manner (TUNEL reaction). Fluorescein labels incorporated in nucleotide polymers are detected and quantified by fluorescence microscopy.

Procedure (Roche kit, USA)

- Prewarmed the citrate buffer

- Placed the slide in a plastic jar containing 200 ml 0.1 M citrate buffer, pH 6.0
- Applied 750 W (high) microwave irradiation for 1 minutes
- Rapidly cooled and immediately added 80 ml distilled water (20°–25°C) to the jar.
- Then transferred the slide into PBS – 1 minutes
- Immersed the slide(for 30 min at room temperature) in a blocking solution containing 0.1 M Tris-HCl, 3 % BSA, and 20 % normal bovine serum, pH 7.5
- Rinsed the slide twice with PBS at RT.
- Excess fluid drained off.
- Applied 50 µl of TUNEL reaction mixture (A + B) to the section and incubated for 60 min at 37°C in a humidified atmosphere.
- Rinsed slide three times in PBS (5 min for each wash).
- Excess fluid drained off.
- Rinse slides three times in PBS for 5 min each.
- Slides are air dried.
- 10 µl of vecta shield was mounted.
- Viewed under fluorescent microscope.
- Positive control DNAse was applied (10 min RT) and proceed by adding Tunel reagent.
- Negative control TUNEL without enzyme (B).

3.39 ELISA FOR NITRIC OXIDE SYNTHASE (eNOS/ NOS3)

Principle

The microtiter plate is pre-coated with an antibody specific to NOS3. Then samples added to the wells forms a complex with the pre-coated antibody, followed by biotin-conjugated antibody specific for NOS3. Avidin conjugated to Horseradish Peroxidase (HRP) binds to the biotin. After TMB substrate solution is added, only those wells that contain NOS3, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of NOS3 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

Materials (USCN, China)

- All the reagents and samples are brought to room temperature (18-25°C) before use.
- Standard - Reconstituted the Standard with 1.0 mL of Standard Diluent, kept for 10 minutes at room temperature, shaken gently (not to foam). The concentration of the standard in the stock solution is 1,000 pg/mL. Double dilution series are done to get working of diluted standard such as 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL.
- Assay Diluent A and Assay Diluent B
- Detection Reagent A and Detection Reagent B
- Wash Solution
- TMB substrate

Assay procedure

- Determined the wells for diluted standard, blank and sample.
- Added 100 µL each of dilutions of standard, blank and samples into the appropriate
- wells. Covered with the Plate sealer and incubate for 2 hours at 37°C.
- Removed the liquid of each well without washing next step is proceeded..
- Added 100 µL of Detection Reagent A working solution to each well. Incubated for 1 hour at 37°C after covering it with the plate sealer.
- Aspirated the solution and washed with 350 µL of 1× wash solution to each well using multi-channel pipette and allowed to sit for 1~2 minutes.
- Totally washed thrice and removed the remaining liquid from all wells completely by snapping the plate onto absorbent paper.
- Added 100 µL of Detection Reagent B working solution to each well. Incubated for 30 minutes at 37°C after covering it with the plate sealer.
- Repeated the aspiration/wash process for total 5 times and blotted well on absorbent paper.
- Added 90 µL of Substrate Solution to each well, covered with a new plate sealer and incubate for 15 - 25 minutes at 37°C which is protected from light.

- The liquid turned to blue by the addition of Substrate Solution is stopped by adding 50 µL of stop solution to each well.
- The liquid turned yellow is mixed thoroughly measured at 450 nm immediately in ELISA reader.

3.40 INTERCELLULAR ADHESION MOLECULE (sICAM)

Principle

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody has been pre-coated onto a micro plate. Standards, control, and samples are pipette into the wells and any ICAM present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color measured is in proportion to the amount of ICAM bound in the initial step. The sample values are then read off the standard curve.

Materials (R&D,USA)

- Micro plate- polystyrene micro plate coated with a monoclonal antibody specific for rat sICAM-1.
- Conjugate - Polyclonal antibody conjugated to horse radish peroxidase .
- Rat sICAM-I Standard.
- Rat sICAM-1 Control.
- Assay Diluent RD1-41 - Buffered protein solution.
- Calibrator Diluent RD5-3 - Buffered protein solution.
- Wash Buffer Concentrate - Buffered surfactant.
- Color Reagent A - Hydrogen peroxide.
- Color Reagent B - Stabilized chromogen (tetramethylbenzidine).
- Stop Solution - Diluted hydrochloric acid.

Assay procedure

- All reagents and samples are brought to room temperature before use.
- Prepared all reagents, standard dilutions, control (undiluted) and retinal lysate (1:50).
- Added 50 µl of Assay Diluent RD1-41 to each well.
- Added 50 µl of Standard, Control, or sample per well. Covered with the adhesive strip provided.
- Incubated for 2 hours at room temperature on a horizontal orbital micro plate shaker.
- Aspirated each well and washed, repeated the process four times for a total of five washes.
- Inverted the plate and blotted it against clean paper towels.
- Added 100 µl of conjugate to each well and incubated for 2 hours at room temperature on the shaker.
- Aspirated and washed the wells for 5 times.
- Added 100 µl of substrate to each well and incubated for 30 minutes at room temperature on the benchtop. Protected from light.
- Added 100 µl of stop solution to each well.
- Gently taped the plate to ensure thorough mixing.
- Determined the optical density of each well within 30 minutes, using a micro plate reader at 450 nm (correction wavelength set at 540 nm).
- Concentration of sICAM-1 in retina was expressed as ***ng/mg protein***.

3.41 ADVANCED GLYCATION END PRODUCTS

3.41.1 Preparation of AGE standards calibrator by thermal glycation (Bhatwadekar and Ghole 2005; Sampathkumar, Balasubramanyam et al. 2005)

The following mixtures of reagents were prepared in separate 2 ml bottles each -

A. **Reaction mixtures**

1. BSA + 0.5 M glucose in 1ml of 0.2 M phosphate buffer(**AGE Standard calibrator**)
2. 0.1 M Aminoguanidine + BSA + 0.5 M glucose in 1ml of 0.2 M phosphate buffer
3. 0.1 M Lysine + BSA + 0.5 M glucose in 1ml of 0.2 M phosphate buffer

B. Controls

1. BSA in 1ml of 0.2 M phosphate buffer
2. 0.5M glucose in 1ml of 0.2 M phosphate buffer.
3. 0.1 M lysine in 1ml of 0.2 M phosphate buffer

- To all of the 6 mixtures prepared, 10 µl of chloroform was added to prevent bacterial growth. The inside of the bottle caps was wiped thoroughly with cotton dipped in Toluene to prevent any contamination.
- The bottles were then placed in the incubator set at a temperature of 50°C for 4 days.
- After 4 days, all the reaction mixtures were analyzed for the formation of AGEs in a spectroflourimeter (Excitation: 370 nm, Emission – 440nm).
- The BSA + Glucose reaction mixture is the AGE standard calibrator. It is used to estimate the amount of AGE present in the test samples.

3.41.2 Processing of samples for AGE estimation

The test samples in which AGEs were estimated were –

- The serum/ retinal lysate samples were diluted 10 times with 0.2 M Phosphate buffer.
- The lens samples were weighed and homogenized using 0.25 N NAOH .The homogenate is cyclomixed to get a clear solution. This was then diluted 10 times with 0.2 M phosphate buffer.
- ***Estimating AGEs in test samples***
- The AGE standard and test samples were taken in a black well ELISA Plate.
- The plate was then read in **SPECTRAMAX M2** at λ_{EX} : 370 nm and λ_{EM} : 440nm.
- Protein estimation by Bradford method

Calculation of AGE concentration in test samples

- The Fluorescence values (arbitrary units) obtained for each of the AGE standard titrations were plotted and a standard graph was obtained.
- Using the slope of the standard graph, the amount of AGEs present in the test samples was calculated by the formula:

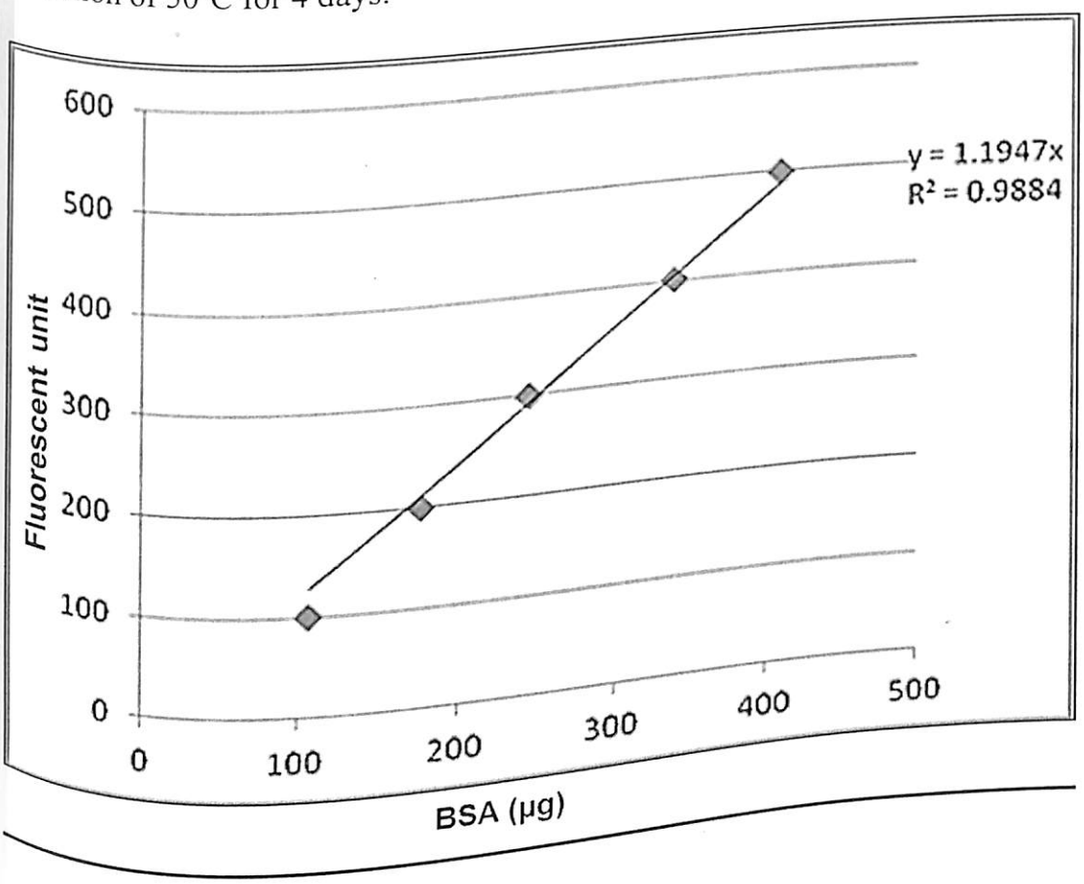
AGE concentration = Test Fluorescence value/ slope of standard Graph*dilution factor

- The AGE concentration is expressed in terms of µg/mg protein by dividing the AGE concentration with the protein concentration of the sample.
- Finally AGE is expressed as **1 unit = 100 µg/mg protein**.

Table 3.15: Standard protocol for AGE estimation.

Content	AGE standard					Lens	Serum / Retinal lysate
Dilution	1:5					1:10	1:5
Volume (µl)	10	20	30	40	50	100	100
Phosphate buffer (µl)	190	180	170	160	150	100	100

Fig 3.4: AGE standard fluorescence value prepared by BSA treated with glucose under thermal condition of 50°C for 4 days.



CHAPTER 4: EVALUATION OF AMINO ACIDS FOR CYTOTOXICITY AND UPTAKE UNDER HIGH GLUCOSE CONDITIONS IN CHO-K1 CELLS

4.1 INTRODUCTION

Amino acids (AA) are basically either glucogenic, ketogenic, or glucogenic and ketogenic. Apart from this nature of AA in metabolism, it is also shown to be beneficial in type 2 diabetes by three possible mechanisms as reported. a) Scavenging the excess glucose in the blood (Ramakrishnan and Sulochana 1993; Ramakrishnan, Sulochana et al. 1996; Ramakrishnan, Sulochana et al. 1997). b) Stimulating the pancreas for insulin secretion (Dixon, Nolan et al. 2003) and c) Up-regulation of the insulin receptor system (Sulochana, Rajesh et al. 2001).

4.1.1 Amino acids transporter

Amino acid transport across the plasma membrane mediates and regulates the flow of these ionic nutrients into cells. The general property includes active transport, stereo specificity (transport is faster for L-stereo isomers), broad substrate specificity (several amino acids share a common transport system) depending on the type of amino acid (acidic, zwitterionic, or basic as well as other characteristics of the side chain) (Palacin, Estevez et al. 1998; Mann, Yudilevich et al. 2003). Neutral amino acid transporter system A shares alanine, serine, glutamine, glycine, 2amino isobutyric acid, system ASC shares alanine, serine, cystine and system L shares leucine, isoleucine, valine, phenylalanine. The cationic amino acid transport system Y^+ transports lysine, arginine, ornithine and anionic transport system transports glutamate and aspartate.

4.1.2 Amino acids metabolism in Diabetes mellitus

It is clear from various studies that there are perturbations in the amino acids in type 2 DM. However understanding the changes in essential amino acid metabolism in the insulin-resistant or diabetic states is still not complete and is still in a stage of infancy (Adams). Questions still remain as to whether amino acids are prognostic markers of the DM and if patterns can differentiate diabetics from non-diabetics.

Protein catabolism in T2DM state can determine the amino acid levels in the blood. Several studies from *Gougeon et al* indicate a lower nitrogen balance and/or greater leucine turnover in untreated T2DM (Pereira, Marliss et al. 2008). However there are other that suggesting no effect in T2DM. One of the important observation is alteration in the insulin-resistant or diabetic state is that only a select number of amino acids)branched chain amino acid (BCAA), sulfur containing amino acids, and phenylalanine and tyrosine are consistently reported to be increased in states of impaired insulin action. Due to altered NADH/NAD ratio by some mechanism there is NADH related depression of branched chain α keto acid (BCKD) activity in metabolically important tissues would attenuate BCAA oxidation, contributing to accumulation of BCAA (Adams,2011).

T2DM participants compared to non-diabetics in obese African-American women showed that the plasma cysteine and methionine levels were 21 and 16 % higher, respectively (Fiehn, Garvey et al.).

Other studies using small numbers of obese non-diabetic individuals also found modest increases of methionine of 9 % to 18-22 % (Menge, Schrader et al.) compared to non-obese controls; increases in plasma cysteine / cystine were 10-14 % (Adibi, 1968).

There were correlations between plasma concentrations of these metabolites and those of the T2DM-predictive BCAA and phenylalanine/ tyrosine analytes in predisease baseline samples derived from the Framingham Offspring Study; however, histidine, lysine, threonine, and tryptophan patterns were not associated with subsequent risk for developing diabetes (Wang, Larson et al.). Clearly further studies are required to unravel the metabolic changes underlying the changes in AA levels in type 2 DM.

Regarding supplements of amino acids, in rodent models, BCAA-rich protein sources when administered in the drinking water have consistently shown improved glucose tolerance and lower adiposity (Eller and Reimer; Pilvi, Korpela et al. 2007). Adipocytes isolated from rats fed a high-protein, low-fat diet were more insulin sensitive with respect to lipolysis compared to animals fed a control diet (Kettelhut, Foss et al. 1985). Glycemic control indices (HbA1c%, fasting blood glucose, and insulin levels) were improved and energy expenditure increased in mice fed with leucine-enriched drinking water (Guo, Yu et al.)

4.1.3 Chinese hamster ovarian cells models in glucose metabolism studies

The cell type used for the study throughout is CHO-K1 cells (derived from Chinese hamster ovary) (Puck and Fisher 1956). CHO cells, in addition to their importance in research, are arguably, the most utilized host for large-scale production of pharmaceutically important proteins, (Chu and Robinson 2001; Andersen and Krummen 2002).

Cultured CHO cells possess an insulin-sensitive facilitated diffusion system for glucose transport, though it does not possess measurable GLUT-4 at protein level (Hasegawa, Anraku et al. 1990). Insulin-regulated amino peptidase (IRAP) is thought to have an important role in the sorting and trafficking of GLUT4 storage vesicles. Recently, it was reported that CHO-K1 cells contained a large concentration of endogenous IRAP which colocalized with the GLUT4 in specific intracellular vesicles, i.e., GLUT4 vesicles, which in response to insulin, resulted in higher glucose uptake in CHO cells (Demaegdt, Smitz et al. 2008). The untransfected CHO cells constitutively express hormone sensitive lipase and peroxisome proliferator-activated receptor γ (PPARγ), a major regulator of adipose differentiation. CHO cells exhibit several adipocyte like features and display insulin-responsive trafficking in DMEM medium supplemented with high glucose (Haney, Slot et al. 1991; Verhey, Hausdorff et al. 1993; Wei, Bonzelius et al. 1998; Bogan, McKee et al. 2001). Both, 3T3-L1 and CHO cells harbor peripheral, vesicular insulin-responsive compartments involved in GLUT4 trafficking to the cell membrane (Bogan, McKee et al. 2001; Ijuin and Takenawa 2003; Katome, Obata et al. 2003). There is genetic evidence that the glucose transporter functions as a major route for glucose entry into CHO-K1 (Hasegawa, Anraku et al. 1990). The addition of amino acids to the growth media for CHO cell cultures was found to mitigate the negative effects of ammonium stress and improved the CHO cell growth as well as important metabolic parameters, including glucose consumption while growing CHO-K1 cells for industrial applications (Chen and Harcum 2005).

Based on the amino acids having the following properties related to diabetes namely alanine, aspartic acid, glutamic acid and lysine showing antiglycating property (Ramakrishnan and Sulochana 1993; Ramakrishnan, Sulochana et al. 1996; Ramakrishnan, Sulochana et al. 1997), the leucine, isoleucine and arginine promoting glucose uptake (Paolisso, Tagliamonte et al. 1997; Doi, Yamaoka et al. 2005; Nishitani, Takehana et al. 2005), the amino acids chosen for the study were, namely alanine, arginine, leucine, isoleucine, aspartic acid, glutamic acid and lysine.

Based on the importance of amino acids in decreasing the blood sugar, decreasing the protein glycation, increasing insulin secretion, the present study aimed to first elucidate the uptake of amino acids in CHO-K1 cells and later chapter its influence on insulin signaling.

4.2 MATERIALS AND METHODS

4.2.1 Cell culture

CHO-K1 cells were maintained in F-12K1 medium in 25 cm^2 flasks with 10 % fetal bovine serum (FBS) in a 5 % CO_2 incubator at 37°C. For the experiments, cells were seeded at 1 X 10^4 cells/well in 24-well plates. When cells reached 80 – 90 % confluence, the cells were serum starved for 24 hours and all experiments were done in triplicates. The basal medium had 7 mM glucose and was used as the control for the various high glucose concentrations studied.

4.2.2 Cell morphology (under phase contrast microscope)

Cells were grown to confluence in 24 well plate and before the experiments cells were serum starved for 4 hours. Different concentration of glucose and amino acids (5-30 mM) + insulin (100 nM) was added and incubated for 24 hours. At the end of 24 hours the cells were viewed under phase contrast microscope.

4.2.3 Cell viability by Trypan blue dye exclusion test

The cells were exposed to various concentration of glucose and amino acid for 24 hours. After the incubation period was over the medium was removed and cells were trypsinized. One volume of 1 % trypan blue in 9 volumes of cell suspension was made. Mixture was kept at room temperature for 10 minutes. The cells were loaded on the haemocytometer. And the cells were counted in the center and 4 corner primary squares of each grid. Unstained and the stained cells were counted and recorded separately.

4.2.4 Cell toxicity assay by MTT assay

Cytotoxicity of the amino acids mixture was studied by MTT assay. CHO-K1 cells were exposed to 7, 12, 17 & 27 mM glucose in the presence and absence of amino acids mixture (5, 10, 20 &

30 mM) with and without insulin (100 nM). At the end of 24 hours the viability of CHO-K1 cells was assayed by the reduction of MTT to formazan. Briefly, cells were seeded in 24-well microtiter plates (1×10^{4} cells per well in 1 ml medium), and left to adhere to the plastic plates overnight before being exposed to condition medium. After 24 h of exposure MTT was added to the wells at a final concentration of 0.5 mg / ml / well and the cells were incubated at 37°C for an additional 4 hours. Thereafter, the medium was removed, the formazan crystals were dissolved in 500 µL of DMSO (37°C, 10 min) and the absorbance was measured at 540 nm in spectrophotometer (Beckman DU640, USA). The MTT assay has also been interpreted as mitochondrial metabolic activity of the cell therefore is a useful mean to determine whether metabolic substrates are used to provide respiratory energy. The absorbance at 540 nm is expressed as % normalized to control (Wu, Lei et al. 2009).

4.2.5 Metabolic activity by Lactic acid estimation

Cells were grown to confluence in 6 well plate. Serum free medium was added and incubated for 4 hrs before the experiments. Different concentrations of glucose and amino acids (5-30mM) + insulin (100nM) was added and incubated for 24 hrs. Cells were lysed with PBS using RIPA buffer. Cells were sonicated, centrifuged and the supernatant was used for lactic acid estimation by spectrophotometric method which was read at 570 nm.

4.2.6 Amino acids uptake by labeled radio isotope

a) Amino acid uptake when in mixture form: Amino acids mixture of 5 mM concentration of each cold amino acids was added along with one of the amino acid in the mixture labeled with $U^{14}C$ of 0.5 µCi to the medium. To this 100 nM insulin and glucose of 7 (normal) , 12 & 17 mM (high glucose) was added and exposed for 8 min. The cells were then lysed using 0.1N NaOH and the 24 well plates were stored at 4°C. The uptake of individual labeled amino acid was measured using Liquid Scintillation System. The same experiment was carried out in the absence of insulin.

b) Amino acid uptake when in free form: Amino acids of free form of 5 mM concentration of each cold amino acids with labeled amino acids of 0.5 μCi of the same (hot) was added to the medium along with glucose of 7, 12 & 17 mM in the presence and absence of insulin for 8 min. At the end of exposure time, the cells were lysed with 0.1 N NaOH and read in liquid scintillation system.

4.2.7 Statistical Analysis

The data were expressed as mean values ± standard deviation. Statistical significance between the treated and untreated groups in all experiments was measured using student's t-test. $p < 0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Cell morphology (under phase contrast microscope)

a) Glucose alone: CHO-K1 cells were serum starved for 4 hours and incubated with different glucose concentrations (5 mM - 20 mM) for 24 hours and the morphology was observed under phase contrast microscope. The normal morphology of cells were spindle shaped and epitheloid. With increase in glucose concentration the change in morphology was observed. The rounding of cells shows cell death. This was seen at 17 mM G and maximal at 27 & 37 mM glucose.

b) With 5 mM AAM: Cells were exposed to 5 mM AAM in the presence of varying glucose for 24 hours and observed under microscope. Cells exposed to varying high glucose concentration with AAM of 5 mM was found to improve the morphology in terms of less number of rounded cells and near normal morphology. In 37 mM G alone a few rounded cells were observed.

c) With 10 mM AAM: With addition of 10 mM AAM rounded cells were observed in high glucose concentrations, of 12 – 37 mM G.

d) With 20 mM AAM: With addition of 20 mM AAM the cell loss was seen at all glucose concentration **(Fig 4.1)**.

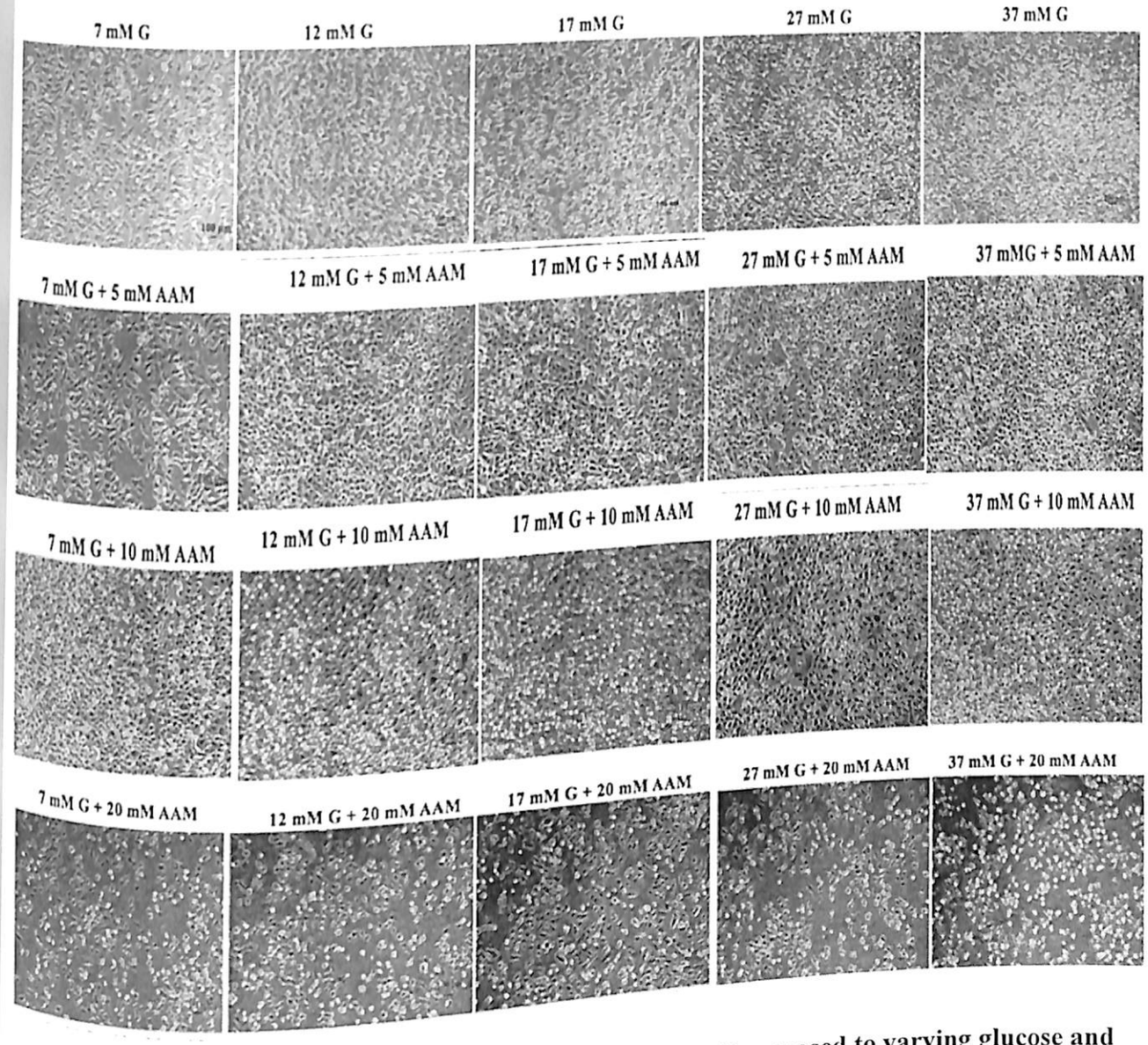


Fig 4.1: Phase contrast microscopy images of CHO-K1 cells exposed to varying glucose and AAM showing rounding at high glucose concentration and at high AAM concentration at the end of 24 hours.

4.3.2 Trypan blue dye exclusion test

To further check the cytotoxic effect trypan blue was done for amino acids at high glucose concentrations.

a) Trypan blue at 24 hours: Cell viability was tested directly by the trypan blue dye test which shows the dead cells as blue. With increase in glucose concentration significant decrease in viability was seen ($p=0.046$ for 27 mM G & $p=0.003$ for 37 mM G). Addition of AAM up to 20 mM did not show any cytotoxic effect in 7 and 12 mM glucose ($p<0.05$). Addition of AAM improved the viability at high glucose concentration. Significantly as seen at 17mM G with 5 mM AAM ($p<0.05$) and at 27 mM with 5 & 10 mM AAM (both showing $p<0.05$). However addition of 30 mM AAM showed significant fall ($p<0.001$) in viability including at normal glucose correlating with the morphology observed under microscope. Thus 30 mM AAM is found to be cytotoxic while cells are able to tolerate up to 20 mM AAM. A significant improvement is observed at as low as 5 mM AAM. At this 5 mM AAM the study was extended up to 48 hours as detailed below **(Fig 4.2A)**.

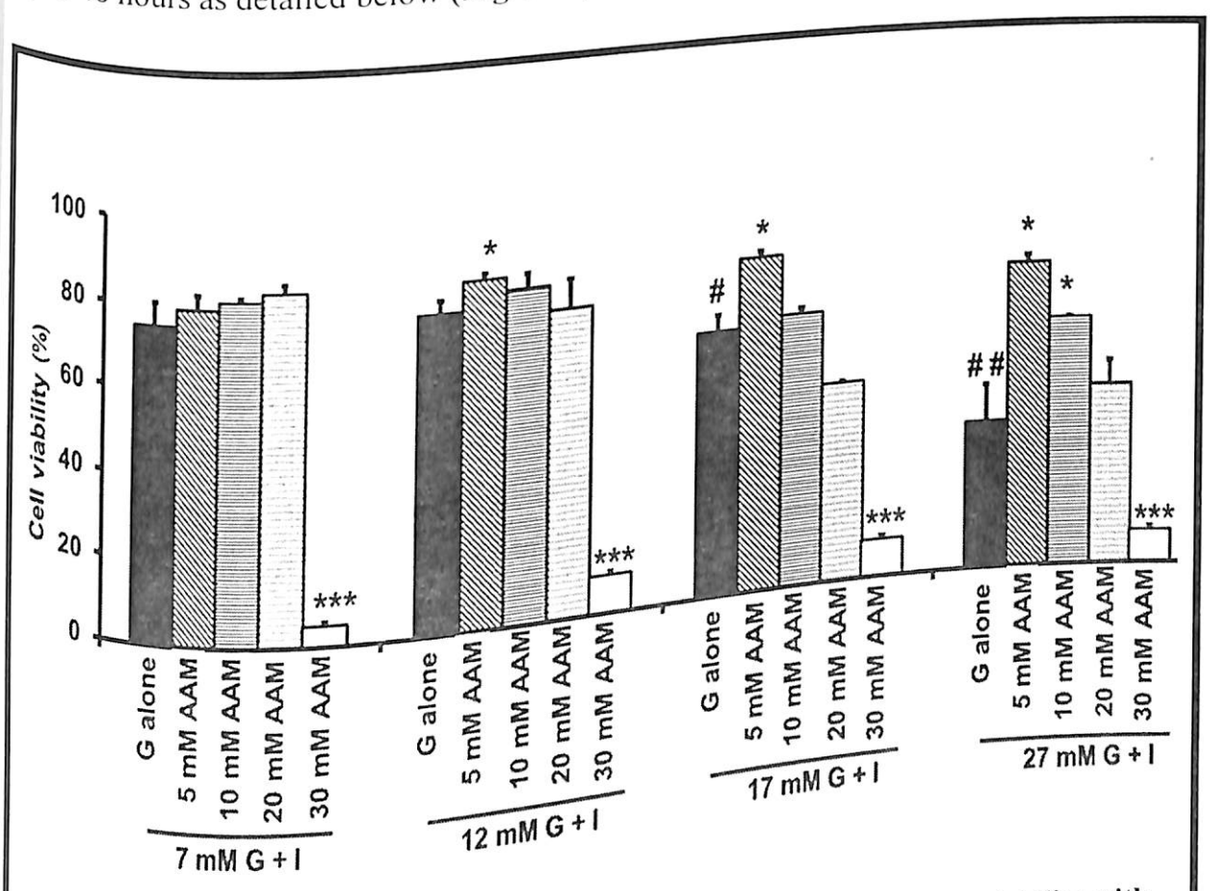


Fig 4.2A: Trypan blue dye exclusion test shows significant increase in the viability with 5 mM AAM and 30 mM AAM to be toxic to cells when exposed with G for 24 hours.

b) Trypan blue at 48 hours: At the end of 48 hours the addition of 5 mM AAM continues to show the cytoprotective effect ($p<0.003$) **(Fig 4.2B)**.

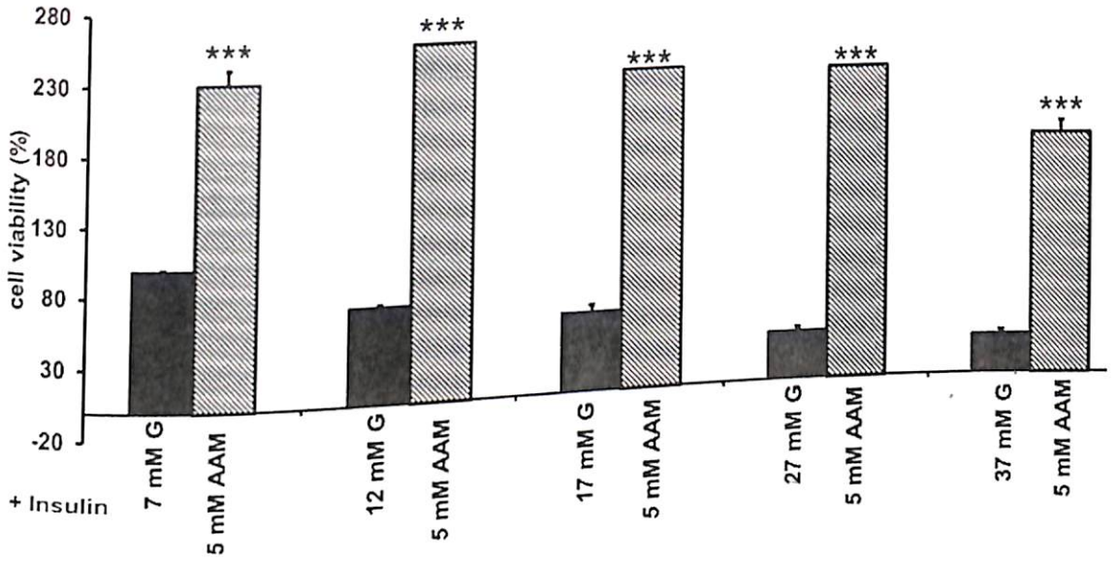


Fig 4.2B: Trypan blue dye exclusion test sowing the significant increase in the viability of 5 mM AAM till 48 hours.

4.3.3 Metabolic activity as studied by MTT assay

MTT assay is also a measure of the metabolic activity. The changes with respect to high glucose and the addition of AAM was studied in the presence and absence of insulin.

a) Effect of insulin on metabolic activity: Fig 4.3A shows that in presence of insulin (100 nM) the metabolic activity is increased as seen at all concentration of glucose compared to absence of insulin ($p=0.000$ at 7 mM G; $p=0.02$ at 12 mM G; $p=0.03$ at 17 mM G, $p=0.02$ for 27 mM G) **(Fig 4.3A)**.

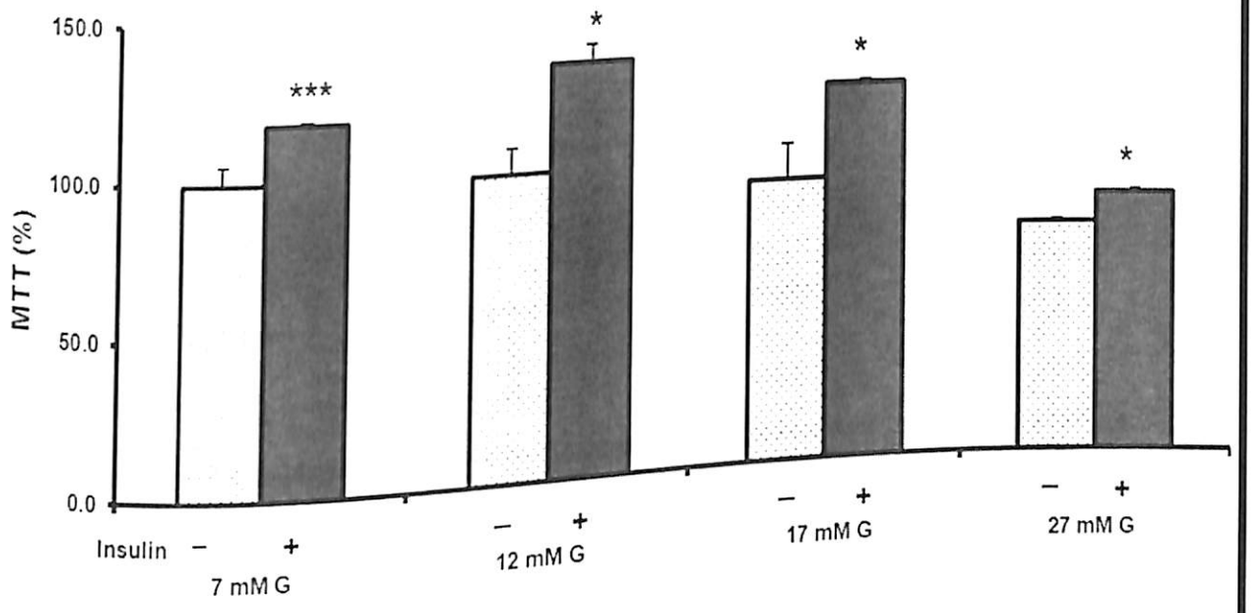


Fig 4.3A: Effect of insulin on metabolic activity as studied by MTT assay: Presence of 100 nM insulin along with glucose showed increased metabolic activity compared to insulin absence. p values were obtained after pair vise comparison between absence and presence of insulin with glucose where *$p<0.05$.

b) **Effect of AAM at varying concentration in absence of insulin on metabolic activity:** In the absence of insulin there was a dose dependent decrease in metabolic activity with increase in AAM concentration as seen in the presence of high glucose **(Fig 4.3B)**. Thus AAM alone cannot replace the effect of insulin.

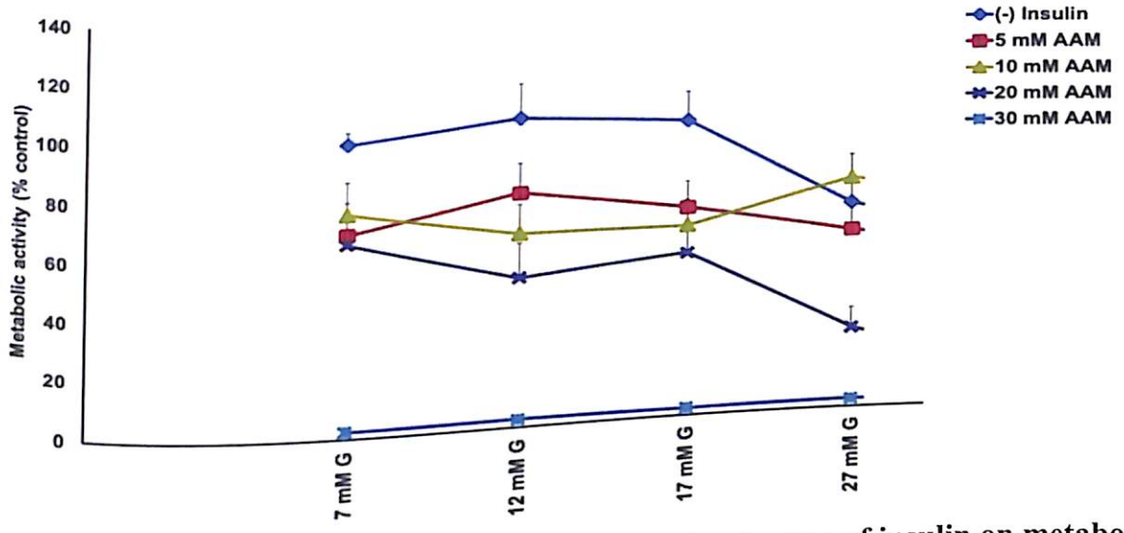


Fig 4.3B: Effect of AAM at varying concentration in absence of insulin on metabolic activity as studied by MTT assay: showing AAM in absence of insulin decreased metabolic activity.

c) **Synergistic effect of AAM along with insulin:** In the absence of insulin, when AAM was added there was a decrease in the metabolic activity compared to insulin alone. AAM and insulin, showed further increase due to additive effect **(Fig 3C)**.

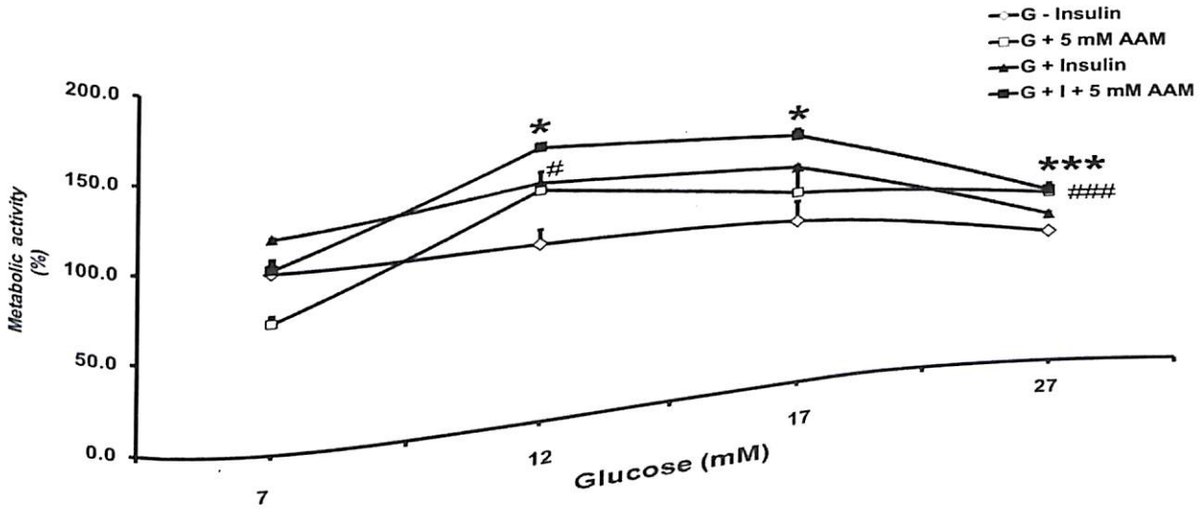


Fig 4.3C: Synergistic effect of AAM along with insulin as studied by MTT assay: showing the synergistic effect of insulin and AAM.

d) **Effect of AAM at varying concentration in presence of insulin on metabolic activity:** The metabolic activity is found to be lowered at 27 mM glucose by 30 %. Addition of AAM, significantly increased the metabolic activity with 5 mM AAM, at all high glucose levels. AAM of 10 mM had the same effect at 17 and 27mM glucose but did not further improve it. Thus 5 mM AAM is found to sufficiently increase the metabolic activity which is beneficial especially at high glucose concentration especially at 27 mM G wherein addition of 5 mM AAM the metabolic activity becomes comparable to that of the control.

To interpret the MTT assay in terms of cytotoxic effect it was found that 30 mM AAM is cytotoxic at all glucose concentration, as observed in trypan blue test and morphology **(Fig 4.3D)**.

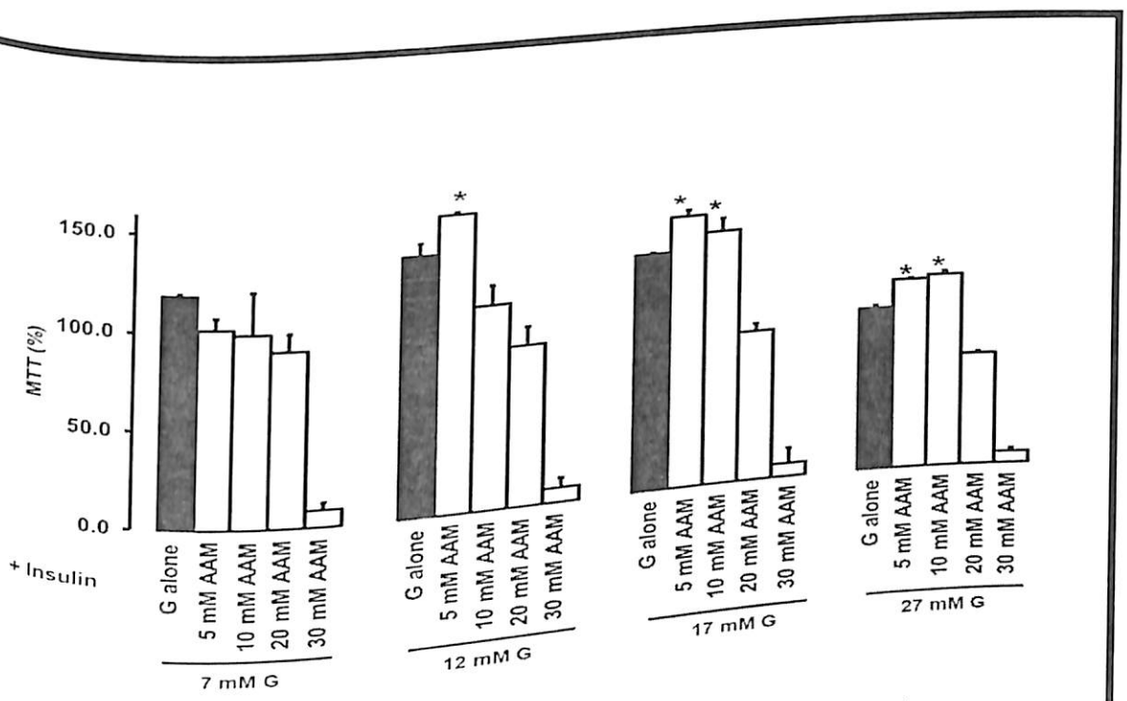


Fig 4.3D: Effect of AAM at varying concentration in presence of insulin on metabolic activity by MTT assay: showing metabolic activity when cells exposed to different concentrations of AAM along with 100 nM insulin and glucose showed a dose-dependent decrease in the viability with the cytotoxic effect seen at 30 mM concentration at all glucose concentration. p values were obtained after pair vise comparison between glucose (G) alone with glucose & amino acids mixture (AAM) where *p<0.05.

e) **Protective effect of AAM on metabolic activity:** Pre, post and co-treatment of AAM with glucose and insulin, was studied to find which of the three shows the maximal changes in

metabolic activity. Fig 3E shows that the co-treatment i.e., glucose + 5 mM AAM showed the maximal beneficial effect in terms of increased activity. This is maximally seen at very high glucose (27 mM) with 30 %. Pre or post treatment did not show any significant change **(Fig 4.3E)**.

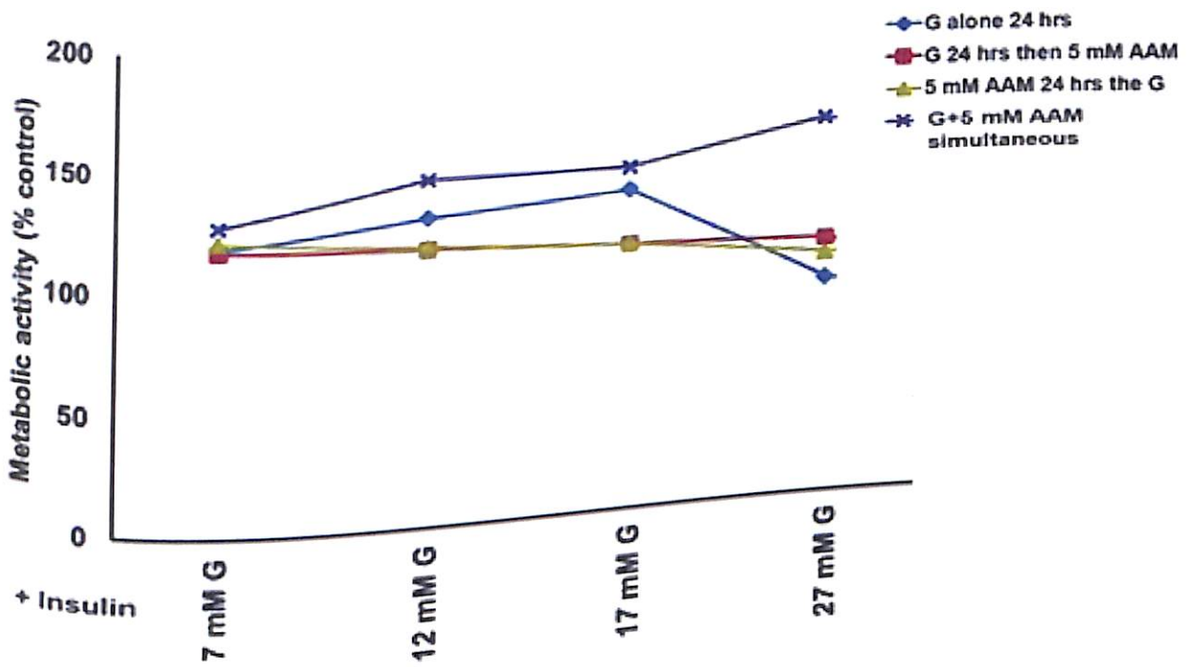


Fig 4.3E: Protective effect of AAM on metabolic activity by MTT assay: showing protective activity of AAM when added with different concentration of glucose showing simultaneous addition of AAM with G is effective than pre and post.

f) **Effect of varying individual amino acids on metabolic activity:** The effect of free amino acids used in the mixture (Ala, Arg, Lys, Leu, Ile, Asp & Glu) on the metabolic activity was tested individually. At 5, 10 & 15 mM concentration of all the free amino acids studied showed increase in the metabolic activity compared to untreated control at all concentration of glucose, in the presence of insulin **(Fig 4.3F)**.

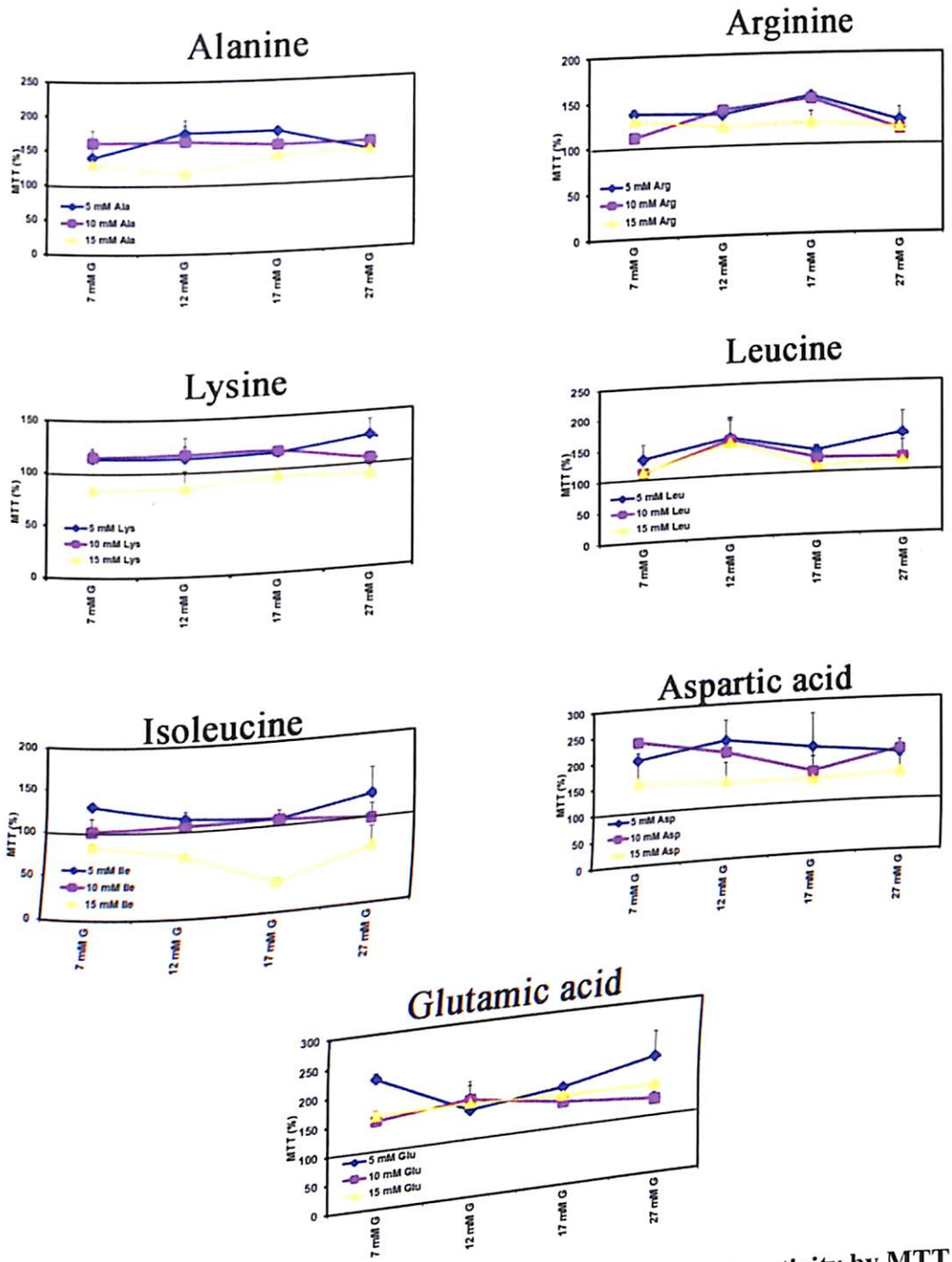


Fig 4.3F: Effect of varying individual amino acids on metabolic activity by MTT assay: showing metabolic activity on exposure to free amino acids of the mixture in varying glucose conditions with insulin.

4.3.4 Metabolic activity by lactic acid estimation

a) **Metabolic activity of insulin on glucose in presence and absence of insulin:** Lactic acid levels are indicative of utilization of glucose. Lactic acid increases with increase in glucose concentration. A 2 - 2.5 fold increase was further seen with addition of 100 nM insulin **(Fig 4.4A)**.

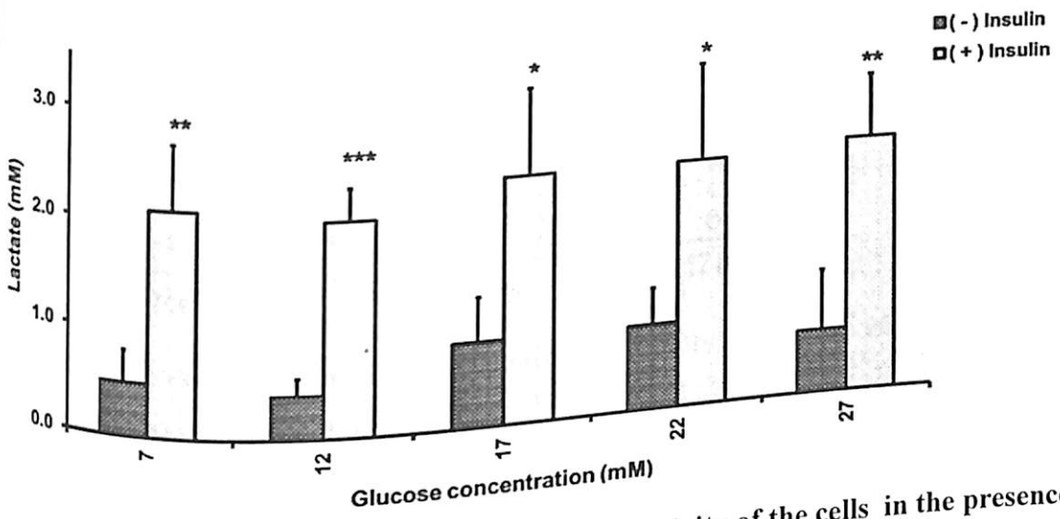


Fig 4.4A: Lactate estimation showing metabolic activity of the cells in the presence and absence of 100 nM insulin.

b) **Effect of AAM at varying concentration in presence of insulin on metabolic activity:** In the presence of insulin, the AAM of varying concentration was studied to see the levels of lactate when added to normal and high glucose. AAM of 5 mM concentration showed increased lactate production in the medium significantly at normal and at all high glucose concentration. 10 & 20 mM AAM did not show significant change at 17, 27 mM G. Thus 5 mM AAM at high glucose concentration showed increased lactate production indicative of increased metabolism of the glucose in the cell **(Fig 4.4B)**.

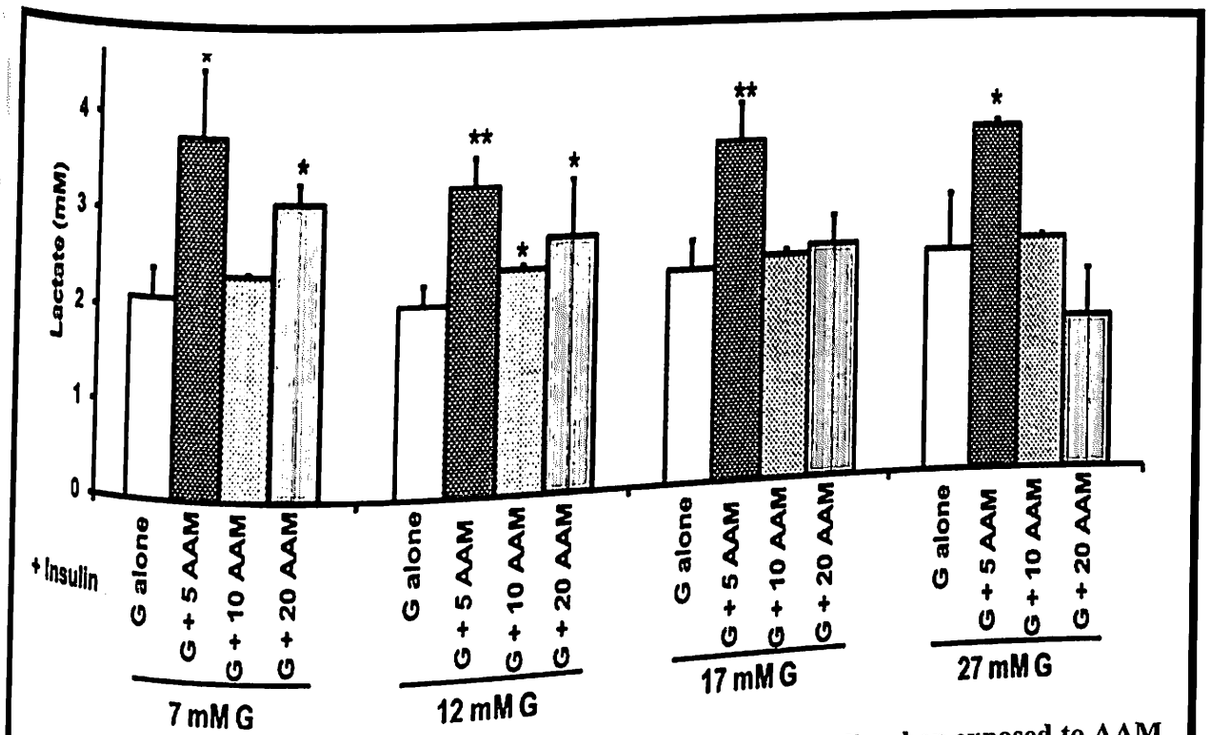


Fig 4.4B: Lactate estimation showing metabolic activity of the cells when exposed to AAM and glucose along with 100 nM insulin.

4.3.5 Amino acids uptake under high glucose condition: In order to see if there is a selective uptake of amino acids exposed under varying glucose concentration, 14C-labelled amino acids uptake was studied in the free form and in mixture form. In the mixture only one of the amino acid will be labeled to see if the uptake is influenced by the other amino acids in the mixture.

a) **Does presence and absence of insulin influences AA uptake?** AA transport was observed to be influenced by the insulin. In the absence of insulin there was a significant uptake of the amino acids namely alanine, arginine, lysine and aspartic acid. However when present as mixture insulin did not seem to influence the uptake, except for the increase in the uptake of arginine **(Fig 4.5A & 4.5B)**.

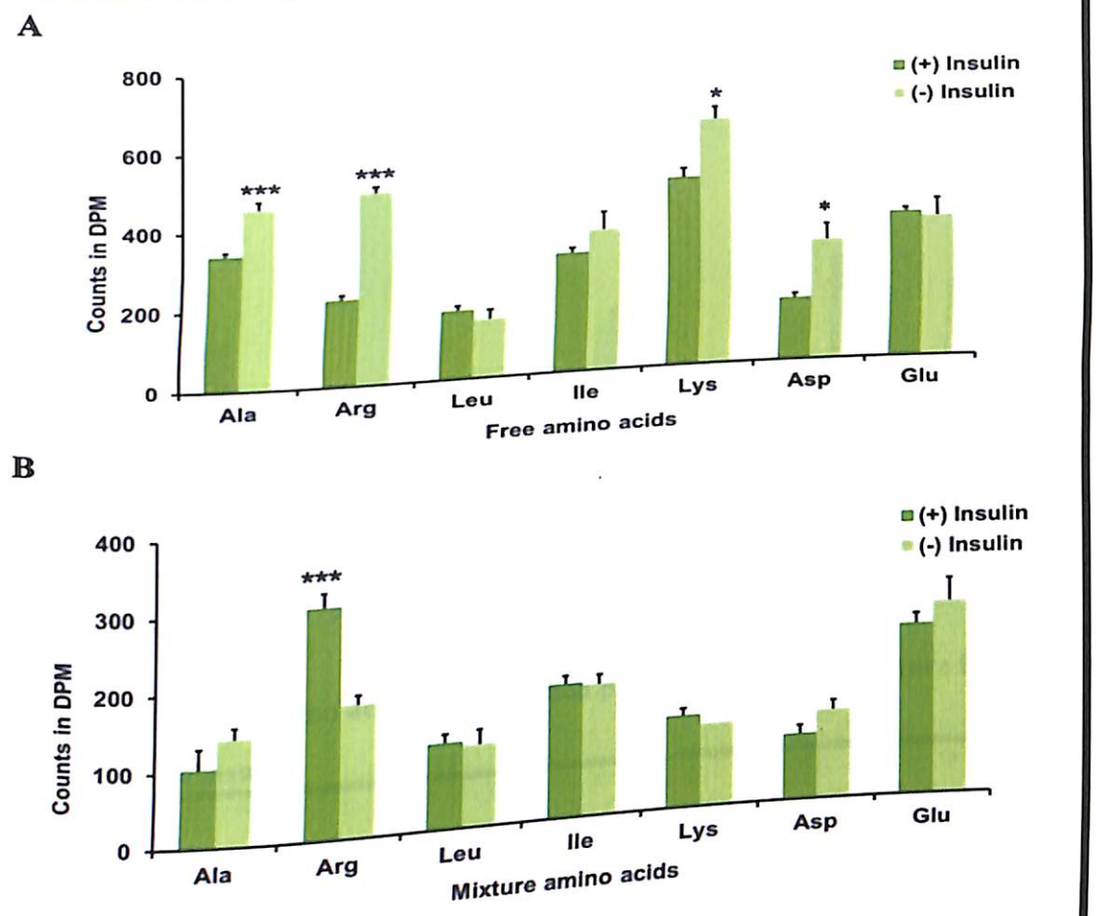


Fig 4.5A&B: Labeled amino acids uptake in the presence and absence of insulin when present in free and mixture form.

b) Is uptake influenced by free or mixture form of AA? With the effect of insulin on AA transport whether in free or mixture, the study was extended to see does the AA present along with other AA influence one another in there transport. With basic amino acids, lysine entry was more than arginine, among branched isoleucine was more than leucine and among acidic glutamic acid was more than aspartic acid. As per the experimental protocol in the study, in the presence of insulin and when present as mixture arginine shows more uptake than in the free form while all others show marginally lesser and probably an optimal uptake than the corresponding free form. Thus arginine alone is influenced by the insulin (**Fig 4.6**).

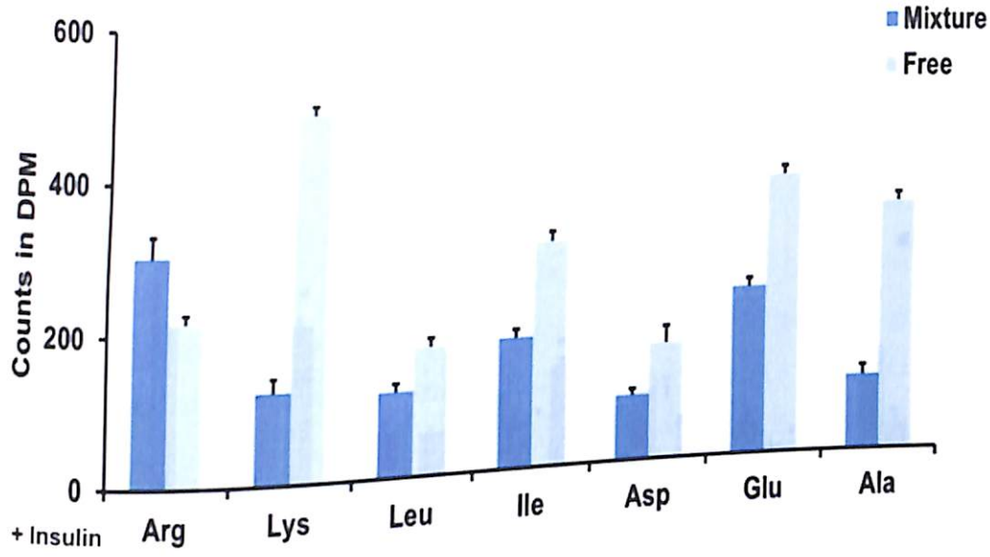


Fig 4.6: Labeled amino acids uptake when present in free and mixture form in the presence of insulin.

c) **Is AA uptake influenced by glucose concentration?** AA uptake was studied in the presence of varying concentrations of glucose. It was found that AA uptake was influenced by the concentration of glucose. Alanine, isoleucine showed an increase with increase in glucose and is significantly increased at 17 mM G ($p=0.001$ & $p<0.05$ respectively), while glutamic acid and arginine showed a decrease dose dependently and is significantly decreased at 17 mM G ($p<0.01$). Leucine did not show a change while others showed varied effects **(Fig 4.7A).** In the free form, the effect of glucose was further seen with more amino acids. Alanine, leucine, aspartic acid showed an increase with increase in glucose and is significantly increased at 17 mM G ($p<0.05$), while arginine, isoleucine and glutamic acid showed a decrease dose dependently and is significantly decreased at 17 mM G ($p<0.05$) **(Fig 4.7B)**.

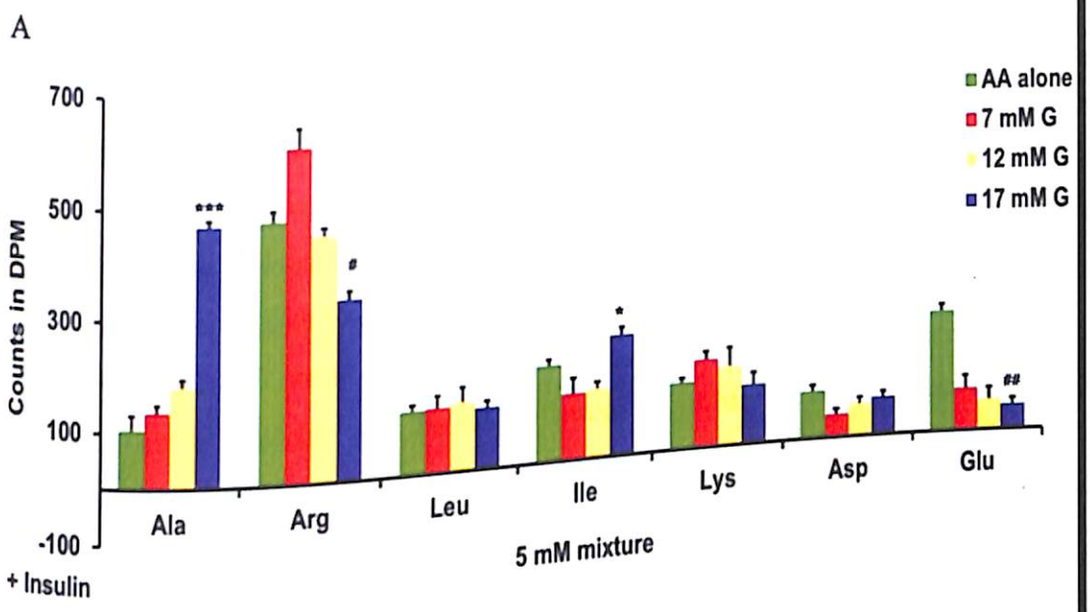


Fig 4.7A: Labeled amino acids uptake when present as mixture form in the presence of glucose. p value is comparison between absence of glucose and presence of glucose with AAM (* shows significant increase and # decrease).

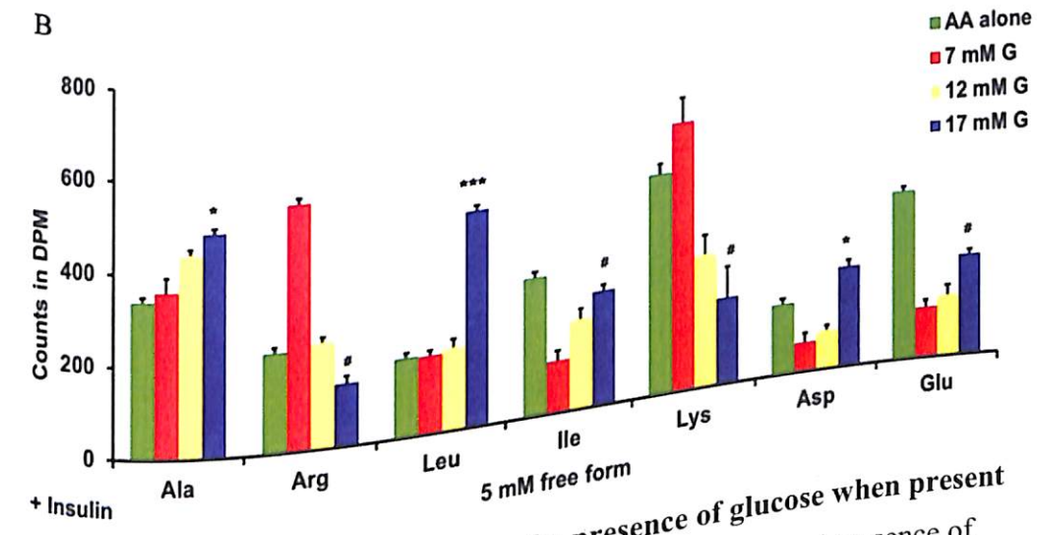


Fig 4.7B: Labeled amino acids uptake in the presence of glucose when present as free form. p value is comparison between absence of glucose and presence of glucose with AAM (* shows significant increase and # decrease).

4.4 CONCLUSIONS

1. Amino acids mixture constituted by alanine, arginine, isoleucine, leucine, aspartic acid, glutamic acid and lysine at 5 mM concentration **are non-toxic** when added to varying high glucose concentrations and significantly increases the cell viability and the metabolic activity in CHO-K1 cells.
2. The amino acids uptake by the CHO-K1 cells was studied by labeling studies ($U^{14}C$-labelled individual amino acids) and it was found to be influenced by both high glucose, insulin and 'free' or 'mixture' form of the amino acid. Amongst the basic AA namely, arginine and lysine, arginine uptake was found be maximal, while it was glutamic acid amongst the acidic ones namely glutamic acid and aspartic acid. Isoleucine amongst the branched namely leucine and isoleucine was found to have the maximal uptake in the mixture studied. Alanine uptake was comparable to that of arginine that showed the maximal uptake. Thus these were the amino acids that showed **preferred uptake based on the charge as they use common transporters while using amino acids mixture** and thus can be predominantly associated with increased glucose uptake seen with amino acids mixture treatment in high glucose conditions.

CHAPTER 5: MECHANISM OF ACTION OF AMINO ACIDS ON INSULIN SIGNALING CASCADE IN CHO-K1 CELLS

5.1 INTRODUCTION

5.1.1 Role of insulin in glucose uptake

Insulin elicits its action by binding to its receptors on the target cells, leading to the autophosphorylation and increased tyrosine kinase activity in the cytoplasmic domain of the β subunit, which in turn switches on various downstream signaling cascades. Insulin receptor tyrosine kinase (IRTK) then phsophorylates the insulin receptor substrate (IRS) which in turn phosphorylates phosphatidylinositol-3-OH-kinase (PI3K) which phosphorylates the mytotubules of GLUT 4 that translocates to the membrane for the glucose uptake. Increased IRTK and PI3K activates GLUT 4 that increased the internalization of glucose into the cell and oxidation to generate ATP (Chang, Chiang et al. 2004). Insulin mediated signal transduction is pivotal for glucose homeostasis, cell proliferation, differentiation, tissue development and growth. At molecular level, IR-mediated signal transduction is regulated by several factors, including nutrients/dietary supplements (Wu 2009). Based on the insulin signaling, several drugs have been designed, which augment IRTK and PI3K activities. The activation of Akt promotes translocation of glucose transporter 4 (GLUT4) to the plasma membrane facilitating glucose entry in the cell (Saltiel and Kahn 2001). By activating IRTK and PI3K, insulin resistance is mitigated and there is better utilization of glucose inside the cells through oxidation. Overall, alterations of the activation status of the insulin signaling enzymes (IR, IRS1/2, PI3K), and downstream targets (PDK, PKB and its targets GSK-3 and AS160, aPKCs, and MAPK-family protein kinases) are attributed to defects in one or more steps of the insulin signaling cascade as studied in muscle and adipose tissue from insulin resistant, obese and type 2 diabetic subjects (Frojdo, Vidal et al. 2009).

5.1.2 Insulin resistance in diabetes

Insulin resistance is a state of adequate insulin with inadequate response to insulin. In compensation of resistance pancreas secrete more insulin leading to hyperinsulinemia. In spite of the high plasma levels of insulin and due to insulin resistance there is an inadequate glucose transport across cells in T2DM and metabolic syndrome (Ohne, Toyoshima et al. 2005; Frojdo, Vidal et al. 2009). At the cellular level, insulin resistance involves a decrease in the activity of the signaling molecules (Caro, Sinha et al. 1987; Goodyear, Giorgino et al. 1995; Bjornholm, Kawano et al. 1997; Zierath, Krook et al. 1998; Cusi, Maezono et al. 2000; Kahn and Flier 2000). A drop in the activation of the signaling molecules in insulin resistance can lead to cellular depletion of GLUT4 (Hissin, Foley et al. 1982; Garvey, Hueckstead et al. 1989).

During the basal state, GLUT4 predominantly localizes in the trans-Golgi-network and recycling endosomes, with minimal presence on plasma membrane. After insulin exposure, GLUT4 relocates to the plasma membrane (Watson, Kanzaki et al. 2004; Kanzaki 2006). Insulin resistance has been associated with small numbers of intracellular glucose transporters despite a normal GLUT4 protein expression

The muscle cells from obese and type 2 diabetic people is characterized by having defects at several steps of the insulin signaling pathway, including decreases in insulin receptor and IRS-1 tyrosine phosphorylation, and phosphatidylinositol PI 3-kinase activity (Goodyear, Giorgino et al. 1995). One approach to increase muscle glucose disposal is to reverse defects in insulin signaling. Weight loss and thiazolidinediones (TZDs) are examples of interventions known to enhance glucose disposal by improving defects in insulin signaling (Kahn and Flier 2000).

AMPK is another pharmacologic target as its stimulation increases insulin-independent glucose transport in muscle and decreases hepatic glucose production, leading to improved blood glucose concentrations (Bergeron, Russell et al. 1999).

5.1.3 Amino acids in glucose disposal

Amino acid levels in plasma have been shown to have disease correlation in diabetes mellitus (Girish, Rajesh et al.). In the context of hyperglycemia, lysine, glycine, alanine, glutamic acid and aspartic acid have been reported to exhibit antiglycating (Ramakrishnan, Sulochana et al. 1996) and anti-cataract properties (Ramakrishnan and Sulochana 1993). Oral supplementation with amino acids for type 2 diabetes patients under anti-diabetic therapy produced a further decrease of post prandial plasma glucose without change in plasma insulin levels. This decrease was attributed to improved insulin sensitivity (Sulochana, Lakshmi et al. 2002). Increase in the glucose transport in CHO-K1 cells was noted with an amino acid mixture (AAM) [Selvi 2010]. The transport kinetics (Palacin, Estevez et al. 1998) and the anabolic role of amino acids (Tipton, Rasmussen et al. 2001) are well established. Our previous study reported that AA increase the glucose uptake in CHO-K1 cells as seen by labeled deoxy glucose uptake and GLUT4 translocation (Selvi, Angayarkanni et al.). But the mechanism through which AAM augmented the GLUT4 recruitment is not clear. The important signaling involved in Glut4 translocation are IRTK , Akt/PKB, Cbl/TC10, Rab dependent pathways (Hou and Pessin 2007). In this study we explored the mechanism involved in the same by looking at the insulin signaling cascade that is involved in glucose uptake.

5.2 MATERIALS AND METHODS

5.2.1 Cell culture and treatment

CHO-K1 cells were maintained in F-12K1 medium in 25 cm^2 flasks with an addition of 1 mg/L of ciprofloxacin, 2.5 g/L $NaHCO_3$ and 10 % fetal bovine serum (FBS) in a 5 % CO_2 incubator at 37°C. For the experiments, cells were seeded at 1 X 10^4 cells in 25 cm^2 flask. When cells reached 80-90 % confluence, they were serum starved for 4 hours. Insulin (100 nM) pretreatment was given for an hour. The cells were then exposed to varying glucose (7, 17, 27 mM) concentration along with varying AAM (0.25, 1, 5 & 20 mM) for 8 min for the glucose uptake, 2 min for IRTK, PI3K and Akt, 5 min for GLUT4 analysis and 30 min for glycogen synthase activity assay.

5.2.2 Glucose uptake study

Glucose uptake was studied by using the tracer as labeled $U^{14}C$ glucose or $U^{14}C$ deoxyglucose or glucose peroxidase kit method. The protein concentrations of whole cell lysates were determined using the bradford method. Deoxy glucose uptake expressed as disintegrations per minute per milligram protein.

5.2.2.1 Labeled $U^{14}C$ deoxyglucose uptake study in the presence and absence of insulin

Cells were serum starved for 24 hours and exposed to varying cold glucose (7, 12, 17, 27 & 37 mM) in the presence and absence of 100 nM insulin along with radiolabeled 0.2 µci of $U^{14}C$ deoxyglucose for 8 min. At the end of 8 min the cells were washed with ice cold PBS to arrest the reaction and then the cells were lysed with 0.1 N NaOH. The radioactivity in lysate was read in a Liquid Scintillation Counter (LSS, Beckman-6500, Fullerton, CA).

5.2.2.2 Labeled $U^{14}C$ glucose uptake at varying time point

To study the glucose uptake by using tracers, the time point need to fixed and was studied by varying the time from 2 min to 1 hour. Cells were serum starved for 24 hours and exposed to varying cold glucose (7, 12, 17, 27 & 37 mM) in the presence of 100 nM insulin along with radiolabeled 0.5 µci of $U^{14}C$ glucose for 2, 8, 30 & 60 min. At the end of respective time the cells were washed with ice cold PBS to arrest the reaction and then the cells were lysed with 0.1 N NaOH. The lysate was read in a LSS.

5.2.2.3 Labeled $U^{14}C$ glucose uptake in the presence and absence of amino acid mixture

To see the effect of amino acids concentration of glucose uptake labeled $U^{14}C$ glucose was used as tracer. CHO-K1 cells in F-12K1 medium were serum starved for 24 hours and then subjected to the experimental conditions. Varying amounts of Glucose (7, 12, 17 & 27 mM) along with or without amino acids mixture (2.5, 5 & 10 mM) were added along with the $U^{14}C$ glucose (0.5 µCi / ml) as a tracer for the glucose uptake in the presence and absence of 100 nM insulin. After 8 minutes of exposure at 37°C, the medium was removed and the reaction arrested by adding ice cold PBS immediately, the cells were washed thrice with PBS and lysed with 0.1 N NaOH and read in a Liquid Scintillation Counter (Henry, Koumanov et al. 1997).

5.2.2.4 Labeled $U^{14}C$ deoxyglucose uptake in the presence and absence of amino acid mixture

In order to see if amino acids mixture augment glucose uptake, deoxy glucose uptake was done in high glucose condition. CHO-K1 cells in F-12K1 medium were serum starved for 24 hours and then subjected to the experimental conditions. Varying amounts of Glucose (7, 12, 17 & 27 mM) along with or without amino acids mixture (2.5, 5 & 10 mM) were added along with the $U^{14}C$ 2-deoxyglucose (0.2 μCi / ml) as a tracer for the glucose uptake in the presence and absence of 100 nM insulin. After 8 minutes of exposure at 37°C, the medium was removed and the reaction arrested by adding ice cold PBS immediately. The cells were washed thrice with PBS and lysed with 0.1 N NaOH and read in a LSS (Henry, Koumanov et al. 1997).

5.2.2.5 Glucose entry as assessed by clinical analyzer in the presence of free amino acids

Individual amino acids influencing the glucose entry was studied by clinical analyzer. Cells were incubated with varying glucose concentration (7, 17 & 27 mM) in the presence of free amino acids at concentrations of 2.5, 5 and 10 mM with insulin 100 nM. After 8 minutes of incubation, the glucose level in the medium was measured by the use of Glucose oxidase/peroxidase assay kit (CPC, Spain) according to the manufacturer's instructions in clinical chemistry system RA50 (Bayer, Germany), to infer the glucose entry in terms of disappearance of glucose in the medium (Krebs, Krssak et al. 2002).

5.2.3 Preparation of Cell Lysate

At the end of exposure time as stated above, the reaction was stopped by adding ice cold PBS, contents were washed thrice with PBS, 1.0 ml of lysis buffer (100 ml PBS, 500 µl Triton X, 300 µl of 1mg/ml protease inhibitor cock tail) was added and the treated cells were kept for 10 min at 4°C. They were then scraped, sonicated for 10 sec, centrifuged at 10,000 rpm for 10 min at 4°C. Supernatant was stored at -80 °C until processed for immunoprecipitation. Protein concentrations were determined by BCA method. The basal medium had 7 mM glucose and was used as control for the various high glucose concentrations studied. All experiments were done in triplicate.

5.2.4 Insulin receptor tyrosine kinase activity by auto radiography

Immunoprecipitation of IRTK: To 500 µg of protein, 2 µg of primary antibody to IRTK was added and the contents were incubated for 1 hour at 4°C followed by 12 hours incubation with agarose conjugate (10µl) at 4°C, centrifuged at 3500 rpm for 10 min at 4°C. A mixture of 200 µl of PBS buffer containing 50 mM TRIS –HCl, pH 7.4, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 100µl of 0.1% Triton X100 , 500 mM LiCl, with 1 mM PMSF and protease inhibitor cocktail was added to the pellet. It was then washed with the buffer thrice and centrifuged. Later it was solubilized with buffer and stored at -80°C, after protein estimation by BCA method.

IRTK Activity: To prepare the cocktail, 10 µl protein kinase assay buffer (50 mM HEPES buffer, 0.1 mM EDTA, 2.5 mg of Brij25, 0.15 M NaCl), 10 µl of 50 mM Cold ATP , 1.5 µl of 2 M $MgCl_2$, 20 µl of pseudo substrate (poly GLU:TYR 4:1), 2 µCi of Radio labeled γ ^{32}P ATP were mixed together. To the immune complex, 41.7 µl of cocktail was added and it was incubated for 30 min followed by addition of sample buffer and boiling. SDS PAGE (7 %) was done at 160 V for 2 hours. The gel was then dried along with whatman no. 1 filter paper and food wrapper with the help of a vacuum dryer at 80°C for 2 hours. The gel was placed in the hyper cassette, exposed to an X-ray film and developed. The amount of radioactivity in the gel was quantified using LSS.

5.2.5 Phosphatidyl inositol 3 kinase activity by auto radiography

After experimental treatment, medium was removed, and cells were rinsed twice in ice-cold PBS and lysed. Immunoprecipitation of PI3-K was done by taking 500 µg of lysates followed by overnight incubation at 4°C with 2 µg of anti-PI3K antibody coupled to protein A/G plus agarose. Immune complexes were washed thrice with buffer (20 mM Tris, pH 7.5, 10 mM NaCl, 0.5 mM EDTA, and 1 mM Na_3VO_4 in PBS). The immune complex as pellet was then resuspended in 50 µl of kinase assay buffer (10 mM Tris HCl, pH 7.5, 10 mM NaCl, 1 mM sodium orthovanadate, 0.5 mM EDTA, 1.5 mM $MgCl_2$) and the kinase reaction was initiated by the addition of 30 µg of phosphatidylinositol and 50 µM ATP containing 2 µCi of [γ-32P]ATP. The samples were incubated for 20 min at 37°C; the reactions were terminated by the addition of 20 µl of chloroform. The samples were then extracted with 160 µl of chloroform / methanol (1:1). The resultant 50 µl lipid fractions were resolved by thin layer chromatography in

chloroform/methanol/ammonium hydroxide (129:114:15). The plate was dried, exposed to X-ray film at -80°C for 24-48 hours, the phosphorylated products were then visualized by autoradiography. The amount of radioactivity from the gel was quantified using LSS.

5.2.6 PKB / Akt expression

5.2.6.1 Akt expression by Western blot

After the experimental treatment, the cells were lysed and stored in -80°C until used. Total protein (50 µg) was separated by SDS-PAGE in 10% polyacrylamide gels and transferred to nitrocellulose membranes. It was developed with specific primary (phospho Ser 473 Akt raised in Rabbit) and secondary antibody (HRP conjugate anti rabbit raised in mouse). Visualization of immunoreactive bands was enhanced chemiluminescence (Millipore Immunobilon Western, USA). Quantification of the band was done by densitometer (GS800, Biorad,USA).

5.2.6.2 Akt by Immuno fluorescence

Intact cell immunofluorescence (IF) was performed by washing the cells once with ice-cold PBS, followed by fixation with 4 % paraformaldehyde for 20 min at RT and 0.1% Triton X-100 in PBS at room temperature for 10 min. The cells were then blocked with 2% BSA in PBS. The cells were then incubated with primary rabbit antibodies (phospho Ser 473 Akt) and were detected with FITC-conjugated anti rabbit raised in Goat for 45 min at room temperature in dark. The IF was also carried out in the presence of the Akt inhibitor wortmannin (2.5 µg/well). The nucleus was counter stained with DAPI then mounted with antifadent Mowiol and examined under 40 X and oil immersion objectives in a Carl Zeiss inverted fluorescent microscope.

5.2.7 Glucose transporter (GLUT4 analysis)

5.2.7.1 GLUT4 analysis by Flow cytometry

Amino acids effect on GLUT4 recruitment was analyzed in CHO-K1 by FACS. Cells were incubated with varying glucose concentration (7, 17 & 27 mM) in the presence and absence of amino acids mixture (5 & 10 mM) with or without insulin (50, 100 nM). After 5 min of exposure at 37 °C, the reaction was arrested by adding ice cold PBS immediately, followed by trypsinisation (0.1%); the cells were transferred and stained in polystyrene tubes. They were spun down such that the supernatant was removed with little loss of cells as possible. The

viability of the cells was not less than 90 %. The cells were resuspended to approximately 1-2x10^6 cells/ml in ice cold PBS, 10 % FCS, 1 % sodium azide. To 100 µl of cell suspension 2 µg of the primary antibody in 3% BSA in PBS was added. It was then incubated for 30 min at 4°C in the dark. The cells were washed thrice in ice cold PBS followed by centrifugation at 400 g for 5 min and resuspended. The FITC-labeled secondary antibody was diluted in 3% BSA in PBS at 1:100 dilution; the cells were resuspended in this solution and incubated at 4°C for 30 minutes in the dark. The cells were washed thrice by centrifugation at 400 g for 5 min and resuspended in ice cold PBS, 3% BSA and 1% sodium azide (Muretta, Romenskaia et al. 2008). Data acquisition and analysis were performed using the BD CELL Quest Pro software (BD Biosciences, USA). The data were expressed as % FITC positive cells of the total 10,000 cells gated.

5.2.7.2 GLUT4 by western blot

Total protein (50 µg) was separated by SDS-PAGE in 10% polyacrylamide gels and transfer to nitrocellulose membranes to be developed with specific primary (anti GLUT4 raised in goat) and secondary antibodies (HRP conjugated anti goat raised in mouse). Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Millipore Immunobilon Western,USA). Quantification of the band was done by densitometer (Biorad, GS800).

5.2.8 Glycogen synthase activity by reverse phase – HPLC

Glycogen synthase (GS) activity was determined by the measure of incorporation of UDP-glucose into glycogen, in the absence and presence of allosteric activator glucose 6 phosphate by the method of Danforth WH with slight modifications (Danforth et al). The *assay* was performed with fresh cell lysate. Total protein concentration was determined using Coomassie Plus Bradford Assay Reagent. To access the GS activity, UDPG was used as the substrate with Glucose 6 phosphate and Glycogen. The final product was purified by solid phase extraction-C18 column cartridge with particle size of 40 µm with a pore size of 60 A° and then used for HPLC.

5.2.9 Statistical Analysis

The data were expressed as mean values ± standard deviation. Statistical significance between the treated and untreated groups in all experiments was measured using student's t-test. One-way ANOVA with Tukey's post hoc test in instances of multiple comparisons was used to evaluate the concentration of amino acid supplementation that is beneficial. Statistical analyses were performed using SPSS 14, $p < 0.05$ was considered statistically significant.

5.3 RESULTS

The CHO-K1 cells were exposed to varying levels of high glucose in the medium, in the presence of insulin, along with varying concentrations of amino acid mixture. In the present study, the mechanism of amino acids mediated translocation of GLUT4 has been investigated.

5.3.1 Glucose uptake in the presence and absence of insulin

Glucose uptake is insulin dependent. This experiment was done to see the glucose uptake when CHO-K1 cells were exposed to varying high glucose in the presence and absence of insulin along with labeled $U^{14}C$ deoxyglucose. The amount of glucose as well as the presence of insulin has influenced the glucose uptake as seen when deoxyglucose is used as a tracer to study the glucose entry **(Fig 5.1)**.

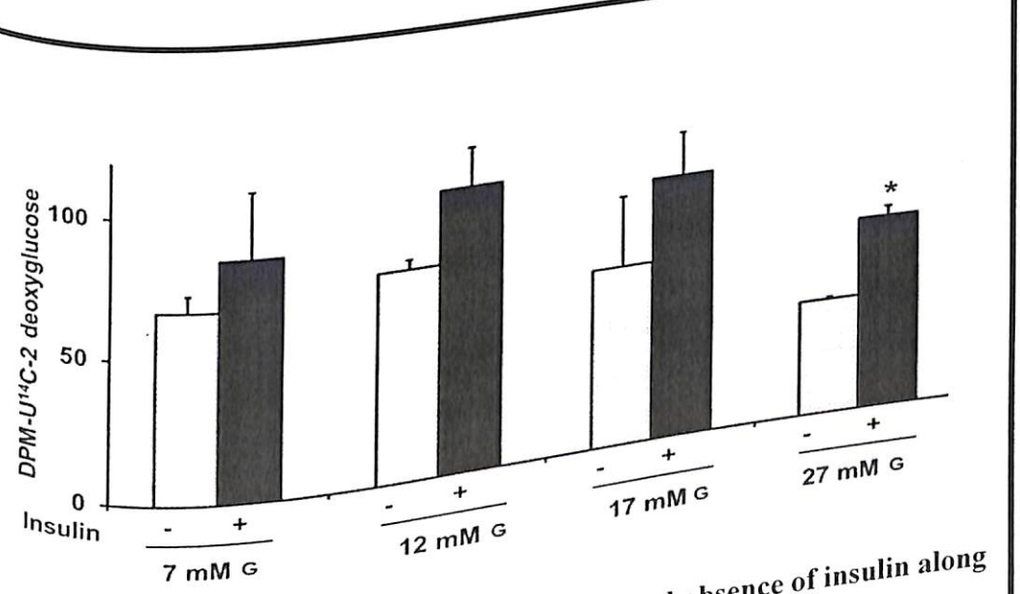


Fig 5.1: 2-deoxyglucose uptake in the presence and absence of insulin along with varying cold glucose.

5.3.2 Glucose uptake in the presence of insulin at varying time

The glucose uptake was studied at 2, 8, 30 min by using labeled glucose as tracer. At 2 min with increase in glucose concentration glucose uptake is increased up to a glucose concentration of 17 mM after which it decreases dose dependently. There was no significant difference between 2 or 8 min or 30 min on the glucose uptake at high glucose concentration. 8 min was chosen for the rest of the studies to assess the glucose uptake **(Fig 5.2)**.

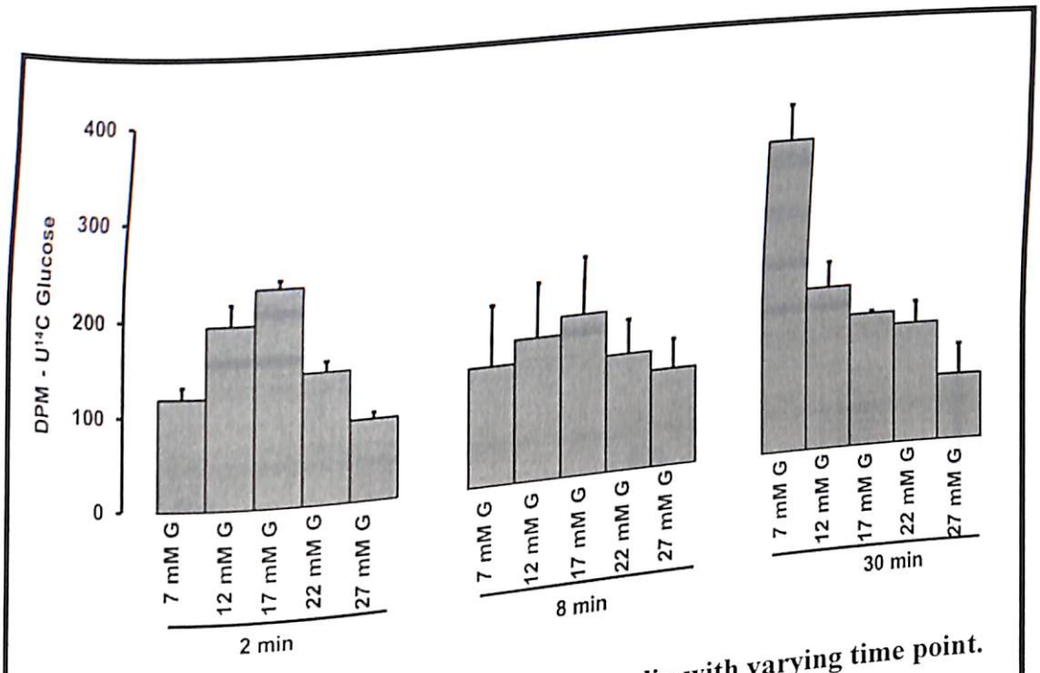


Fig 5.2: Glucose uptake in the presence of insulin with varying time point.

5.3.3 Effect of AAM on glucose uptake in the presence of insulin

To study the effect of AAM on glucose entry in the cells, two methods were used one using tracers such as $U^{14}C$ glucose, $U^{14}C$ 2-deoxyglucose and the other disappearance of glucose in the medium by using spectrophotometer analysis. Though it is obvious from the previous chapter, the maximal glucose uptake was studied in concentrations below and above 5 mM. Therefore the experiments were conducted at 2.5, 5.0 & 10 mM in the presence of varying glucose.

a) **$U^{14}C$ glucose uptake in the presence of AAM:** In the presence of varying AAM (2.5, 5.0, 10.0 mM), the effect of AAM on glucose uptake was seen at 5.0 & 10.0 mM significantly ($p<0.05$) at all the glucose concentration from normal (7 mM) to high glucose (12, 17 & 27 mM). This was studied by measuring the $U^{14}C$ glucose entering the cell **(Fig 5.3A)**.

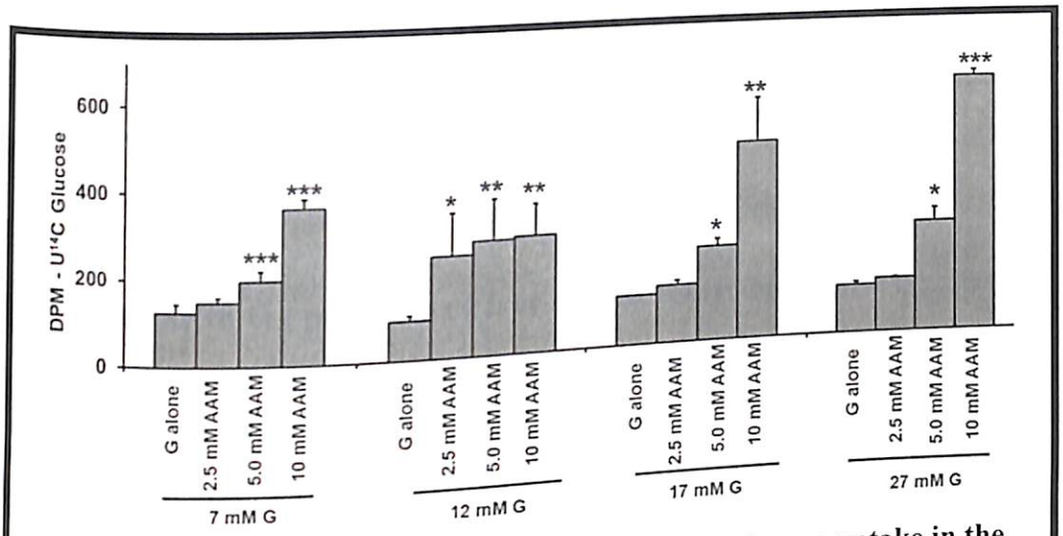


Fig 5.3A: Effect of varying amino acid mixture on glucose uptake in the presence of insulin along with the tracer $U^{14}C$ glucose.

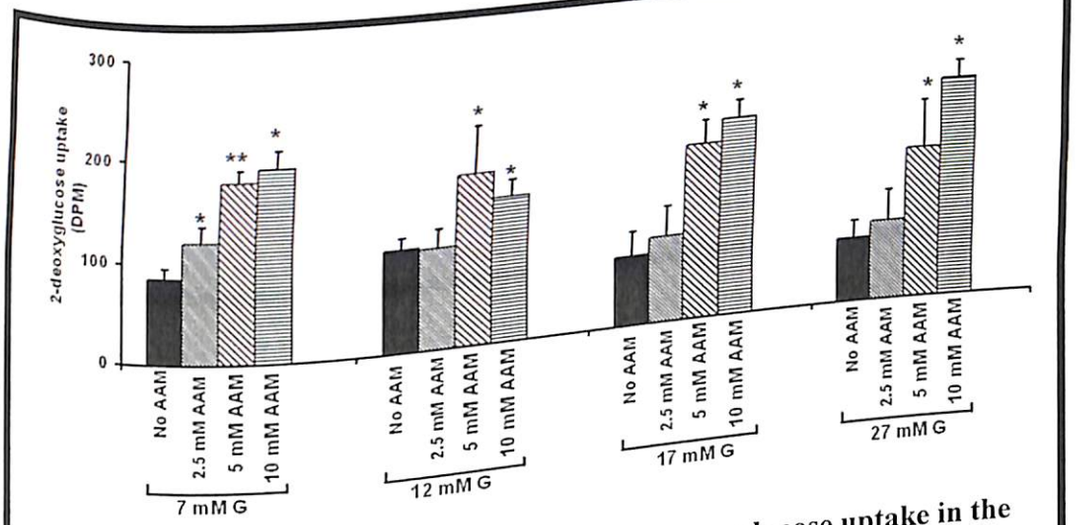


Fig 5.3B: Effect of varying amino acid mixture on glucose uptake in the presence of insulin along with the tracer $U^{14}C$ 2-deoxyglucose.

b) **$U^{14}C$ deoxyglucose uptake in the presence of AAM:** The effect of AAM on glucose uptake was further confirmed by the study using 2-deoxyglucose as the tracer, which is a glucose metabolite that will not further get metabolized as it enters the cell. AAM of 5.0 & 10.0 mM concentration was found to be effective in increasing the glucose uptake significantly *($p<0.05$)* **(Fig 5.3B)**.

Thus based on the above results it is found that glucose uptake is increased significantly in the presence of both 5 & 10 mM AAM. Further in order to see the effect of free amino acids the following experiment was done.

c) Glucose uptake in the presence of free amino acids: In order to compare the glucose uptake promoted by mixture with that of the free amino acids, the effect of individual amino acids on glucose entry was measured by the clinical analyzer. Glucose entry was measured indirectly by estimating the loss of glucose from the medium of cells treated with free amino acids, as well as mixture, compared with that of the control. Of the free amino acids studied, alanine, arginine and lysine showed a significant increase in the entry of glucose at normal (7 mM) and high glucose concentrations (12 and 17 mM), independent of the dose. Isoleucine, at all concentrations, was found to increase the glucose entry at 17 mM glucose ($p<0.05$). Glutamic acid showed significance only at 17 mM glucose when the concentration was 5.0 mM. In all these conditions AAM was found to be promoting maximum glucose entry compared to free amino acids at 5.0 mM concentration in the high glucose levels of 12 and 17 mM **(Fig 5.3C).**

The study has shown a significant increase in glucose uptake by amino acid mixture at high glucose concentration. In order to see the effect of AAM on the insulin signaling cascade that culminates in the glucose uptake by the cell, the major components of insulin signaling pathway namely IRTK, PI3K, Akt GLUT4 and GS was studied to assess if AAM potentiates insulin signaling thereby improving the glucose uptake.

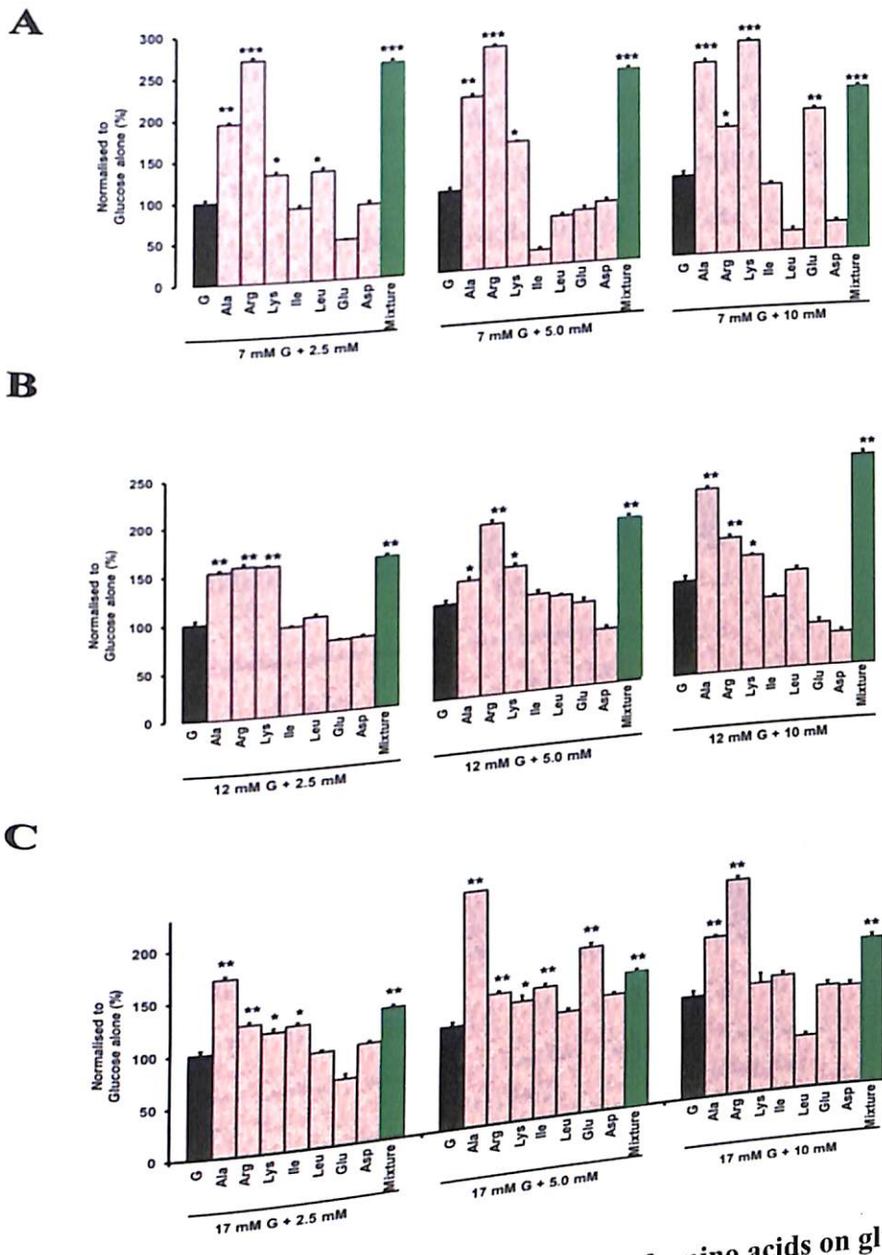


Fig 5.3C: Effect of varying individual & mixture of amino acids on glucose uptake in terms of glucose entry as measured by clinical analyzer in the presence of insulin.

5.3.4 IRTK activity on high glucose and amino acid mixture exposure

Insulin binding triggers the IRTK tyrosine phosphorylation which was estimated by the tyrosine kinase activity. The addition of amino acids mixture increased the IRTK activity significantly both at normal *(p=0.03)* and in elevated glucose (17 and 27 mM) levels as studied by

autoradiography **(Fig 5.4A)**. With addition of lower concentration of AAM (2.5 mM) no significant increase in the IRTK activity was observed. With increase of AAM to 5 mM, significant increase in activity was observed at 7, 17 & 27 mM G *($p<0.05$)*. With the addition of 20 mM AAM too *($p<0.05$)* at 7 &17 mM, there was a significant increase in activity. At 27 mM glucose, addition of 20 mM AAM showed higher activity of IRTK in terms of the phosphorylation observed by the autoradiography. *($p=0.002$)* **(Fig 5.4B)**.

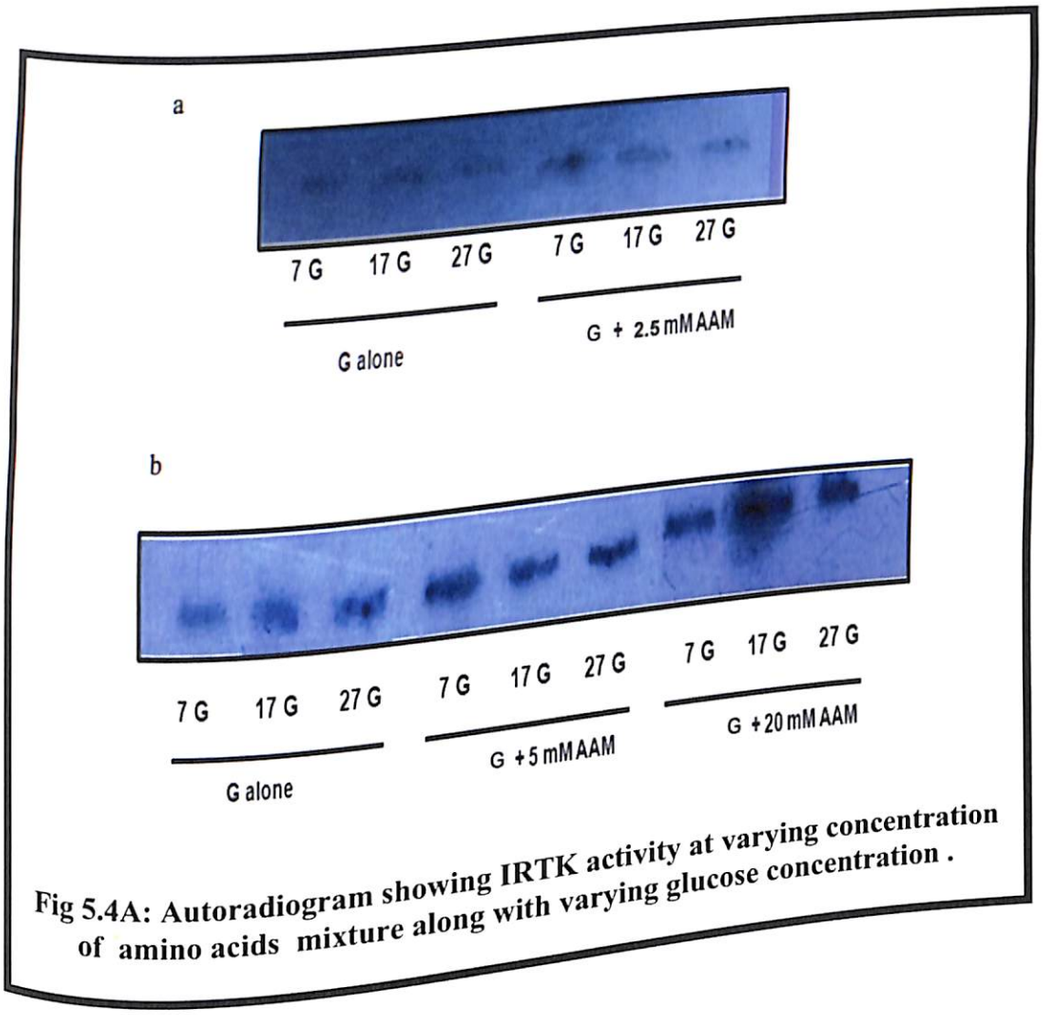


Fig 5.4A: Autoradiogram showing IRTK activity at varying concentration of amino acids mixture along with varying glucose concentration .

Statistical analysis by ANOVA revealed that AAM significantly improves IRTK activity especially at 5 & 20 mM concentration *($p=0.025$)* with increase in glucose concentration.

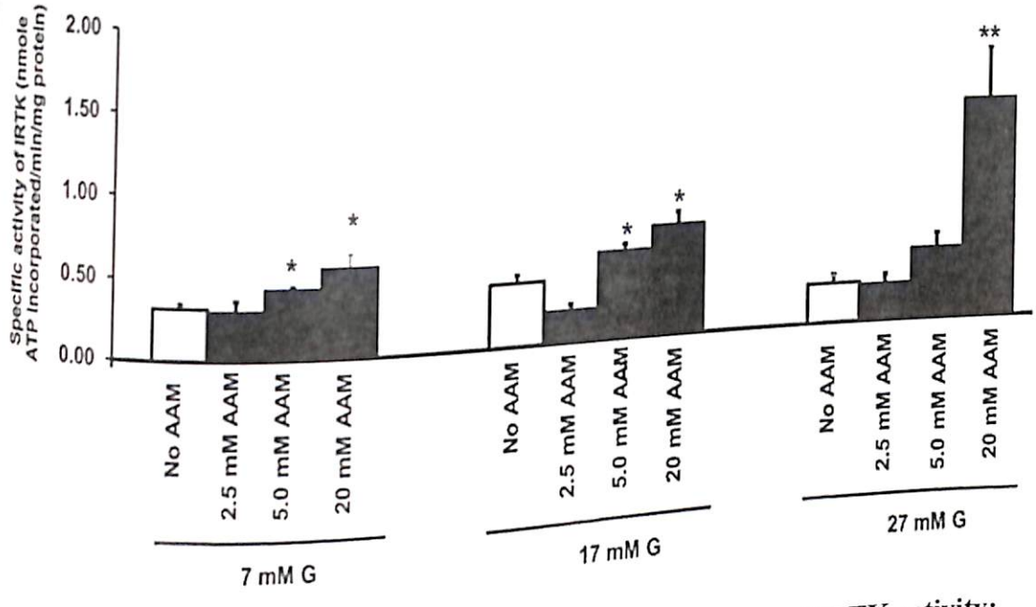


Fig 5.4B: Graphical representation of autoradiogram results of IRTK activity: Showing increased activity at addition of 5 mM AAM at 7 & 17 mM G and at 20 mM AAM at 27 mM G in the presence of insulin.

5.3.5 PI3K activity on high glucose and amino acid mixture exposure

PI3K activity was studied by the autoradiogram developed followed by thin layer chromatography development by iodine stain **(Fig 5.5A)**. With increase in glucose concentration to 17 and 27 mM, significant increase in PI3K activity was seen compared to the normal glucose of 7 mM (*p=0.01 & 0.05* respectively). Addition of 5 and 20 mM AAM to normal glucose (7 mM) showed increased PI3K activity *(p=0.05)*. However only 20 mM AAM showed significance at a high glucose concentration of 27 mM glucose *(p=0.037)* **(Fig 5.5B)**. Statistical analysis by ANOVA revealed that AAM significantly improves PI3K activity with increase in glucose concentration *(p=0.003)*.

Fig 5.5A: PI3K activity by thin layer chromatography followed by autoradiogram: Showing the product formation after the enzyme had reacted with substrate to give product in the presence of glucose with 2.5, 5 & 20 mM AAM. This was done by passing iodine fumes to the TLC plate followed by developing X-ray film.

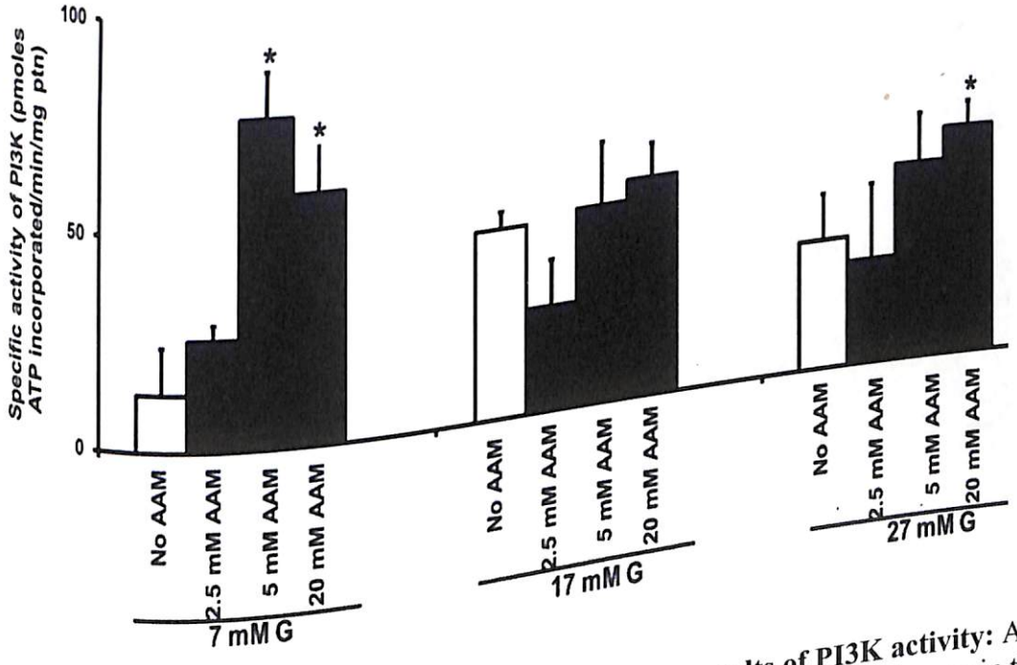


Fig 5.5B: Graphical representation of autoradiogram results of PI3K activity: At varying effect of amino acids addition in mixture from along with varying glucose in the presence of insulin showing significant increase by addition of 5 mM AAM to 7 mM G.

There is an increase in the PI3K activity at 17 mM G irrespective of AAM and this is in response to the increase in glucose. AAM does not seem to improve the activity over and above this and in fact at 17 mM there is a fall in the activity which then increases dose dependently with increase in AAM. The need for AAM is felt at a high glucose concentration of 27 mM, when there is a fall in the PI3K compared to that seen at 17 mM as there is a significant improvement with addition of AAM both at 5 and 20 mM though significant at 20 mM alone. Thus AAM seems to activate PI3K at normal glucose maximally as seen at 5 and 20 mM and though there is a decrease at high glucose, still the presence of AAM has made a difference at 5 and 20 mM dose dependently.

5.3.6 Akt phosphorylation at ser473 on high glucose and AAM exposure

Akt mediates insulin-dependent glucose uptake in insulin-sensitive tissues through GLUT 4 translocation and is the downstream of the PI3K. Therefore the changes in phosphorylated Akt expression were analyzed by immunofluorescence stain and western blot analysis.

*a) **Immunofluorescence stain:*** With increase in glucose concentration increased Akt

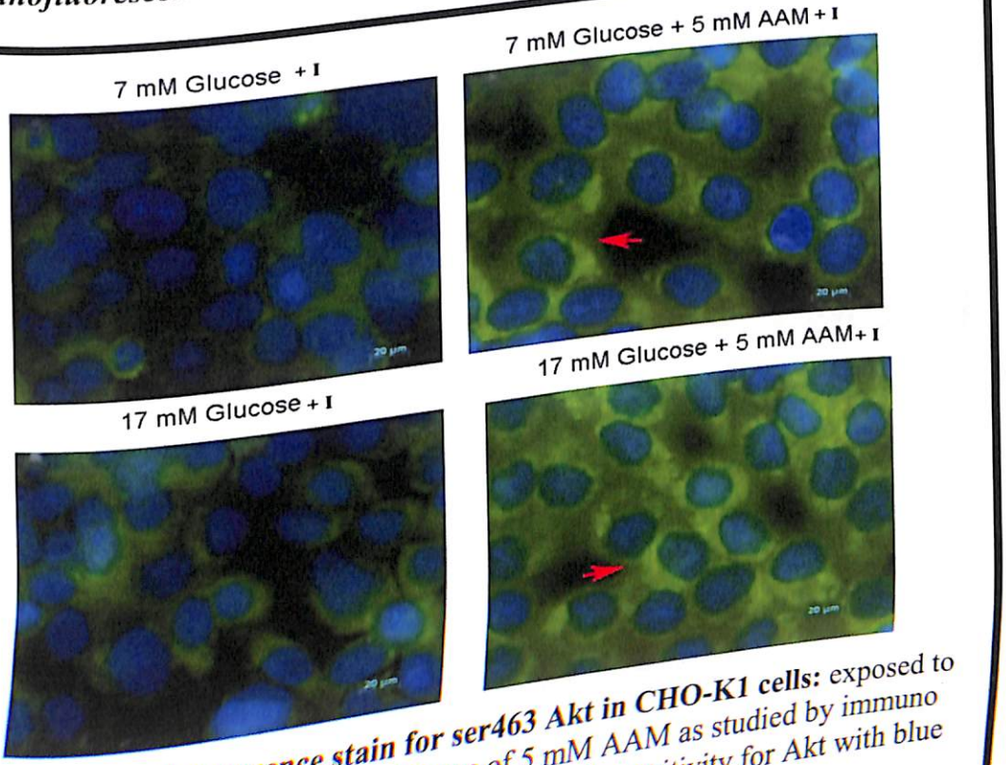


Fig 5.6A: Immunofluorescence stain for ser463 Akt in CHO-K1 cells: exposed to varying glucose in the presence and absence of 5 mM AAM as studied by immuno fluorescence microscopy. *Green fluorescence shows the positivity for Akt with blue nuclei of DAPI.*

expression was seen by immunofluorescence. Addition of AAM further increased the expression of the Akt only at normal glucose **(Fig 5.6A)**. The inhibitor of PI3K, upstream of Akt namely wortmannin, showed inhibition of Akt expression, thus showing the involvement of Akt in signaling at normal glucose **(Fig 5.6B)**.

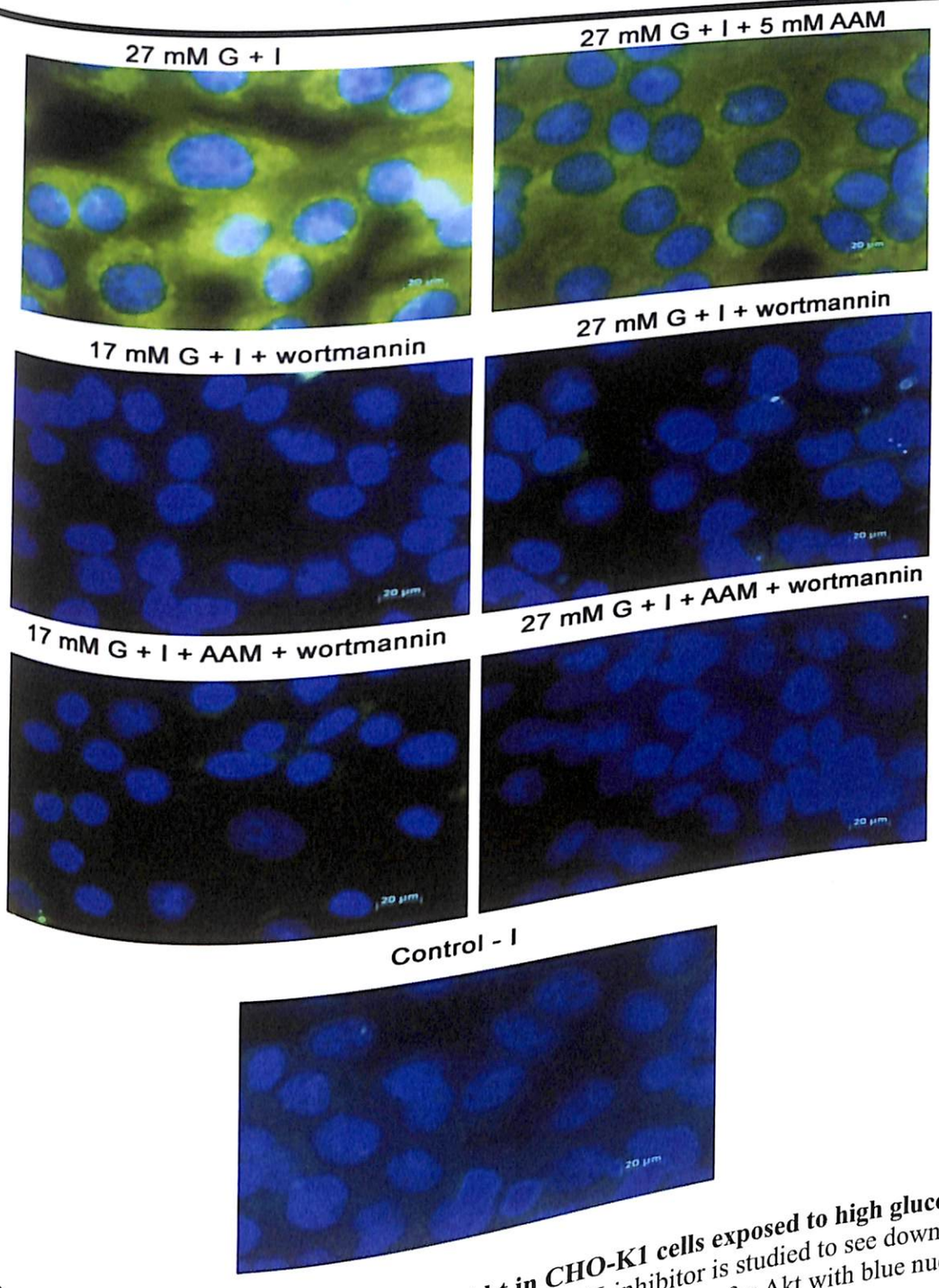


Fig 5.6B: Immunofluorescence stain for ser463 Akt in CHO-K1 cells exposed to high glucose (27 mM G) and 27 mM G + 5 mM AAM: The wortmannin, PI3K inhibitor is studied to see downstream effector molecule Akt inhibition. Green fluorescence shows the positivity for Akt with blue nuclei of DAPI.

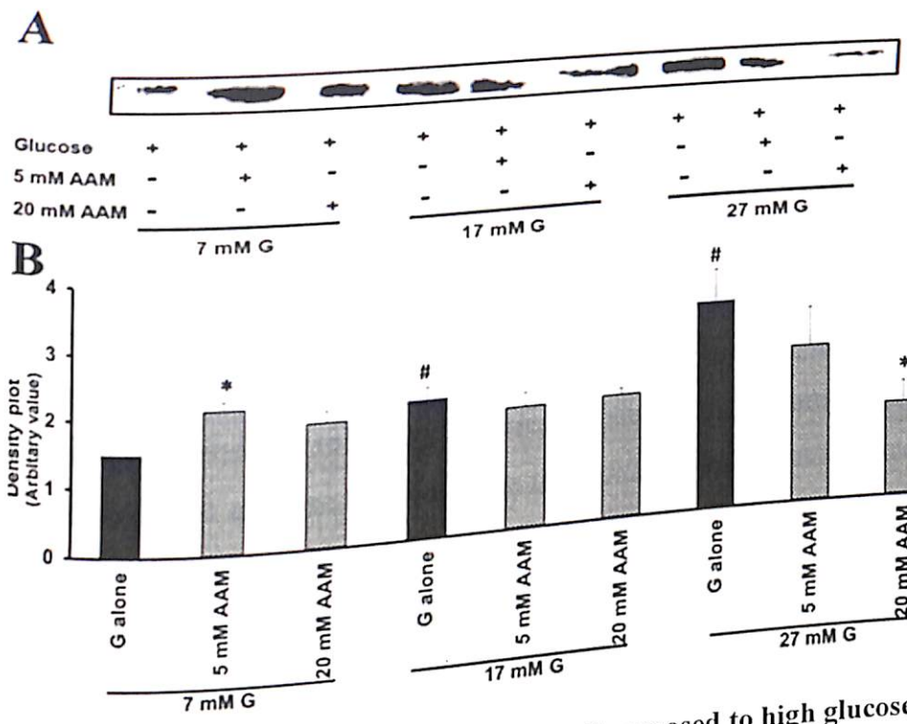


Fig 5.6C: Western blot for ser463 Akt in CHO-K1 cells exposed to high glucose with AAM.

*b) **Western blot:*** With increase in glucose concentration there was a significant increase in the levels of Akt as seen by the western blot (17 & 27 mM G; *p=0.01*) with the maximum at 27 mM. Addition of 5 mM AAM in the presence of 7 mM glucose significantly increased the levels of phosphorylated Akt Ser473 *(p=0.02)* correlating with IF data. However, at high glucose concentration there was no significant change. However at 20 mM AAM there was a significant decrease in Akt phosphorylation *(p=0.035)* **(Fig 5.6C)**.

5.3.7 GLUT4 recruitment by AAM is concentration dependent

The ultimate molecule in insulin signaling that helps in glucose uptake is the translocation of GLUT4 protein which was studied by western blot and FACS analysis.

*a) **GLUT4 translocation in plasma membrane studied by Flow cytometry:*** Analysis of GLUT4 translocation in plasma membrane was done by Flow cytometry analysis. The addition of 5 mM AAM, in the absence of insulin, showed a significant decrease *($p < 0.05$)* in the

GLUT4 translocation, compared to glucose alone **(Fig 5.7A-a)**. In the presence of insulin (100 nM), the addition of 5 mM AAM showed a significant increase in the GLUT4 translocation seen at all glucose concentration compared to the cells to which no amino acid treatment was given *($p<0.05$)* **(Fig 5.7A-b)**. Even after decreasing insulin by 50 % (i.e. 100 nM to 50 nM), the GLUT4 recruitment was found to be 1.2 fold and 1.7 fold higher in 50 nM and 100 nM insulin respectively (seen at 17 mM glucose). At 27 mM glucose, a 2.4 fold and 2.5 fold increase was seen at 50 nM and 100 nM insulin respectively **(Fig 5.7A-c)**. Increasing the AAM to 10 mM did not result in further increase in the GLUT4 recruitment compared to 5 mM AAM *($p < 0.05$)*, **(Fig 5.7A-d)**. Thus, rather than AAM or insulin alone, AAM along with insulin augmented GLUT4 translocation. 5 mM & 10 mM AAM increased the GLUT4 translocation at all glucose levels over and above that of glucose treated alone *($p < 0.05$)*. 5 mM AAM showed 64 %, 23 %, 138 % increase in fluorescence when added to 7 mM, 17 mM, 27 mM glucose respectively as studied by FACS is shown as histogram **(Fig 5.7B)** and density plot **(Fig 5.7C)**.

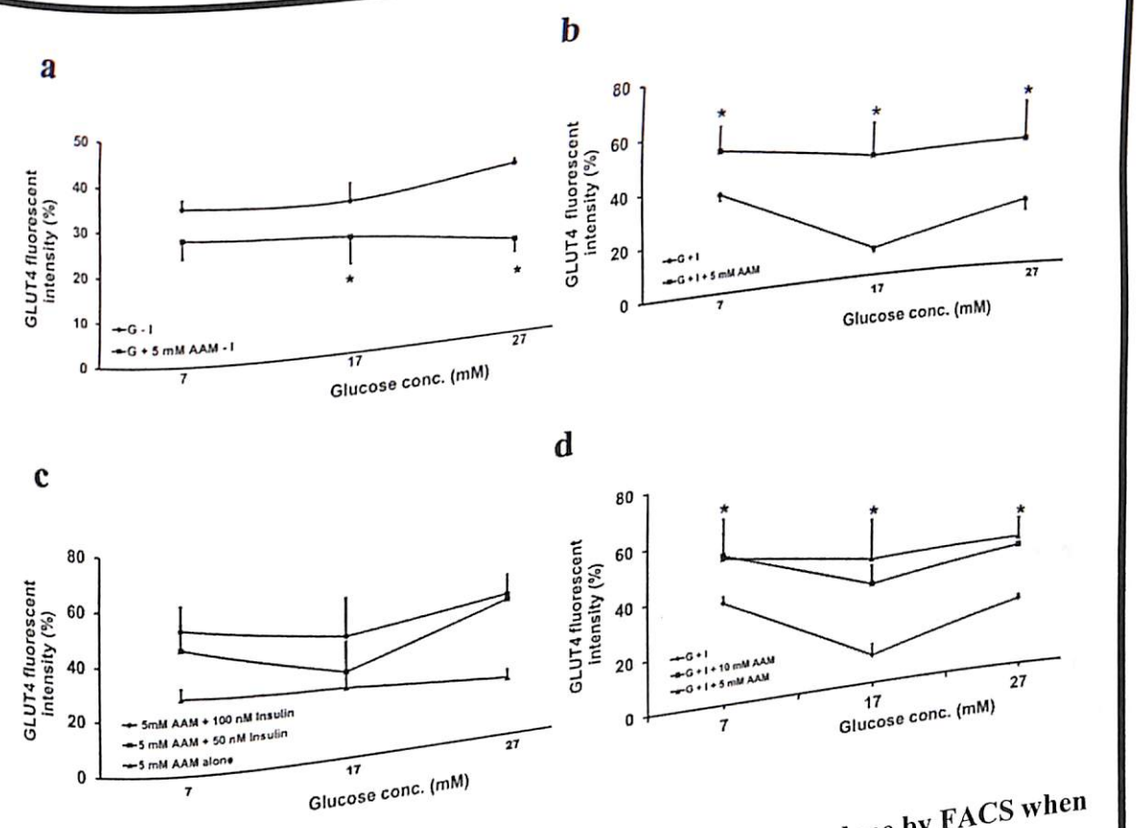


Fig 5.7A(a-d): Graphical representation of the GLUT4 analysis done by FACS when exposed to AAM and glucose along with 100 nM insulin.

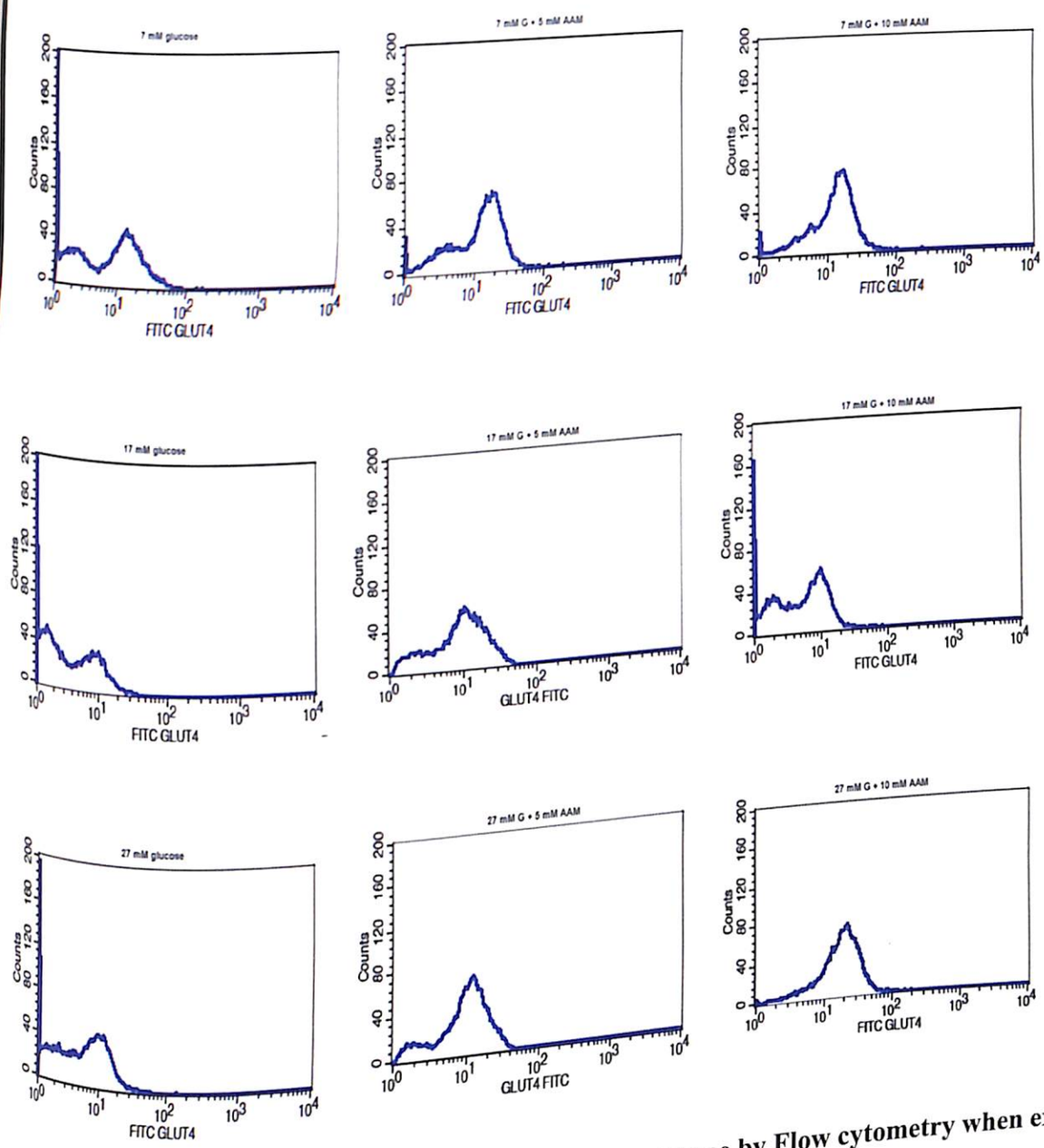


Fig 5.7B: Histogram of GLUT4 analysis in plasma membrane by Flow cytometry when exposed to AAM and glucose along with 100 nM insulin.

GLUT4 protein study by western blot: WB analysis showed a fall in GLUT4 levels at 17 (NS) and 27 mM G *($p=0.049$)* compared to normal glucose. The addition of 5 mM AAM showed a significant increase in the levels of GLUT4 at all concentrations of glucose *($p<0.05$)* **(Fig 5.7D)**.

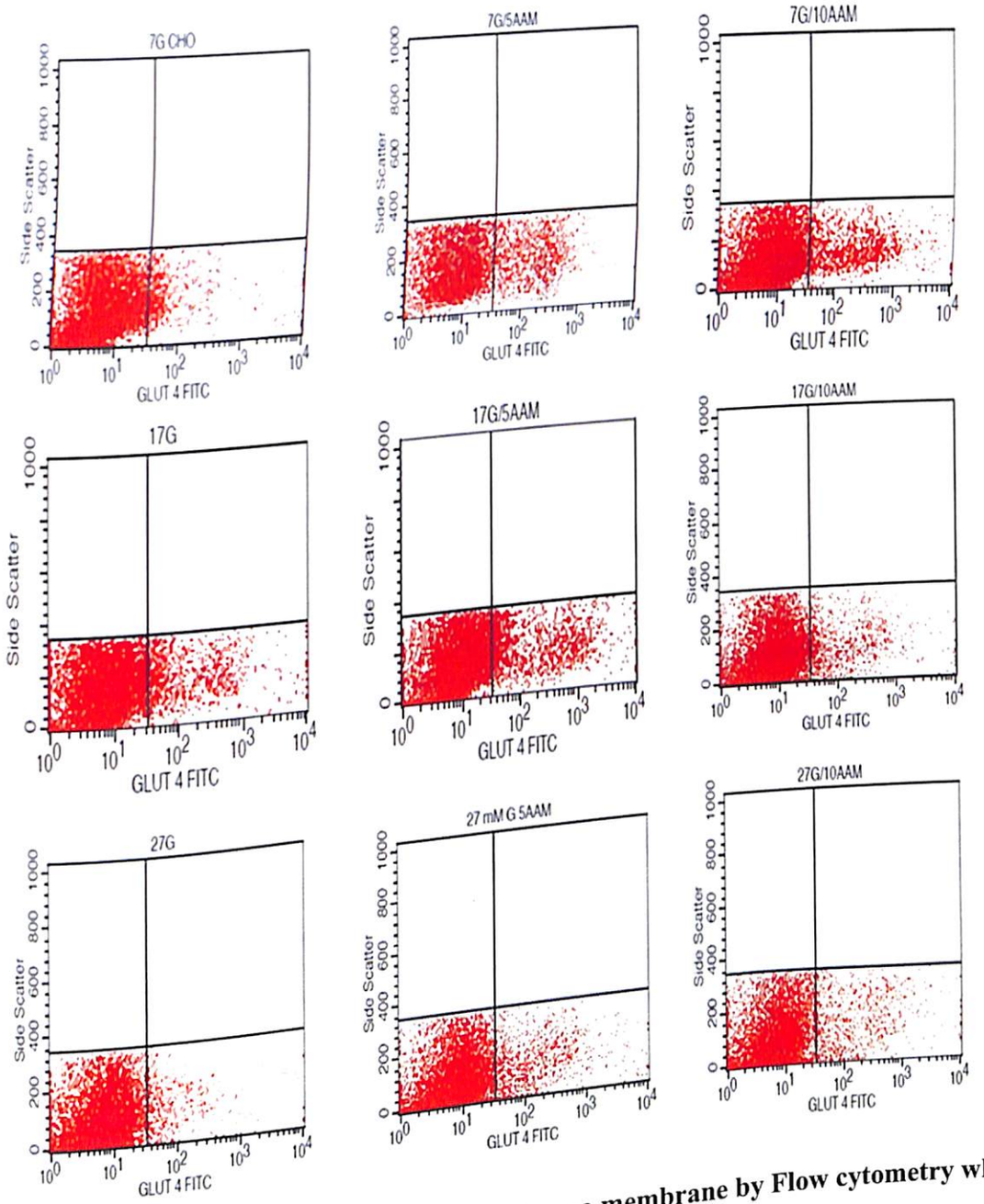


Fig 5.7C: Density plot of GLUT4 analysis in plasma membrane by Flow cytometry when exposed to AAM and glucose along with 100 nM insulin.

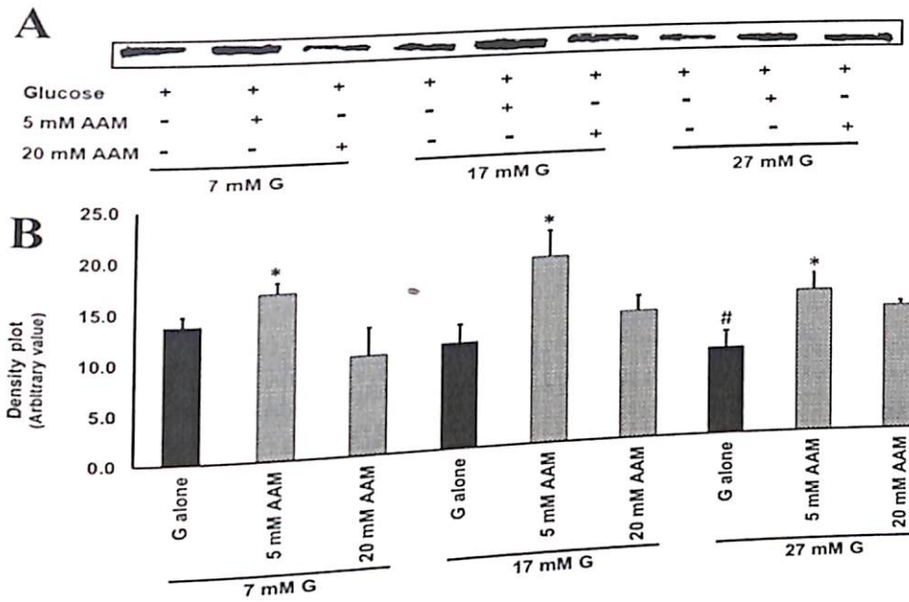


Fig 5.7D: Western blot analysis for the antibody against GLUT4 raised in goat when exposed to amino acid mixture and glucose along with 100 nM insulin.

However at 20 mM AAM it was found that GLUT4 levels are not significantly altered correlating with the glucose uptake data as shown in **table 5.1**.

Table 5.1: Glucose uptake by 2-deoxy glucose as tracer is added along with the glucose of varying concentration and amino acids of 5 & 20 mM.

Glucose conc. (mM)	G alone	G + 5 mM AAM	G + 20 mM AAM
7	85 ± 11	179 ± 13	319 ± 20
17	70 ± 25	176 ± 25	133 ± 22
27	64 ± 19	153 ± 49	115 ± 39

5.3.8 *Glycogen synthase on high glucose and AAM exposure*

*GS activity was measured in terms of increase in UDP formation from the substrate UDPG. UDPG was detected by HPLC at 9th minute **(Fig 5.8A)** and UDP is detected at 16th minute **(Fig 5.8B)**.* Though not significant, GS activity increases with increase in glucose in the medium. Increased GS activity is indicative of the utilization and storage of glucose in the cells. By the addition of 5 mM AAM, increase in UDP levels was seen in terms of peak area at all the

concentrations of glucose which was dependent on the glucose levels **(Fig 5.8C,D)** and the same as graphical representation **(Fig 5.8E)** shows increase in GS activity with addition of 5 mM AAM significantly ($p<0.05$).

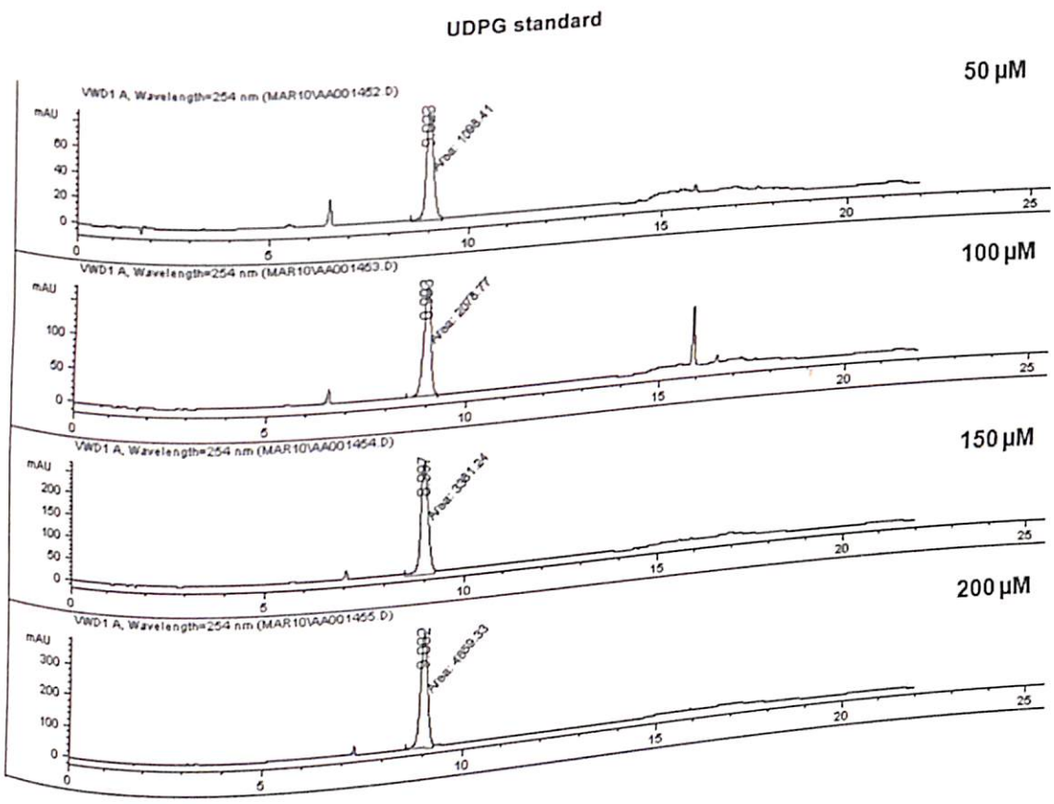


Fig 5.8A: Uridine di phosphate glucose standard of 50, 100, 150 & 200 µM detected at 9th minute as done by HPLC.

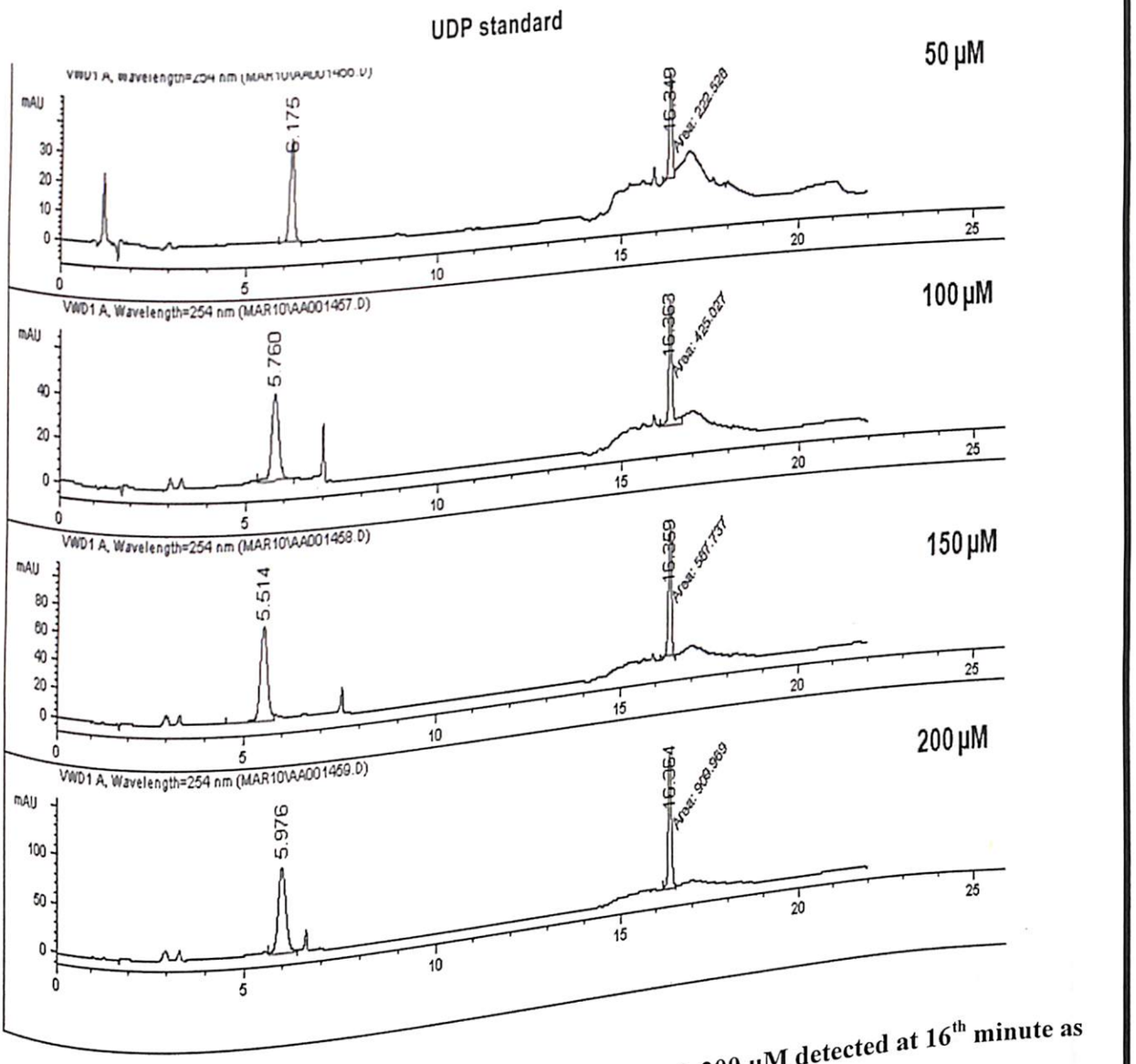


Fig 5.8B: Uridine di phosphate standard of 50, 100, 150 & 200 µM detected at 16th minute as done by HPLC.

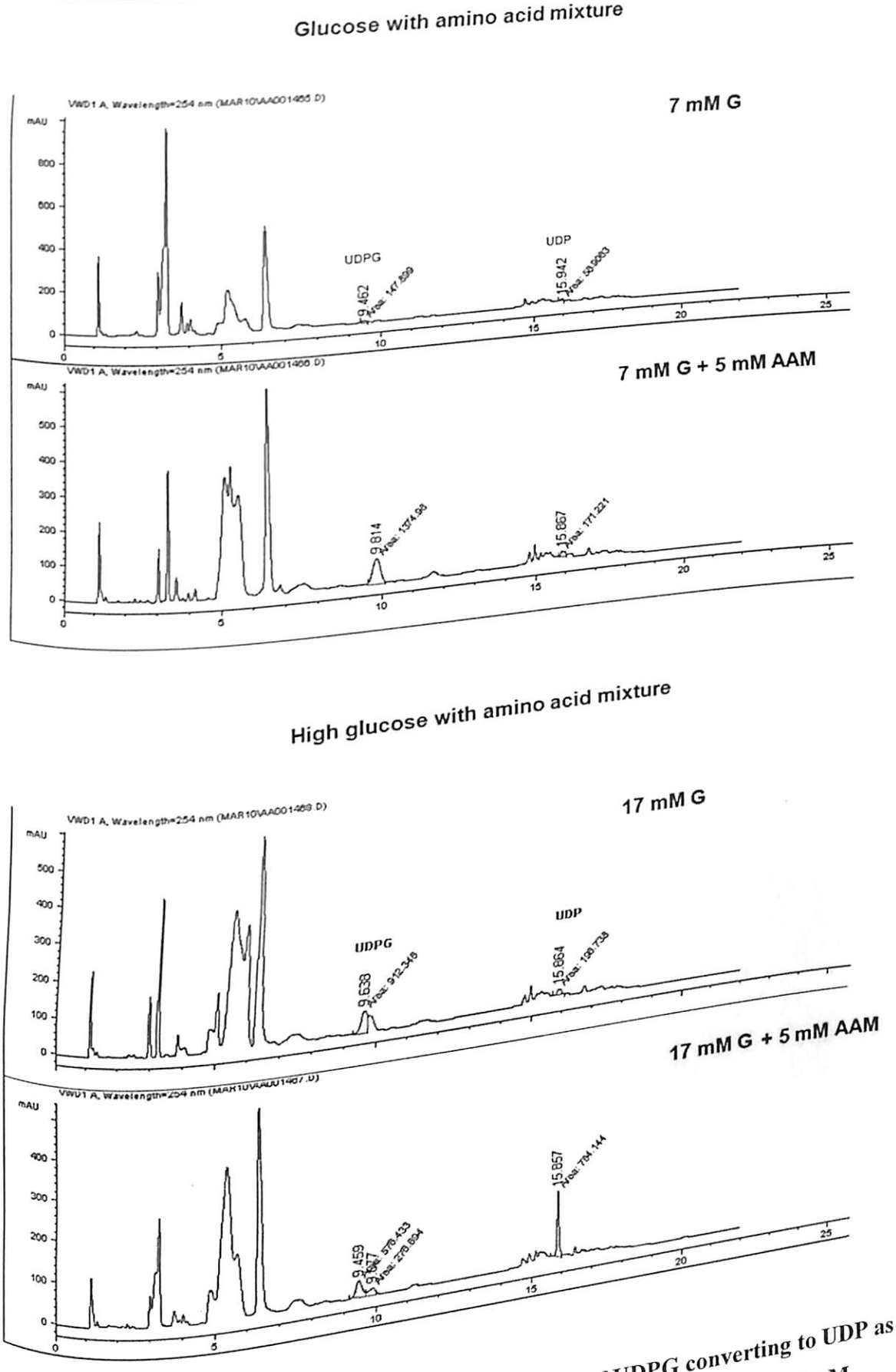


Fig 5.8C: Glycogen synthase activity measurement in terms of UDPG converting to UDP as done by HPLC when exposed to normal 7 & 17 mM glucose alone and glucose + 5 mM AAM.

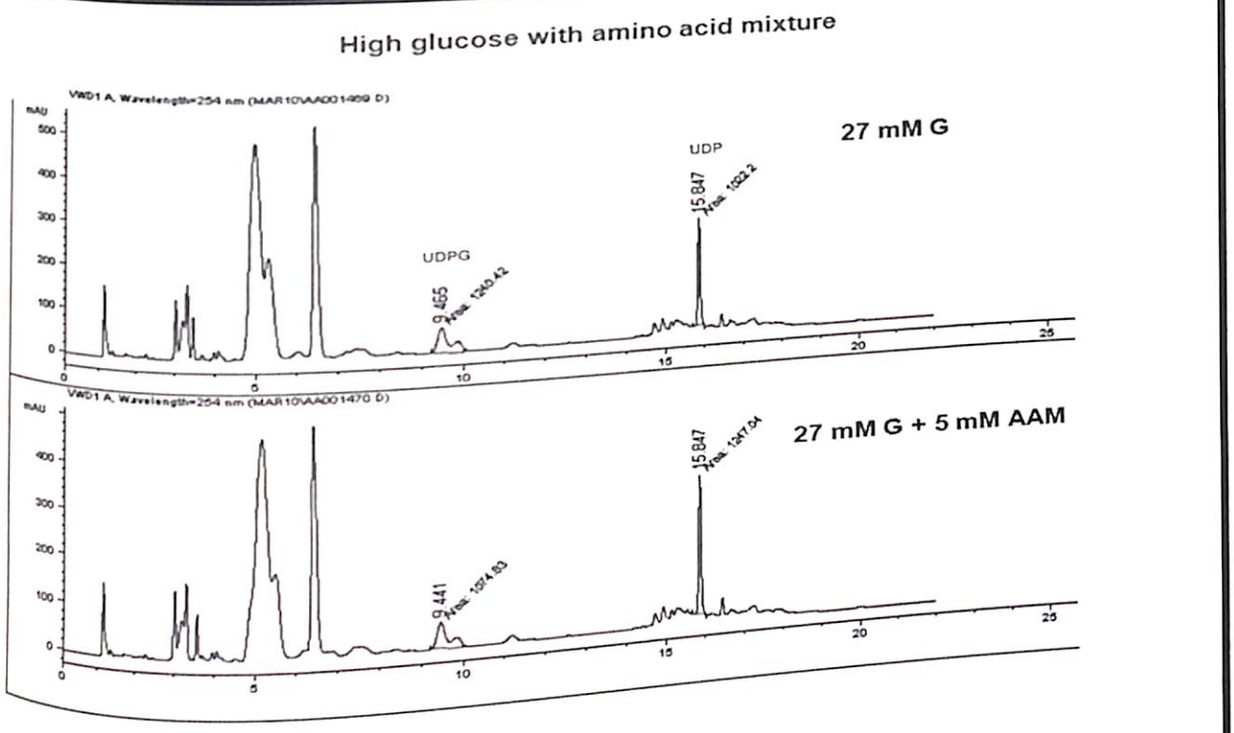


Fig 5.8D: Glycogen synthase activity measurement in terms of UDPG converting to UDP as done by HPLC when exposed to high 27 mM glucose alone and 27 mM glucose + 5 mM AAM.

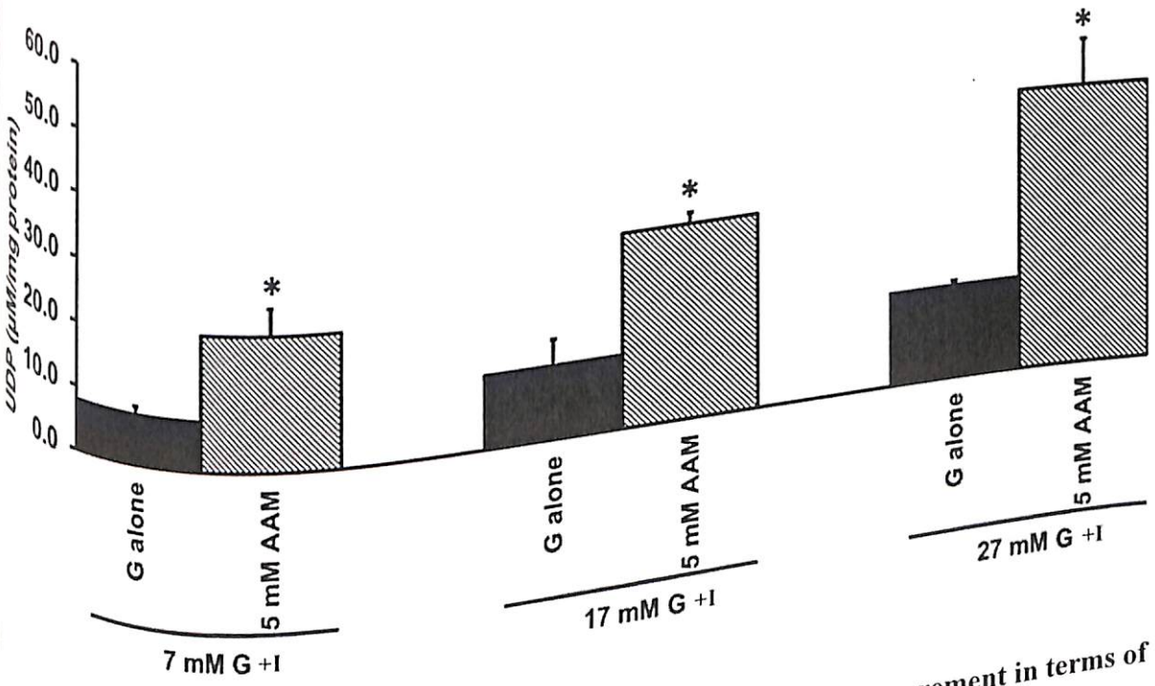


Fig 5.8E: Graphical representation of glycogen synthase activity measurement in terms of UDPG converting to UDP as done by HPLC when exposed to varying glucose alone and glucose with 5 mM AAM.

Amino acids can act at two levels, one as a secretogogue of insulin in the pancreas and the other on the enhancement of glucose uptake. Our study showed that amino acid mixture triggered the insulin signaling cascade as IRTK/PI3K/Akt/GLUT4 and increase the glucose uptake. Glycogen synthase activity is increased in support of the improved glucose availability in the cell, apart from Akt mediated effect. In summary, our data suggests that amino acid mixture can provide a beneficial effect by increasing glucose uptake which can help in the management of diabetes mellitus.

5.4 CONCLUSION

1. This study showed that amino acid mixture **increases glucose uptake** in high glucose by labeled $U^{14}C$-Glucose uptake studies, 2DeoxyGlucose uptake studies, GLUT 4 translocation studies. This is the first report showing free amino acids namely lysine, glutamic acids apart from alanine, arginine, isoleucine increasing the glucose uptake.
2. Amino acids mixture **potentiates the insulin signaling cascade** as IRTK / PI3K / Akt / GLUT4. AA mixture also **increases the Glycogen synthase activity** indicative of increased glucose availability in the cell. In summary, the data suggests that amino acid mixture can provide a beneficial effect by increasing glucose uptake under high glucose conditions thereby explaining the **anti-diabetic** property.

CHAPTER 6: EFFECT OF AMINO ACIDS ON ROS SIGNALING UNDER HIGH GLUCOSE CONDITION IN CHO-K1 CELLS

6.1 INTRODUCTION

Amino acid mixture (AAM) increases glucose transport in CHO-K1 cells with a decrease in Akt expression (Selvi, Angayarkanni et al.). Akt is the center for many diversifying signal, downstream of PI3K, which can cause migration and angiogenesis. When activated by cell surface receptors, PI3K regulates the phosphoinositide lipid metabolism and the production of phosphatidylinositol 3,4,5-triphosphate (PIP3) at the plasma membrane. The production of PIP3 facilitates the recruitment of AKT via its pleckstrin homology domain, leading to its phosphorylation at Thr308 and Ser473 (Shiojima and Walsh 2002; Song, Ouyang et al. 2005).

6.1.1 Akt in apoptotic pathway

Akt/PKB is critical in the signal transduction pathways and is activated in response to growth factors or insulin contributing to several cellular functions including nutrient metabolism, cell growth, transcriptional regulation and cell survival (Shiojima and Walsh 2002; Song, Ouyang et al. 2005). As the primary downstream mediator of PI3K, AKT transmits the PI3K signal to a large number of molecules thereby diversifying the PI3K signal into various functional outcomes. (Renner 2008). Akt/PKB has been shown to regulate IKK activity in both direct and indirect manner. This leads to the nuclear translocation and activation of NF-κB (Song, Ouyang et al. 2005). NF-κB activation can down-regulate pro-apoptotic JNK signaling thus preventing apoptosis in cells (Tang, Minemoto et al. 2001). Thus activation of the ROS/PI3K/Akt/eNOS signaling pathway in early phase exerts protective effects against the induction of apoptosis by high glucose as studied in HUVEC cells (Hao, Linb et al. 2006).

6.1.2 Akt in angiogenesis

Anti-apoptotic property of Akt contributes to cell proliferation which is a hall mark of angiogenesis. The potent factor involved in angiogenesis is VEGF (Nyberg, Xie et al. 2005).

Amongst the molecules involved in VEGF signal transduction pathways, PI3 kinase (PI3K), Akt/PKB and eNOS are involved in NO-induced endothelial cell migration (Kawasaki, Smith et al. 2003). It is known that endothelial-derived NO, catalyzed by eNOS, is a critical mediator of angiogenesis. Stimulation of eNOS results in increased production of NO, which promotes EC migration and angiogenesis (Murohara, Witzenbichler et al. 1999; Cooke and Losordo 2002; Fan, Sulochana et al. 2008).

6.1.3 Vascular endothelial growth factor (VEGF)

VEGF, a 40-kD a glycoprotein, is a proinflammatory molecule that plays a well-recognized role in neovascularization and increased permeability. VEGF is a primary regulator of blood vessels in all the tissues, including the retina. It is a cell survival factor that mediates through its receptor as Flk1/VEGFR2-PI3K-Akt pathway (Fujio and Walsh 1999). Angiopoietin-1 (Ang-1), insulin, insulin-like growth factor-I (IGF-I), ROS also activates PI3K-Akt signaling, illustrating the central role of this pathway in controlling cell viability (Thomas 1995; Morales-Ruiz, Fulton et al. 2000).

6.1.3.1 Angiogenesis and vasoactive compounds

Angiogenesis is the principal factor responsible for disease pathology, such as is the case in diabetic retinopathy and macular degeneration apart from tumor angiogenesis. The levels of VEGF in ocular fluid correlates with the activity of neovascularisation in retinopathy, breakdown of the blood-retinal barrier, increased microvascular permeability (Yamagishi, Nakamura et al. 2006). In addition to hyperglycemia, overproduction of reactive O_2 species by mitochondria, advanced glycation end products (AGEs), hexosamines and increased polyol metabolism of glucose leads to altered signaling of pathways involving protein kinase C, nuclear factor kappa-B (NFK-B) (Mahabeleshwar, Feng et al. 2006), hemeoxygenase and MAP kinase. These changes can damage retinal endothelial cells, pericytes, neurons, glia and pigment epithelial cells and recruit inflammatory cells which produce vasoactive compounds, growth factors (VEGF), coagulation factors and adhesion molecules that eventually lead to angiogenesis and tissue remodeling (Pelikanova 2007).

6.1.4 Oxidative stress in diabetes

Oxidative stress has long been associated with the complications of diabetes including nephropathy, retinopathy and other macro vascular complications due to the chronic hyperglycemia (Pagel-Langenickel, Bao et al.). The oxidative stress seems to play a causative role in diabetic retinopathy (Brownlee 2001).

The prolonged hyperglycemia is known to deplete cellular antioxidants and cause increase in free radicals. It plays a key direct role in the pathogenesis of late diabetic complications (Rosen, Nawroth et al. 2001; Houstis, Rosen et al. 2006). ROS can function as signaling molecules to activate a number of cellular stress-sensitive pathways that cause cellular damage, and are ultimately responsible for the late complications of diabetes and IR (Nishikawa, Edelstein et al. 2000; Evans, Goldfine et al. 2003). Thus increased oxidative stress through protein alterations and redox modifications is reported to contribute to pathogenesis of diabetic microangiopathy (Sampathkumar, Balasubramanyam et al. 2005).

6.1.4.1 Peroxynitrite species

VEGF binding to its receptor and autophosphorylation stimulates the formation of nitric oxide, superoxide anion, and peroxynitrite. Peroxynitrite can modify proteins via tyrosine nitration or thiol oxidation. Peroxynitrite mediates VEGF signaling by an oxidation-dependent mechanism. Peroxynitrite sustains VEGFR2 phosphorylation and propagates VEGF's signal to downstream target, c-Src, via nitration-independent and oxidation-dependent mechanisms.

Peroxynitrite, a non-radical species which is present ubiquitously has a very short half-life, is formed by the interaction between NO and superoxide anion, modifies the proteins by either nitration of tyrosine residues or oxidation of protein-associated thiol groups. VEGF stimulates endothelial cells to produce peroxynitrite. Peroxynitrite serves as a second messenger to activate multiple signaling pathways leading to endothelial migration, proliferation, and tube formation *in vitro* and angiogenesis in vivo (El-Remessy, Al-Shabrawey et al. 2006).

6.1.4.2 Nitric oxide and eNOS in angiogenesis

It is known that endothelial-derived NO, catalyzed by eNOS, is a critical mediator of angiogenesis. Stimulation of eNOS results in increased production of NO, which promotes EC migration and angiogenesis (Murohara, Witzenbichler et al. 1999; Cooke and Losordo 2002; Fan,

Sulochana et al. 2008). The nitric oxide radical (NO•) is an important mediator of both physiological and pathophysiological processes. NO• is produced by inducible nitric oxide synthase (iNOS). iNOS is induced by cytokines and (or) endotoxin during inflammatory and infectious processes and produces abundant amounts of NO• for extended periods. iNOS can be induced in many cell types. NO also reacts with ROS or H_2O_2 generated in mitochondria to form peroxynitrate ($ONOO^-$), an even more active radical. During oxidative stress insult, the formed peroxynitrite is converted to NO. The NO can upregulate VEGF and VEGF in turn can bind to the receptor and produce NO and there by cell migration and proliferation (Brune and Cantoni 2000). NO has been associated with a reduction in cytochrome oxidase activity that could increase ROS and H_2O_2 formation. Oxidative stress causes IkB heterodimer phosphorylation, subsequent NF-kB translocation to the nucleus and transcription of iNOS, in a positive feedback mechanism (Stoppiglia, 2002).

6.1.4.3 Superoxide dismutase

Hyperglycemia produces oxidative stress within cells which concomitantly could induce an increase in the intracellular antioxidant enzyme levels (Ceriello, Giacomello et al. 1995). Hyperglycemia is known to produce an increase in ROS and lipid peroxidation while also increasing the accumulation of advanced glycation end (AGE) products in bovine endothelial cells (Giardino, Edelstein et al. 1996). The formed NO along with superoxide is removed from system by the enzyme SOD.

6.1.5 Amino acids in angiogenesis and apoptosis

There are many peptides identified in inhibiting angiogenesis (Mehta, Yamada et al.). However there are limited studies on effect of individual amino acids. A study by Zhong et al have showed L-arginine have an anti-senescence effect via the PI3K/Akt pathway in HUVECs exposed to high glucose and it might be a therapeutic agent for diabetic vascular complications (Zhong, Zou et al.). Prolonged exposure of a clonal pancreatic β-cells line to L-alanine resulted in co-ordinated expression of a number of key genes related to metabolism, signal transduction and oxidative stress and modulating secretory function, against cytokine-induced apoptosis (Cunningham, McClenaghan et al. 2005). L-leucine when supplemented to pancreatic islets β

cells, it increased cytoplasmic catalase (CAT) activity in response to H_2O_2, with no decrease in NO (Stoppiglia, Nogueira et al. 2002).

Amino acids have been shown to be accumulated to combat the oxidative stress when there is associated ER stress (Harding,2003). A signaling pathway initiated by eIF2α phosphorylation protects cells against metabolic consequences of ER oxidation by promoting the linked processes of amino acid.

From our previous experiments, an increase in PI3K/Akt with increase in glucose concentration was found. The addition of amino acids in the form of mixture increased the PI3K when added to normal glucose alone, with addition to high glucose there was no further increase. Similarly, with relation to Akt, only to normal glucose conc. there was an increase in phosphorylated Akt levels when AAM was added. One of the objective in this study was to elucidate the effect of AA downstream of PI3K at the level of VEGF in order to see if they have anti-angiogenic potential.

6.2 MATERIALS AND METHODS

6.2.1 Cell culture and treatment

CHO-K1 cells were maintained in F-12K1 medium in 25 cm2 flasks with the addition 10 µg/ml of ciprofloxacin, 2.5 g/L NaHCO3 and 10% fetal bovine serum (FBS) in a 5% CO2 incubator at 37°C. For the experiments, cells were seeded at 1 X 10^4 cells/well in 25 ^{2}cm flask and 96 well plate. When cells reached 80 – 90 % confluence, they were serum starved for 4 hours. Insulin (100 nM) pretreatment was given for 1 hour. The cells were then exposed to varying glucose (7, 17, 27 mM) along with or without individual amino acids and AAM (SNAAM) for 1 hour and the assay of VEGF, ROS, Hydrogen peroxide, NO, SOD were done.

6.2.2 Preparation of Cell Lysate

At the end of respective time exposure, the reaction was stopped immediately by adding ice cold PBS, washed thrice in the same. PBS was then removed, added 1.0 ml of Lysis buffer (100 ml PBS, 500 µl Triton X, 300 µl of 1mg/ml protease inhibitor cock tail). Kept for 10 min at 4° C, the cells were scraped and sonicated for 10 sec, centrifuged at 10,000 rpm for 10 min at 4° C. Supernatant was stored at -80° C until processed for immuno-precipitation. Protein

concentrations were determined by Bradford coommassie plus method. The basal medium had 7 mM glucose and was used as the control for the various high glucose concentrations studied. All experiments were done in triplicates.

6.2.3 Intracellular ROS measurement by DCF method

To determine the amount of ROS generation, DCFH diacetate was used as it penetrates the cells and becomes hydrolyzed by an intracellular esterase to form DCFH (non-fluorescent), which reacts with intracellular ROS to form the highly fluorescent DCF. The cells were incubated in dark for 30 mins with 5 μM of DCFDA, the fluorescence intensity was determined at 485 nm (Excitation) and at 535 nm (Emission) using Dynex Triad Elisa reader.

6.2.4 Nitric oxide by Griess test

This assay determines nitric oxide based on the enzymatic conversion of nitrate to nitrite by NADH dependent enzyme nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product which absorbs light at 540 nm (Bio Vision, California).

6.2.5 Super oxide dismutase by kinetic assay

After the experimental treatment the cells were lysed and the ability of super oxide dismutase to inhibit the autoxidation of epinephrine was estimated.

6.2.6 VEGF expression by Western blot

After the experimental treatment the cells were lysed and stored in -80°C until used. Total protein (50 μg) was separated by SDS-PAGE in 10 % polyacrylamide gels and transferred to nitrocellulose membranes. Then developed with specific primary (anti VEGF raised in Rabbit) and secondary antibodies (HRP conjugate anti rabbit raised in mouse). Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Millipore Immunobilon Western,USA). Quantification of the band was done by densitometer GS800.

6.2.7 Statistical Analysis

The data were expressed as mean values ± standard deviation. Statistical significance between the treated and untreated groups in all experiments was measured using student's t-test. $p < 0.05$ was considered statistically significant.

6.3 RESULTS

The CHO-K1 cells were exposed to varying concentrations of glucose, in the presence of insulin, and the beneficial effect of amino acids were studied at the level of cell survival and apoptotic signaling by looking at ROS and NO levels, as well as the expression of Akt and VEGF.

6.3.1 Amino acids decrease reactive oxygen species

One of the causative factors to trigger the VEGF is the oxidative stress. As glucose can induce ROS generation, reactive oxygen species was measured in terms of peroxynitrite species and the effect of amino acids on the levels of these was studied.

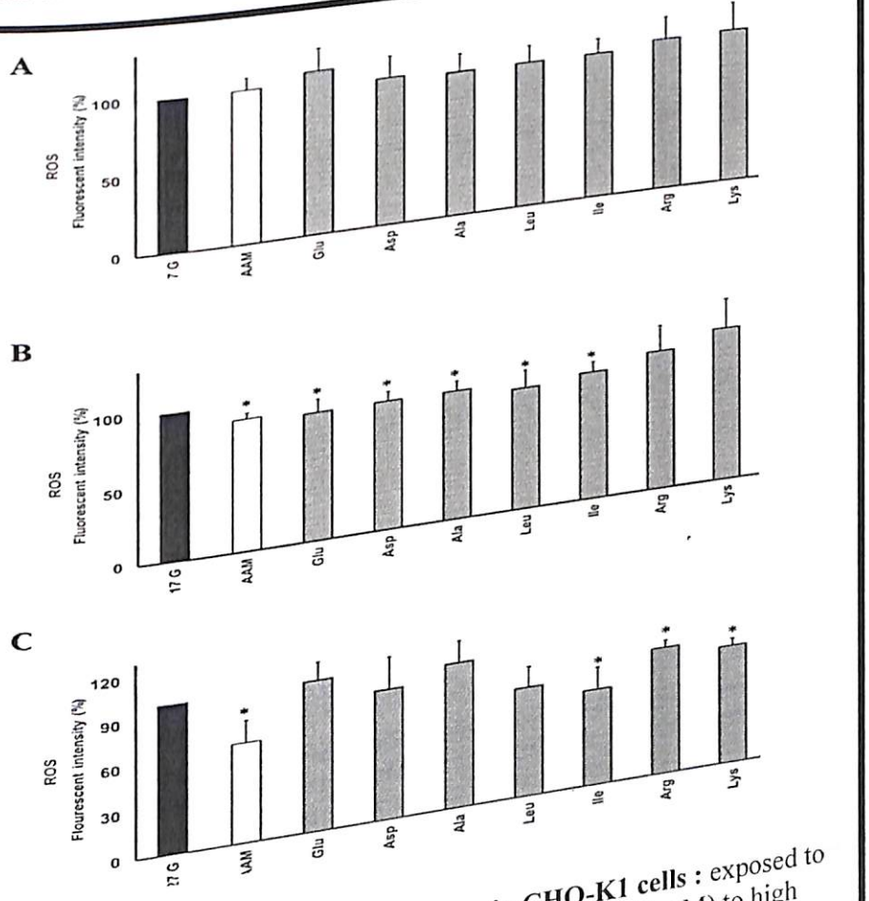


Fig 6.1: ROS analysis by DCF fluorescence in CHO-K1 cells : exposed to amino acids (mixture and free form) along with normal (7 mM) to high glucose (17 & 27 mM). p values were obtained after pair vise comparison between glucose (G) alone with glucose + amino acids mixture (AAM) *p<0.05 is considered significant.

No significant change was detected when AAM was added to normal glucose concentration. At high glucose concentration the ROS levels were decreased significantly *($p<0.05$)* (17 mM G- 15 % & 27 mM G – 35 % decrease). Glutamic acid, aspartic acid, alanine, leucine showed a similar significant decrease like AAM at 17 G while arginine and lysine showed decrease at 27 G *($p<0.05$)*. Isoleucine showed significant decrease at both 17 and 27 G *($p<0.05$)* **(Fig 6.1).**

6.3.2 Amino acids mixture decreases nitric oxide

During oxidative stress, the peroxynitrite is converted to nitric oxide. The NO level which is upstream of VEGF was estimated in the experimental conditions of normal and high glucose with and without AAM.

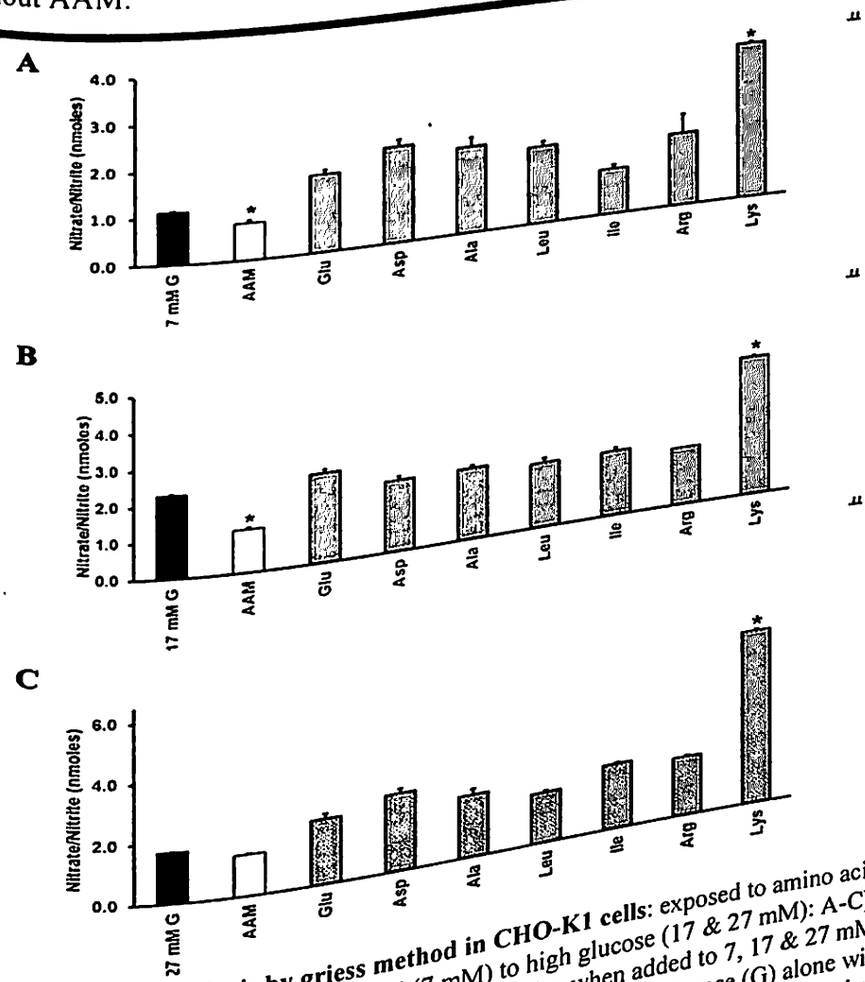


Fig 6.2: Nitric oxide analysis by griess method in CHO-K1 cells: exposed to amino acids (mixture and free form) along with normal (7 mM) to high glucose (17 & 27 mM): A-C) Decrease in NO seen at 5 mM AAM and increase with lys when added to 7, 17 & 27 mM glucose. p values were obtained after pair vise comparison between glucose (G) alone with glucose & amino acids mixture (AAM) where *p<0.05 are considered significant and *shows decrease & # shows increase.

High glucose alone showed an increase at 17 mM and decrease at 27 mM. Addition of AAM rather than free AA decreased the NO levels significantly (27 % with 7 mM G, 47 % with 17 mM G and 17 % with 27 mM G). This can be beneficial in terms of decreasing VEGF. Lysine as free AA showed significant increase in NO levels (2 fold) and therefore could be a good vasodilator While arginine is known to produce NO, lysine is not reported so far **(Fig 6.2)**.

6.3.3 Amino acids mixture decreases SOD which produces H_2O_2

Nitric oxide formed from peroxynitrite was converted to H_2O_2 by the enzyme SOD. The effect of AA on the activity of SOD was studied with varying concentrations of glucose. With increase in glucose, at 17 mM there was a increase in SOD corresponding to the increase in NO and ROS. Addition of AAM showed a fall in SOD activity when added to all glucose concentration significantly with respect to the corresponding glucose concentration (7 mM *p=0.01*, 17 mM G *p=0.006*, 27 mM G *p=0.009*) **(Fig 6. 4)**.

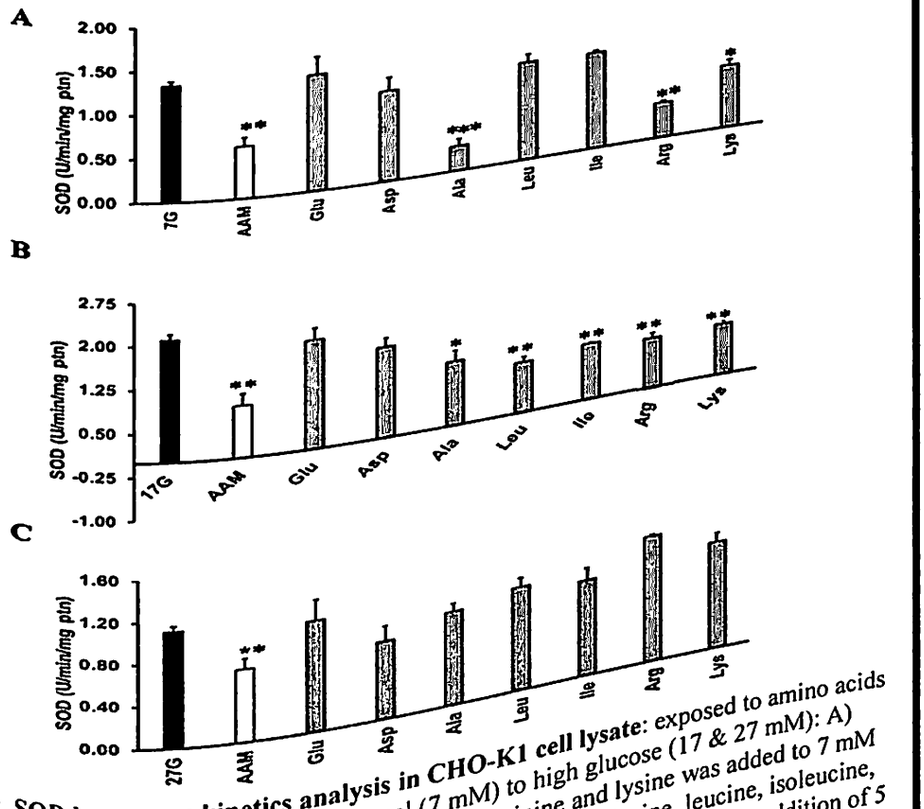


Fig 6.3: SOD by enzyme kinetics analysis in CHO-K1 cell lysate: exposed to amino acids (mixture and free form) along with normal (7 mM) to high glucose (17 & 27 mM): A) Decrease in SOD seen when 5 mM AAM, alanine, arginine and lysine was added to 7 mM glucose. B) Decrease in SOD seen at addition of 5 mM AAM, alanine, leucine, isoleucine, arginine and lysine when added to 17 mM glucose. C) Decrease in SOD seen at addition of 5 mM AAM when added to 27 mM glucose. p values were obtained after pair vise comparison between glucose (G) alone with glucose & AAM where *p<0.05, **p<0.01, ***p<0.001 are considered significant.

6.3.4 Amino acids mixture decreases the VEGF levels

The downstream signaling of Akt pathway was further looked at, by measuring the levels of VEGF, a cell survival signaling molecule. VEGF was studied in the presence of amino acids mixture and the free amino acids. Addition of AAM showed significant decrease in the levels of VEGF at all concentration of glucose (7 mM $p=0.004$, 17 mM $p=0.037$ & 27 mM $p=0.004$). Arginine and lysine showed varied effect with respect to varied glucose concentration with significant decrease at 7 & 17 mM G ($p<0.05$). However, glutamic acid and aspartic acid showed significant increase at 27 mM G. Alanine, leucine and isoleucine showed significant decrease at all glucose concentration with $p< 0.01$ at 7 & 17 mM G and $p<0.05$ at 27 mM G **(Fig 6.5)**.

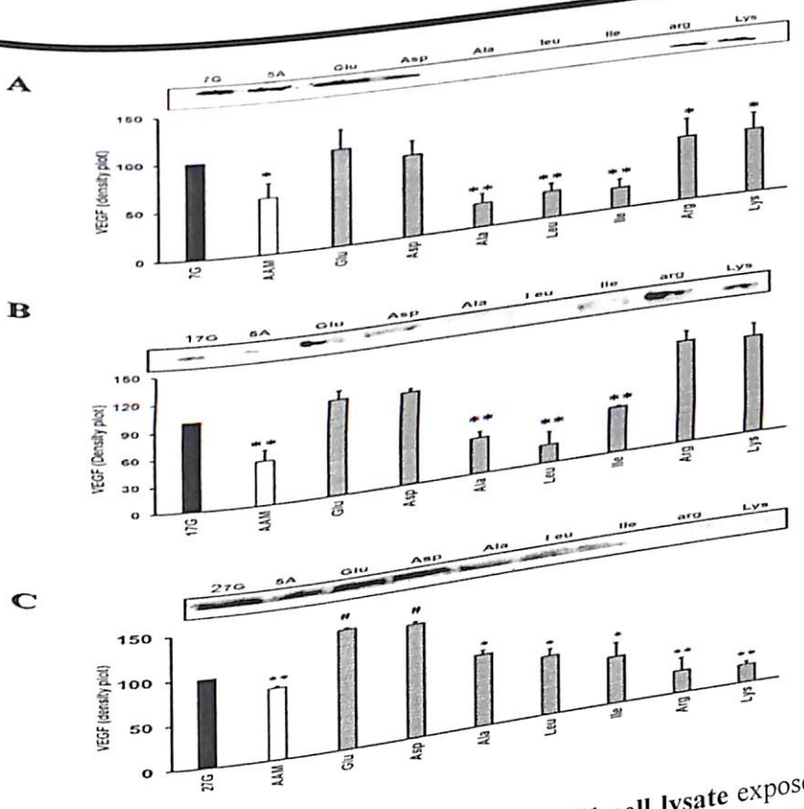


Fig 6.4: VEGF analysis by western blot in CHO-K1 cell lysate exposed to amino acids (mixture and free form) along with normal (7 mM) to high glucose (17 & 27 mM): A) Decrease in VEGF seen when 5 mM AAM, alanine, leucine, isoleucine, arginine and lysine was added to 7 mM glucose. B) Decrease in VEGF seen at addition of 5 mM AAM when added to 17 mM glucose. C) Decrease in VEGF seen at addition of 5 mM AAM, alanine, leucine, isoleucine, arginine and lysine when added to 27 mM glucose with increase in glutamic acid and aspartic acid. p values were obtained after pair vise comparison between glucose (G) alone with glucose & amino acids mixture (AAM) where *p<0.05, **p<0.01, ***p<0.001.

6.3.5 Effect of Amino acids mixture on cell proliferation/Apoptosis

The increase in Akt and VEGF increases the cell proliferation and thereby can be proangiogenic. Addition of AAM to normal glucose increased Akt which is a serine/threonine protein kinase that functions as a critical regulator of cell survival. AAM showed no significant change in proliferation as revealed by G0-G1 phase of cycle studied by FACS **(Fig 6.5A & 6.5B)**. AAM was found to show a tendency to anti-apoptotic activity as seen by FACS analysis of annexin V as seen at early and at late phase. The normal 7 mM G + AA compared to G alone showed a significance of ($P<0.01$, with 70 % decrease) and at high glucose 27 mM G + AA compared to G alone (50 % decrease) though not significant **(Fig 6.6A & B)**.

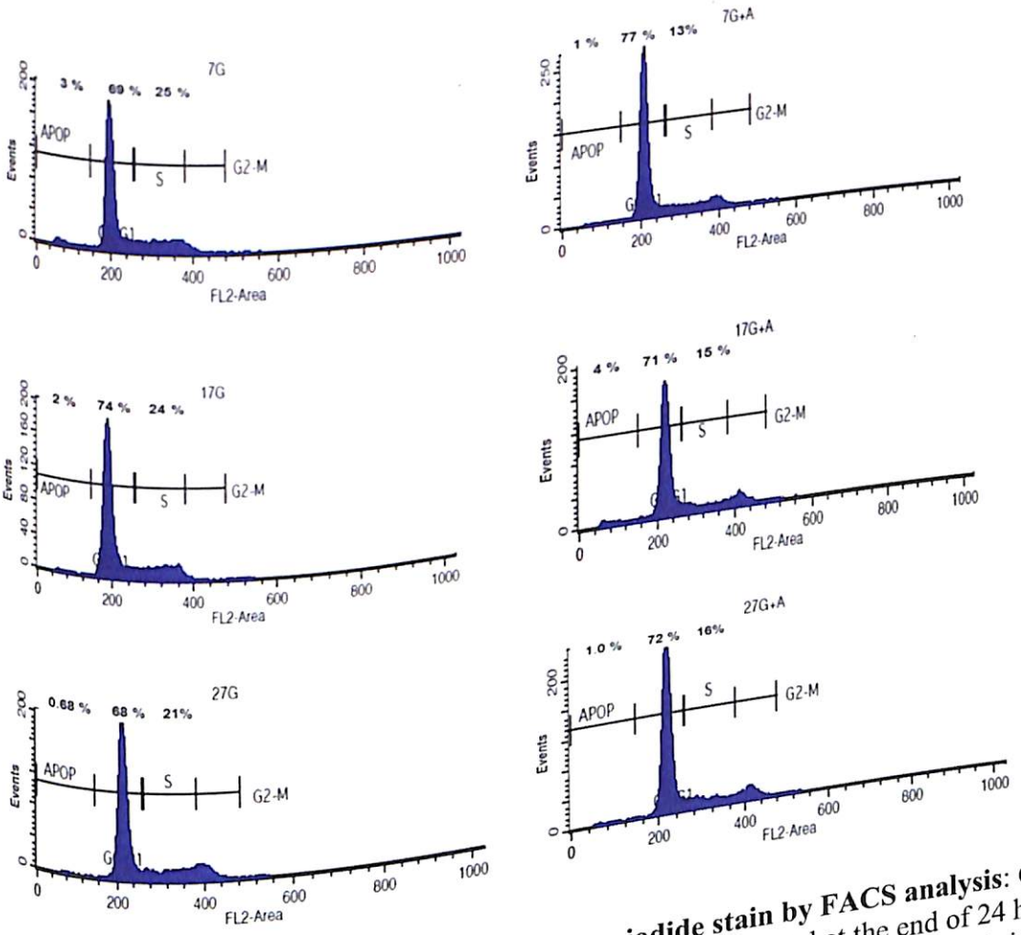


Fig 6.5A: Cell proliferation assay by propidium iodide stain by FACS analysis: CHO-K1 cells were exposed to the experimental condition and analysed at the end of 24 hours with PI for cell cycle analysis. Histogram of cell cycle showing the % of apoptosis in G0-G1 phase.

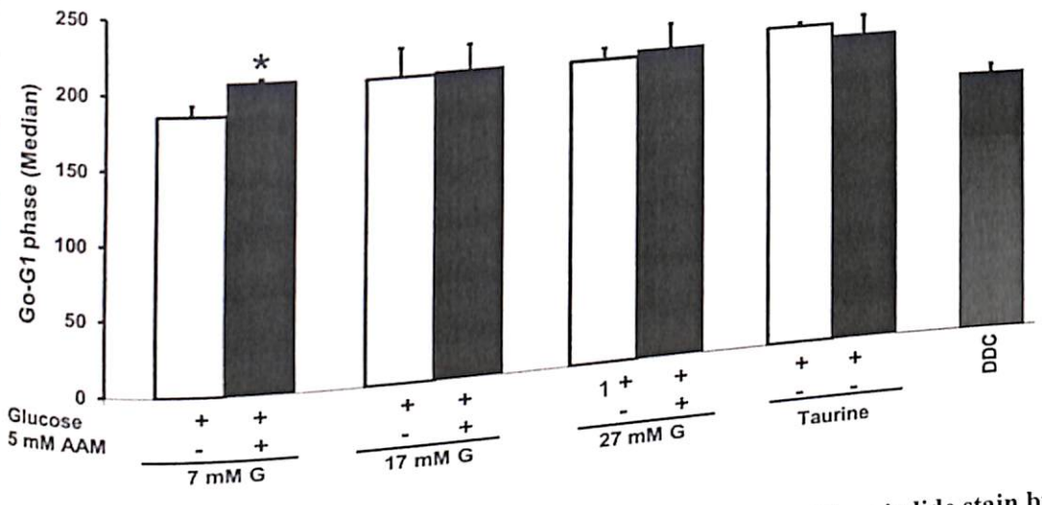


Fig 6.5B: Graphical representation cell cycle analysis of propidium iodide stain by FACS analysis in CHO-K1 cells. *Taurine is a positive control for cell proliferation and DDC for cell proliferation inhibition.*

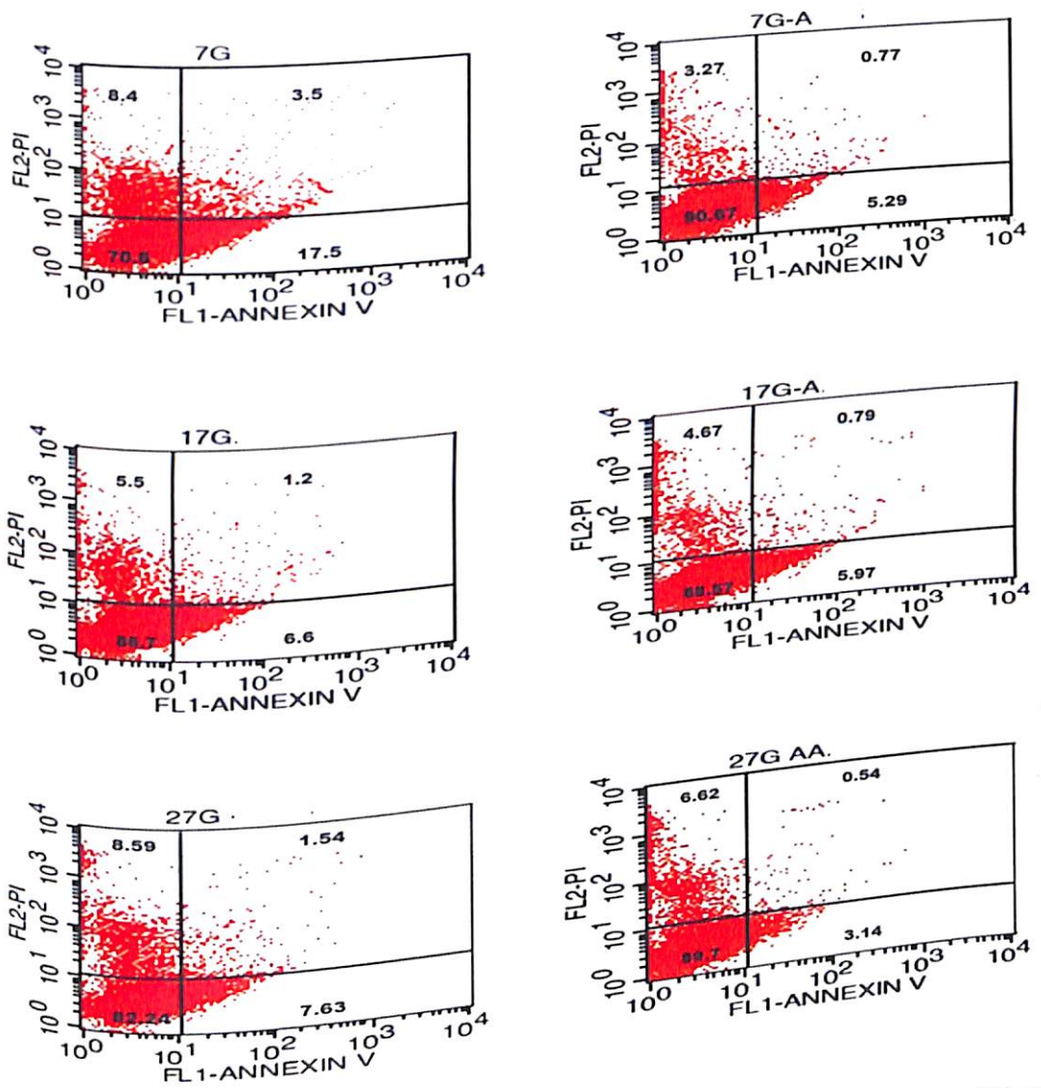


Fig 6.6A: Cell apoptosis study by annexin V stain by FACS analysis: CHO-K1 cells was grown and exposed to the experimental condition and at the end of 24 hours stained with Annexin V. Scatter plot of flow cytometry showing AAM addition decreasing the events of early apoptosis. The quadrent of FL1 & FL2 are lower left – Live, Lower right – Early apoptosis, Upper left – Necrotic, Upper right – Late apoptosis.

Thus, amino acids by decreasing the oxidative signaling pathway (ROS/NO/SOD) could be beneficial in decreasing the VEGF and there by anti-apoptotic.

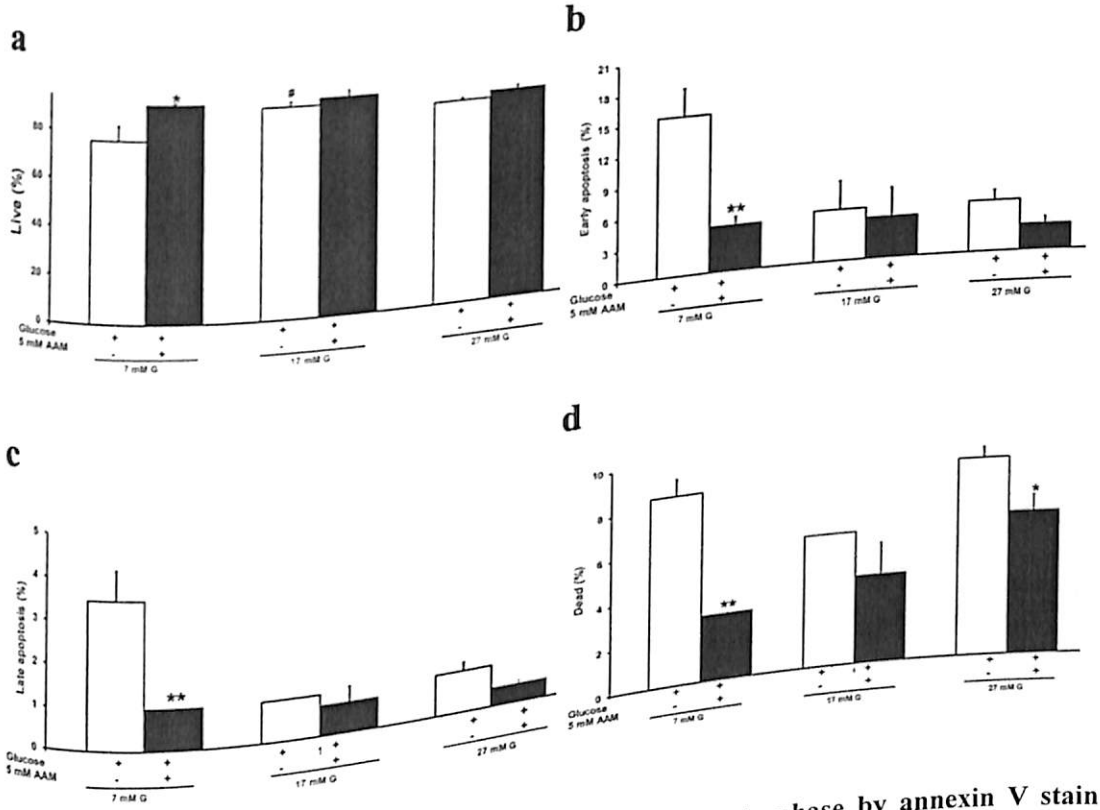


Fig 6.6B: Graphical representation of percentage of apoptotic phase by annexin V stain by FACS analysis in CHO-K1 cells. *p value is comparison between glucose alone with glucose + AAM and # among glucose.

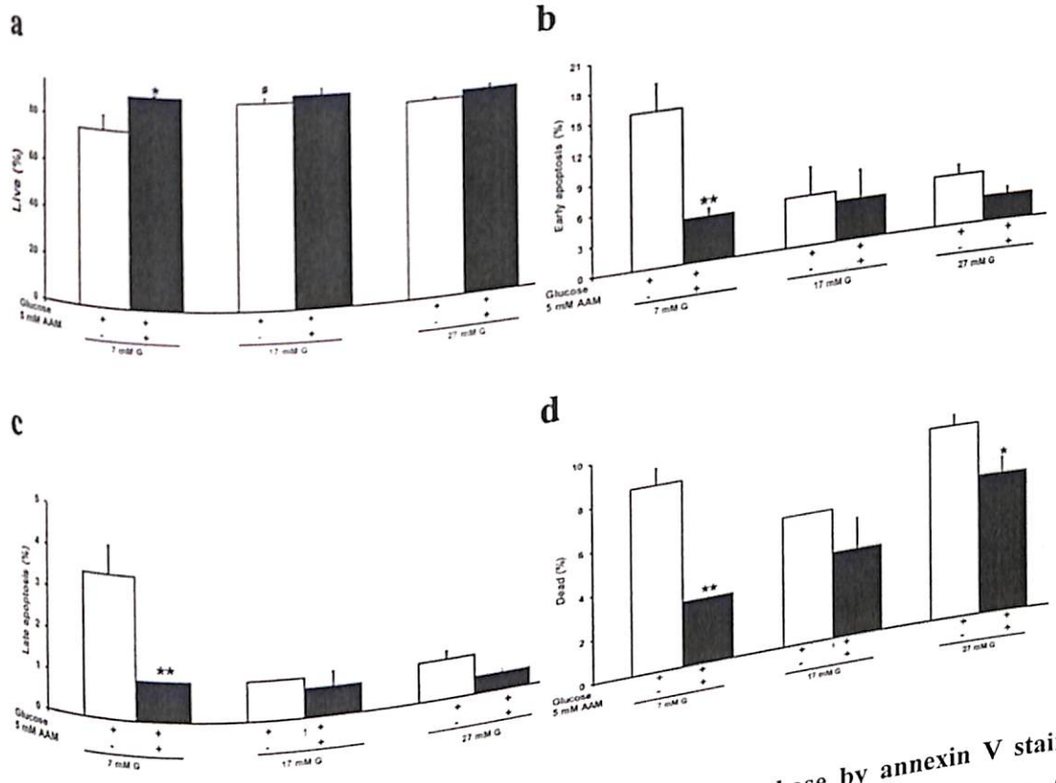


Fig 6.6B: Graphical representation of percentage of apoptotic phase by annexin V stain by FACS analysis in CHO-K1 cells. *p value is comparison between glucose alone with glucose + AAM and # among glucose.

6.4 CONCLUSION

Oxidative stress has long been associated with the complications of diabetes (nephropathy, retinopathy & other vascular complications) owing to chronic hyperglycemia. The study indicates that amino acids mixture can reduce the levels of ROS / NO /SOD which were increased under high glucose conditions **(Fig 9.1)**. A novel AAM was also found to decrease the VEGF, thus having implications on cell migration, proliferation and angiogenesis characteristic of diabetic retinopathy. However this has to be concluded based on studies in retinal endothelial cells. Thus the results show that AAM decrease the VEGF levels by controlling the oxidative stress via ROS/NO/SOD and therefore is possibly having **anti-angiogenic** property. Amongst the free amino acids studied, this is the first report showing lysine to have a NO promoting activity which can have vasodilating effect. The vasodilating property if any for lysine needs to be established.

CHAPTER 7: EFFECT OF AMINO ACIDS ON GLUCOSE UPTAKE IN HUMAN ADIPOCYTES UNDER HIGH GLUCOSE CONDITION

7.1 INTRODUCTION

The pathophysiology of type 2 diabetes is characterized by defects in insulin action and insulin secretion. Metabolic actions of insulin are mediated by the insulin receptor/IRS/PI 3-kinase signaling pathway in insulin's target organs including the liver, skeletal muscle and adipose tissue. Dysregulation of fatty acid metabolism, abnormalities of the function and the secretion of adipokines, as well as the increase in stress signaling might contribute to the development of insulin resistance (Ogawa and Kasuga 2006). With the onset of insulin resistance and diabetes there is insulin signaling defects, especially in adipocytes (Reaven 1995). Insulin signaling pathways involving the activation of PI3-kinase are responsible for the metabolic regulation of carbohydrate, lipid, and protein utilization (Cheatham and Kahn 1995). The activation of PI3 kinase leads to translocation of GLUT4-containing vesicles and subsequently increased glucose uptake in skeletal muscle and adipocytes (Brozinick, Hawkins et al. 2004; Martin, Lee et al. 2006). Reduced GLUT4 protein expression is a common characteristic of adipose tissue from animal models of diabetes and insulin resistance (Musi and Goodyear 2006).

7.1.1 Adipose tissue/ Adipocytes

Adipose tissue is a highly active metabolic and endocrine organ (Havel 2002). In addition to being a fat storage depot, adipose tissue has been shown to synthesize and secrete several biologically active molecules such as adiponectin that influence glucose metabolism. a hormone that regulates energy homeostasis, glucose and lipid metabolism. Adiponectin reduces tissue triglycerides (TG) content and up-regulates insulin signaling because increased tissue TG interferes with insulin-stimulated phosphatidylinositol (PI) 3-kinase activation and thereby glucose transporter 4 translocation (Kadowaki, Yamauchi et al. 2006; Blumer, van Roomen et al.

2008). Study on electron microscopy showed adipocytes have increased small membrane invaginations associated with lipogenesis. (Fan, carpentier et al. 1983) Microscopically oil droplets are visible which can be stained by Oil red O.

7.1.2 Adiponectin in glucose uptake

Adiponectin (also known as 30-kDa adipocyte complement-related protein; Acrp30, apM1, AdipoQ and Gbp28) (Scherer, Williams et al. 1995) is a unique adipocyte-derived hormone with anti-diabetic, anti-inflammatory, and anti-atherogenic properties (Goldstein and Scalia 2004). Adipose tissue has been increasingly recognized as an important endocrine organ that secretes a number of biologically active "adipokines" (Kadowaki, Yamauchi et al. 2006) such as free fatty acid, adipsin, leptin, plasminogen activator inhibitor-1, resistin, and TNF-α (Lazar 2006). Some of these adipokines have been shown to directly or indirectly affect insulin sensitivity by influencing the glucose and lipid metabolism (Kershaw and Flier 2004). Adiponectin secreted by adipocytes regulates energy homeostasis and glucose and lipid metabolism.

7.1.3 Mechanisms for adiponectin's metabolic effects

Several mechanisms for adiponectin's metabolic effects have been described. In the liver, adiponectin enhances insulin sensitivity, decreases influx of non-essential fatty acids, increases fatty acid oxidation, and reduces hepatic glucose output. In muscle, adiponectin stimulates glucose use and fatty acid oxidation(Hotta, Funahashi et al. 2001; Yamauchi, Kamon et al. 2003). Phosphorylation and activation of the 5'-AMP-activated protein kinase (AMPK) are stimulated with both globular and full-length adiponectin in skeletal muscle, in liver. Only with full-length adiponectin it increases glucose uptake (Kadowaki, Yamauchi et al. 2006). It switches off the ATP-consuming pathways and switching on ATP-generating pathways resulting in increased glucose uptake and fatty acid oxidation in muscle. (Hardie 2004). In parallel with its activation of AMPK, adiponectin stimulates phosphorylation of acetyl coenzyme A carboxylase (ACC), fatty-acid oxidation, glucose uptake and lactate production in myocytes and reduction of molecules involved in gluconeogenesis in the liver, and reduction of glucose levels in vivo (Yamauchi, Kamon et al. 2003) **(Fig 7.1).**

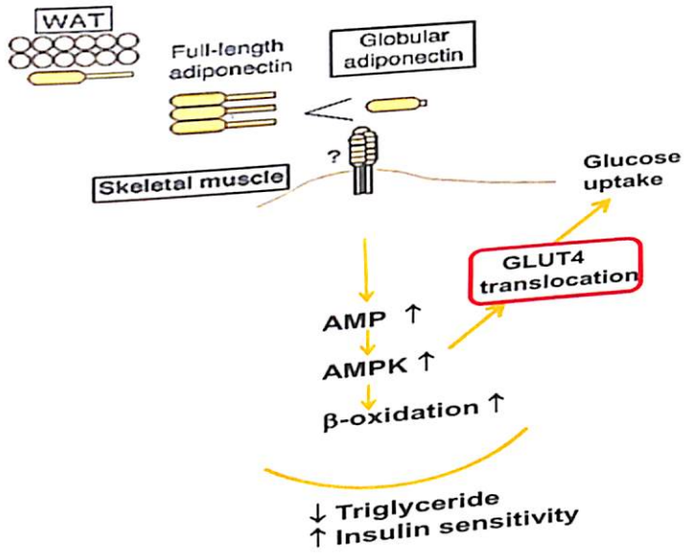


Fig 7.1: Schematic representation of AMPkinase pathway translocating the GLUT4 in adipose tissue.

Amino acids in the present study was observed to be beneficial in improving the insulin signaling cascade and there by glucose uptake as studied in CHO-K1 cells. The present study is on the adipocytes which is of human origin. Adipocytes are one of the metabolically active cells which utilizes glucose for the metabolism. They are the first cell to become insulin resistant in diabetes (Arner,2003). Increased triglycerides formed undergoes increased β-oxidation, indicating that the cell is metabolically active and has high insulin sensitivity. This study is aimed to look at the effect of amino acids on the glucose uptake and GLUT4 translocation in human adipocyte cell line.

7.2 MATERIALS AND METHODS

7.2.1 Cell culture and treatment

Human visceral Preadipocytes (LONZA) of passage 1 were retrieved and passage 2 culture were grown in 24wells for glucose uptake, 6 wells for GLUT4 RNA extraction. Cells were grown in 24 well plates on cover slips for GLUT4 immuno fluorescence. Amino acids of 5 mM of free alanine, arginine, aspartic acid, glutamic acid, leucine, isoleucine & lysine and mixture were used for the study to evaluate glucose uptake, GLUT4 translocation and mRNA expression in the

presence of high glucose condition. With respect to adipocytes glucose concentration of 5 mM is taken as normal and as the preadipocytes and adipocytes are maintained at 27 mM glucose, 33 mM is considered as high glucose.

7.2.2 2-Deoxy Glucose uptake

Serum starved cells for 2 hours were preincubated with 100 nM insulin added followed by exposure to experimental conditions such as glucose alone (normal -5 mM and high glucose – 33 mM with and without free amino acid/mixture. To all the conditions 100 nM insulin, hot and cold deoxyglucose was added and incubated for 30 mins. Washed with ice cold 1X PBS, cells lysed with 1 % SDS in 0.2 N NaOH and measured radio labeled glucose levels in the cell by LSS. Proteins were estimated by Bradford method.

7.2.3 Immunofluorescence stain for GLUT4 translocation

Adipocytes were grown on cover slips, serum starved, preincubated with insulin and exposed to various conditions for 30 min. Followed by fixation, permeabilisation and primary antibody (Human Anti Glut raised in rabbit) 1:100 for overnight. Washed thrice with PBS and secondary antibody-anti rabbit-FITC 1:200 was added and incubated for 2 H. Washed thrice with PBS and mounted with Vectashield (mountant with counter stain DAPI and anti-fade). The cells were visualized under confocal microscope.

7.2.4 GLUT4 expression by RT-PCR

After serum starvation, exposure of cells to various conditions, RNA was extracted by TRIZOL method. RNA was converted to cDNA, quantified by nano drop and PCR was done for GLUT4 mRNA expression. Gel was run for the PCR products and visualized under transilluminator.

7.2.5 Oil Red O staining of preadipocyte and adipocyte cells

Oil red O staining was performed on day 8. Briefly, cells were washed twice with PBS and fixed with 7.5% formaldehyde for 15 minutes. After washing twice with PBS, cells were stained for at least 1 hour in freshly diluted oil red O solution at 37°C. Cells were photographed using a bright field microscope with digital camera at 40 X &100 X magnification.

7.2.6 Statistical Analysis

The data were expressed as mean values ± standard deviation. Statistical significance between the treated and untreated groups in all experiments was measured using student's t-test. $p < 0.05$ was considered statistically significant.

7.3 RESULTS

In order to see the beneficial role of amino acids in glucose uptake and GLUT4 translocation, human cell line adipocytes were utilized for the study.

7.3.1 Differentiation of preadipocytes to adipocytes

The preadipocytes were converted to adipocytes for the first time in the lab. The preadipocytes were fibroblastic cells which were converted to adipocyte by the addition of IBMX and Dexamethasone. Where IBMX decreases cell proliferation and increases differentiation whereas dexamethasone is a gluco corticoid anti-inflammatory agent.

Human preadipocyte cells are fibroblastic-like precursor cells isolated from adipose tissue. After 8 days of culture in post adipocyte differentiation medium, the cytoplasm is filled with numerous lipid droplets of various size as shown in phase contrast microscopy and the oil red O stain are taken by the fat droplets in adipocyte cells **(Fig 7.2).**

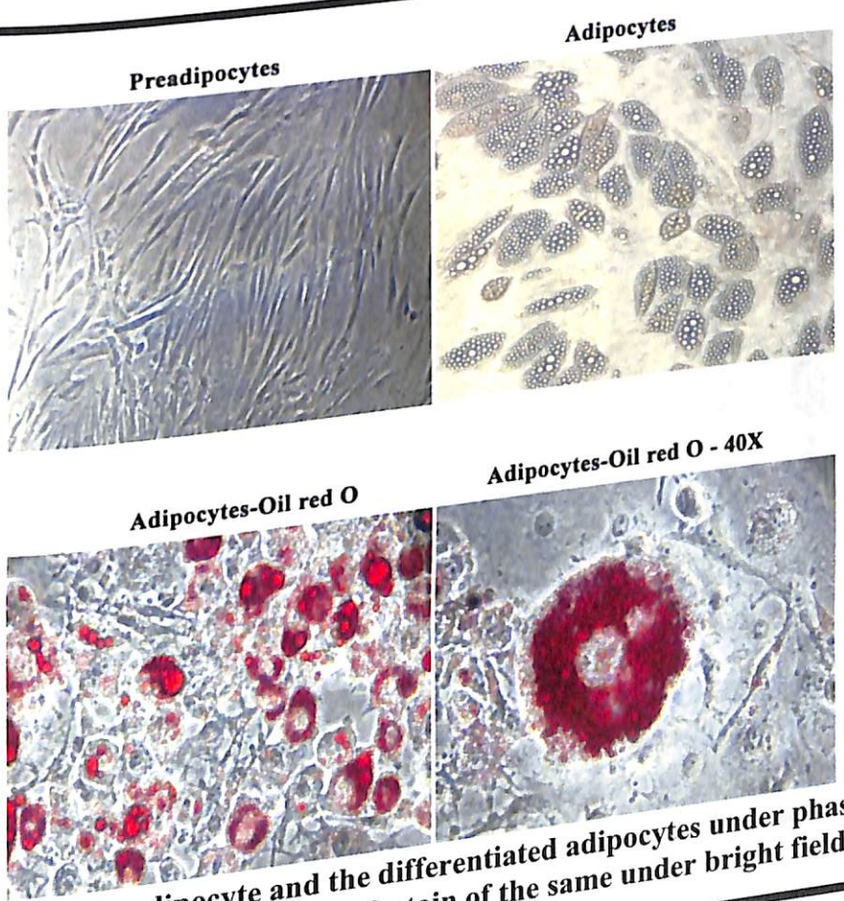


Fig 7.2: Human preadipocyte and the differentiated adipocytes under phase contrast microscope and the oil red O stain of the same under bright field.

7.3.2 Glucose uptake influenced by amino acids

Glucose uptake and the effect of amino acids on the same was studied in human adipocytes cell line by 2-deoxy glucose uptake. Glucose uptake was increased with the addition of AA and free amino acids namely glutamic acid, isoleucine and lysine of which lysine showed statistical significance *(p=0.05)* when compared to 33 mM glucose alone. Leucine and arginine did not promote glucose uptake and was found to decrease with 2-deoxy uptake as seen in the adipocytes **(Fig 7.3)**.

7.3.3 GLUT4 translocation influenced by amino acids

Glucose transporter namely GLUT4 is the protein which gets translocated to the plasma membrane to transport the glucose. Addition of free amino acids and mixture to 5 mM normal glucose showed increased translocation of GLUT4 as seen by immunofluorescence, with respect to alanine, glutamic acid, leucine, isoleucine, lysine and not mixture of AA as well as free amino acids arginine and aspartic acid at normal glucose **(Fig 7.4)**.

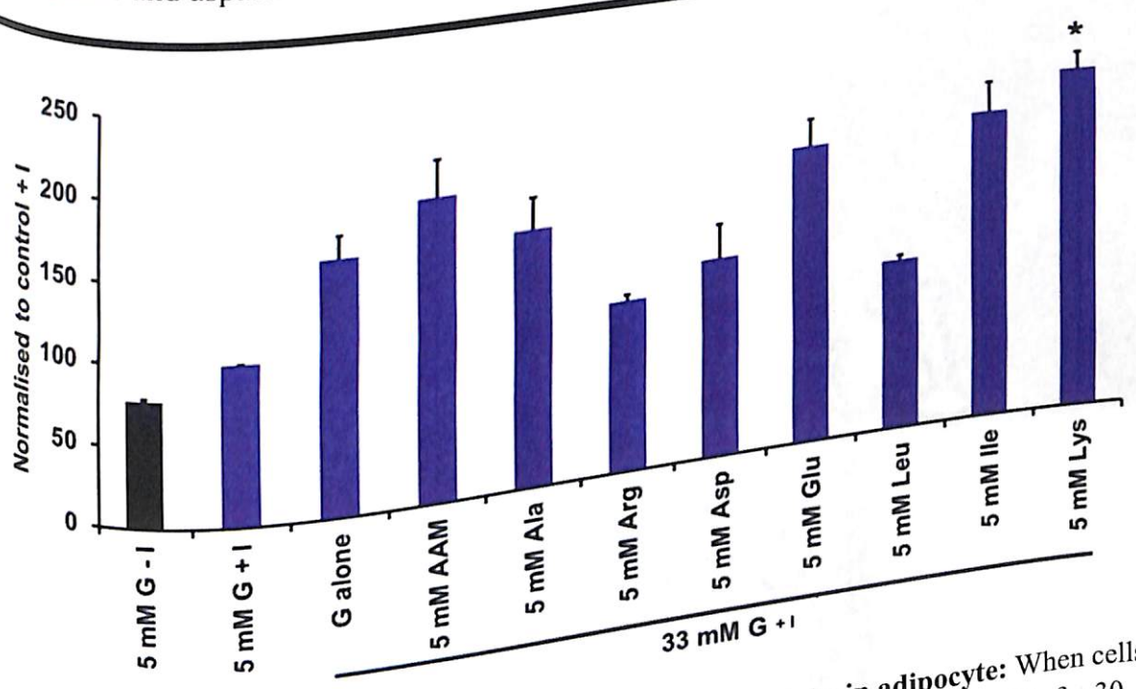


Fig 7.3: Bar diagram showing the labeled 2-deoxy glucose uptake in adipocyte: When cells were exposed to normal and high glucose along with free and mixture of amino acids for 30 min. p value is comparison between 33 mM glucose with 33 mM G + 5 mM AAM.

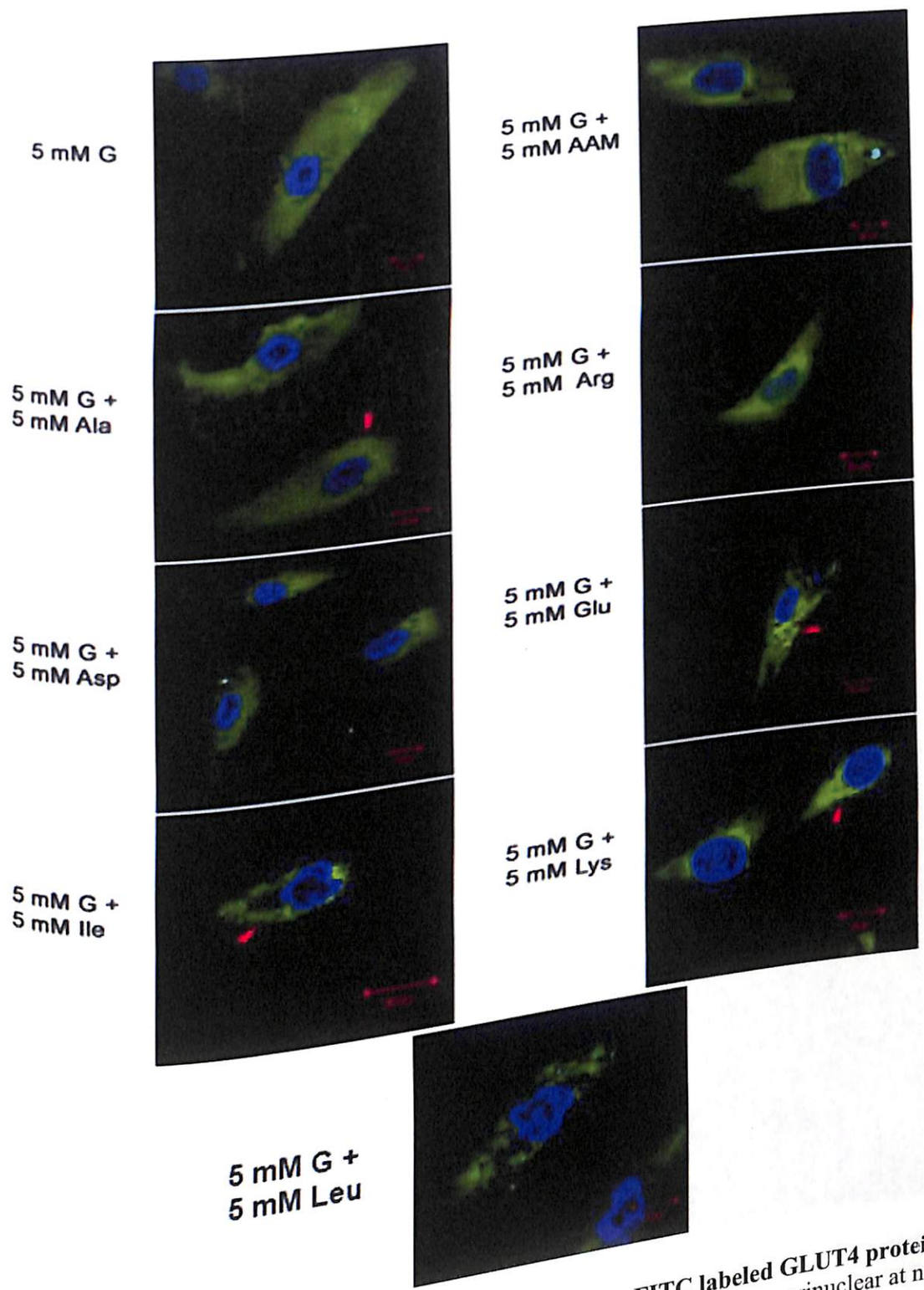


Fig 7.4: Confocal imaging of the GLUT4 translocation for FITC labeled GLUT4 protein in adipocyte when exposed to 5 mM G: Green fluorescence of GLUT4 is seen perinuclear at normal glucose and towards the membrane shows the translocation of GLUT4 as seen when exposed to AAM and individual amino acids namely glutamic acid, isoleucine, lysine & leucine.

At 33 mM high glucose, the free amino acids namely alanine, aspartic acid, isoleucine, lysine as well as mixture form was observed to promote the translocation of GLUT4 as seen by the fluorescence stain for GLUT4 protein. Arginine and leucine did not show any change in immunofluorescence at high glucose **(Fig 7. 5)**.

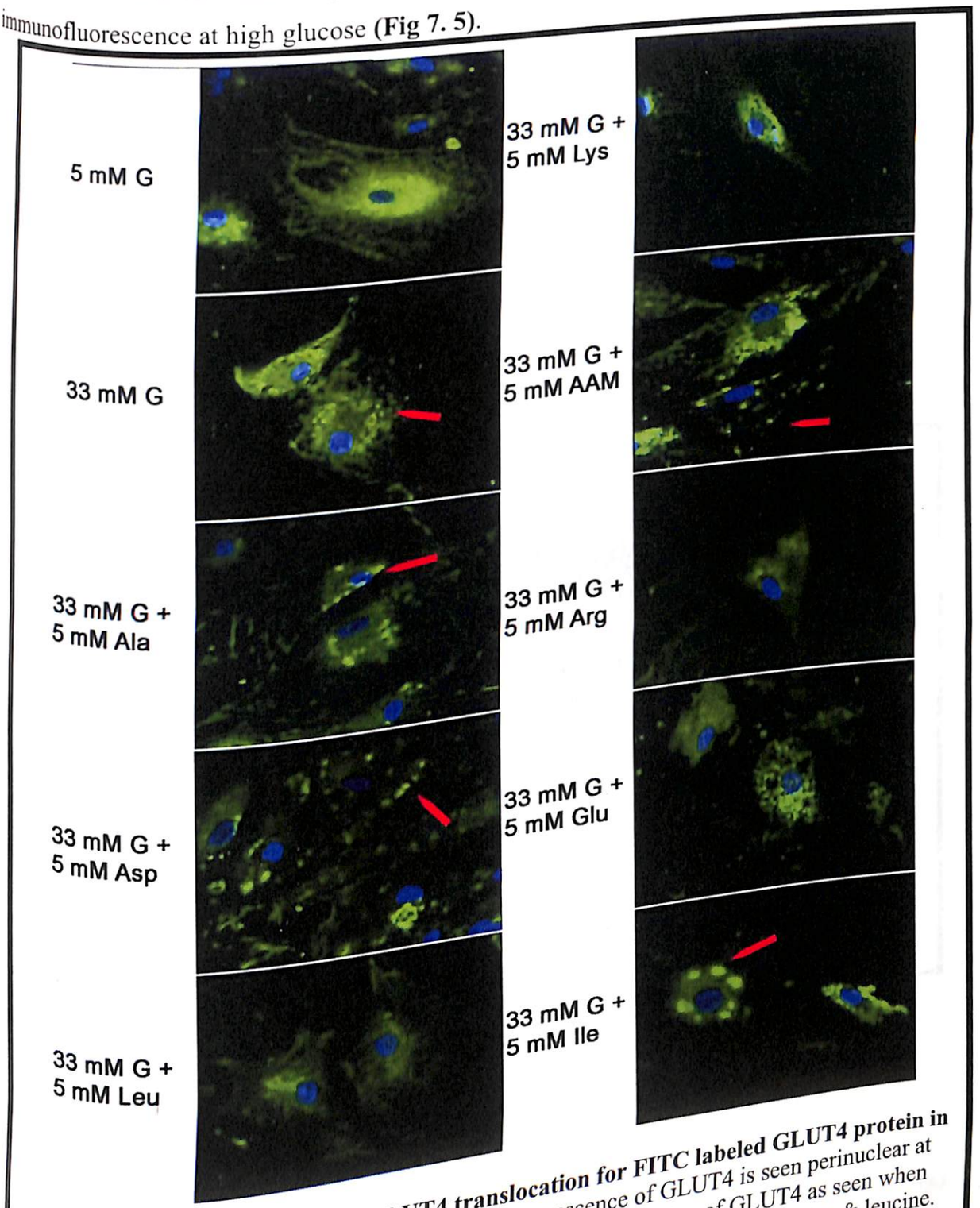


Fig 7.5: Confocal imaging of the GLUT4 translocation for FITC labeled GLUT4 protein in adipocyte when exposed to 33 mM G: Green fluorescence of GLUT4 is seen perinuclear at normal glucose and towards the membrane shows the translocation of GLUT4 as seen when exposed AAM and individual amino acids namely glutamic acid, isoleucine, lysine & leucine.

In this study, the effect of amino acids as studied by glucose uptake and GLUT4 translocation showed AAM had an effect and free amino acids namely aspartic acid, glutamic acid, isoleucine, lysine showed increased glucose uptake and GLUT4 translocation.

7.3.4 GLUT4 mRNA expression in the presence of amino acids

During the insulin signaling cascade the GLUT4 transporter vesicle in only translocated to the membrane in order to facilitate glucose entry. But if there is enhancement of the expression of GLUT4 it is beneficial in the context of insulin resistance. Therefore the effect of amino acids on the mRNA expression was studied. At normal glucose amino acids namely alanine, aspartic acid and leucine showed increased expression while at high glucose concentration, alanine alone showed increase in GLUT4 expression. AAM did not influence the GLUT4 at mRNA level (**Fig 7.6**). The graphical representation of densitometer analysis of GLUT4 mRNA expression is shown in **Fig 7.7.**

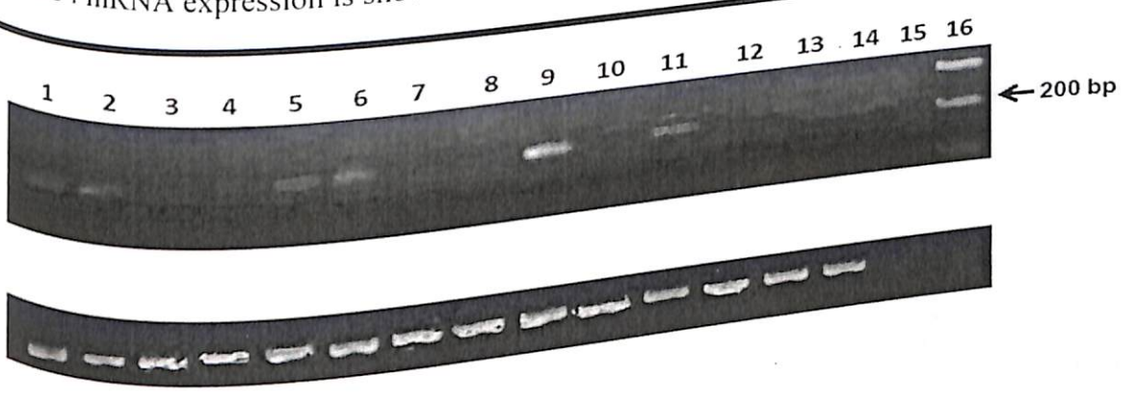


Fig 7.6: RT PCR for expression of GLUT4 in adipocyte

1 - **7 mM G**
2- **27 mM G**
3- 7 mM G + 5 mM AAM
4- 27 mM G + 5 mM AAM
5- **7 mM G + 5 mM Ala**
6- **27 mM G + 5 mM Ala**
7- 7 mM G + 5 mM Arg
8- 27 mM G + 5 mM Arg
9- **7 mM G + 5 mM Asp**
10- 27 mM G + 5 mM Asp
11- **7 mM G + 5 mM Leu**
12- 27 mM G + 5 mM Leu
13- 7 mM G + 5 mM Ile
14- 27 mM G + 5 mM Ile
15 - NC

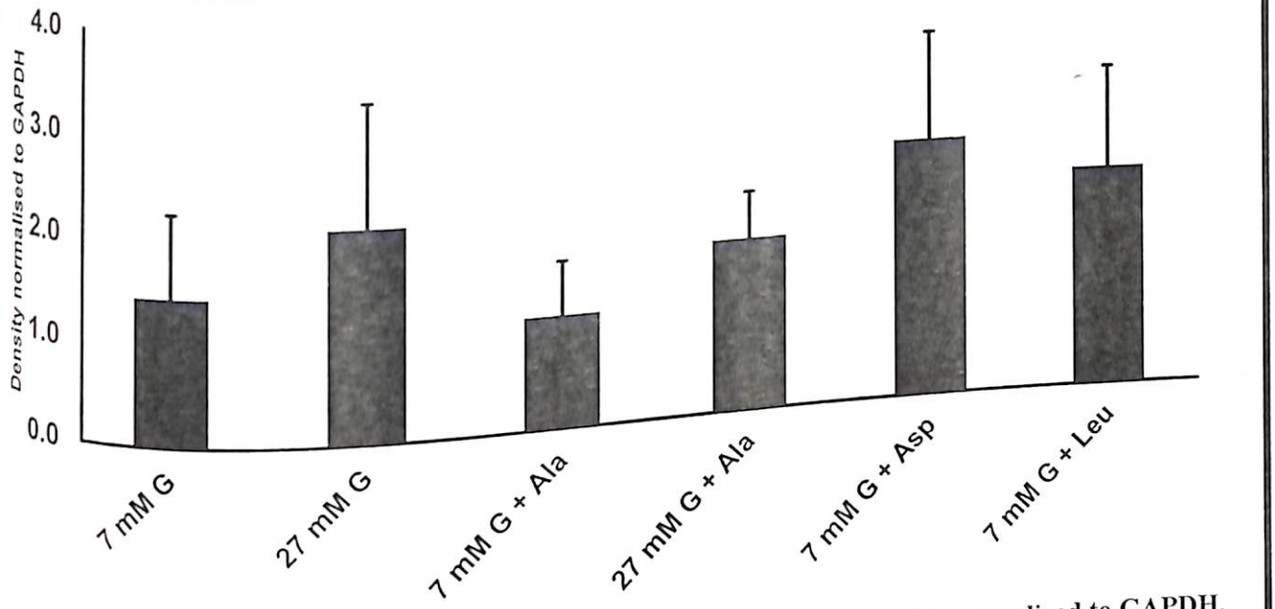


Fig 7.7: Graphical representation of densitometer analysis of GLUT4 mRNA normalized to GAPDH.

In this study the beneficial effect of amino acids in enhancing the glucose transport and GLUT4 translocation was confirmed in adipocytes.

7.4 CONCLUSION

The increased glucose uptake promoted by AAM seen in CHO-K1 cells was also evaluated in human Adipocytes cell line which when exposed to high glucose, in the presence of AAM, as well as the free amino acid lysine showed a significant increase in glucose uptake as seen by 2-deoxyglucose uptake. AAM as well as the free amino acids alanine, glutamic acid, aspartic acid, isoleucine & lysine increased the GLUT4 translocation towards the membrane at high glucose as seen by immunofluorescence. Alanine alone showed increased GLUT4 mRNA expression at high glucose concentration.

CHAPTER 8: EXPERIMENTAL STUDY ON THE EFFECT OF AMINO ACIDS MIXTURE ON RETINAL CHANGES IN THE DIABETIC RATS

8.1 INTRODUCTION

8.1.1 Diabetes and diabetic complications

Diabetes is a chronic illness that requires continuing medical care and patient self-management education to prevent acute complications and to reduce the risk of long-term complications. Diabetic retinopathy is a highly specific vascular complication of both type 1 and type 2 diabetes, with prevalence strongly related to duration of diabetes. Diabetic retinopathy is the most frequent cause of new cases of blindness among adults aged 20–74 years (American diabetes association, 2010). Intensive diabetes management with the goal of achieving near normoglycemia has been shown in large prospective randomized studies to prevent and/or delay the onset and progression of diabetic retinopathy (1998)(UKPDS study).

Proliferative diabetic retinopathy is characterized by neovascularization originating from the retina and/or optic disk in patients with diabetes mellitus. Non-enzymatic glycation has been recognized as a major biochemical cause for diabetic complications such as progression of cataract and development of proliferating diabetic retinopathy (Sulochana, Indra et al. 2001).

8.1.2 Advanced glycation end product

AGEs are a heterogeneous group of molecules formed from the nonenzymatic reaction of reducing sugars with free amino groups of proteins, lipids, and nucleic acids. The initial product of this reaction is called a Schiff base, which spontaneously rearranges itself into an Amadori product, as is the case of the well-known hemoglobin A_{1c}. A series of subsequent reactions, including successions of dehydrations, oxidation-reduction reactions, and other arrangements

lead to the formation of AGEs. Several compounds, e.g., $^{\varepsilon}N$-carboxymethyl-lysine, pentosidine, or methylglyoxal derivatives. serve as examples of well-characterized and widely studied AGEs (Peppa, Uribarri et al. 2003).

A key characteristic of certain reactive or precursor AGEs is their ability for covalent crosslink formation between proteins, which alters their structure and function, as in cellular matrix, basement membranes, and vessel-wall components [Shimizu, 2011]. Other major features of AGEs relate to their interaction with a variety of cell-surface AGE-binding receptors, leading either to their endocytosis and degradation or to cellular activation and pro-oxidant, pro-inflammatory events (Vlassara 2001; Vlassara and Palace 2002). AGEs are found in retinal vessels of diabetic patients, and their levels correlate with those in serum as well as with severity of retinopathy (Koga, Yamagishi et al. 2002). Aminoguanidine, an inhibitor of AGE formation (Mene, Festuccia et al. 2003), is shown to prevent retinopathy in diabetic animals. Also, it is known that AGEs accumulate in peripheral nerves of diabetic patients and that the use of anti-AGE agents improves nerve conduction velocities (Dewhurst, Omawari et al. 1997) and neuronal blood flow abnormalities (Kihara, Schmelzer et al. 1991).

Increased AGE accumulation in the diabetic vascular tissues has been associated with changes in endothelial cell, macrophage, and smooth muscle cell function. In addition, AGEs can modify LDL cholesterol in such a way that it tends to become easily oxidized and deposited within vessel walls, causing streak formation and, in time, atheroma. AGE-crosslink formation results in arterial stiffening with loss of elasticity of large vessels. This arterial stiffness has recently been shown to be reversed by the administration of another anti- AGE class of compounds called AGE breakers (Peppa, Uribarri et al. 2003).

8.1.3 Molecular markers in diabetes

Chronic inflammation is characterized by increased vascular permeability, edema, inflammatory cell infiltration, cytokine and chemokine expression, tissue destruction, neovascularization, and attempts at repair, and diabetic retinopathy exhibits most of these features. In diabetes, prosurvival (neurotrophic) inputs may be reduced and proinflammatory cytokines, chemokines, and cellular responses increased (Boyle 2005). Together, these processes accelerate retinal cell death and increase vascular permeability and occlusion, thus impairing vision.

Interaction of AGEs with RAGE on macrophages causes oxidative stress and activation of nuclear factor-kB (NF-kB) via activation of the p21ras and the mitogen-activated protein (MAP) kinase signaling pathway (Yan, Schmidt et al. 1994). NF-kB modulates gene transcription for endothelin-1, tissue factor and thrombomodulin and generation of pro-inflammatory cytokines such as interleukin-1a (IL-1a), interleukin- 6 (IL-6) and tumour necrosis factor- a (TNF-α) (Wang, Vom Hagen et al. 2008). There is also enhanced expression of adhesion molecules including vascular cell adhesion molecule- 1 (VCAM-1) and intercellular adhesion molecule- 1 (ICAM-1), in addition to other effects such as increased vascular permeability.

8.1.4 Anti-glycating property of amino acids

AGEs are one of the main causative factor for retinal changes in the diabetic condition. In previous studies amino acids were found to mitigate the AGE formation in vitro. Amino acids could be one such breakers in significantly removing the adduct formation. To avert or delay cataract formation or DR, the basic reaction of glycation of proteins has to be arrested. In our previous study free lysine, Alanine, Aspartic acid, or Glutamic acid reduce the glycation of human lens proteins (Ramakrishnan and Sulochana 1993; Ramakrishnan, Sulochana et al. 1996; Ramakrishnan, Sulochana et al. 1997). The beneficial effects of amino acids in cataract should be due to its competitive reaction with glucose and scavenging it from inside the cells particularly in diabetes. i.e. anti-glycation. Once glucose is removed, the chances of early onset of cataract as well as DR could be delayed or avoided.

Animal and human studies have shown that AGEs has been associated with changes in endothelial cell, macrophage, and smooth muscle cell function as seen in diabetic retinopathy. To avert or delay cataract formation or DR, the basic reaction of glycation of proteins has to be arrested. With good control of glucose, the onset of cataract as well as DR could be delayed or avoided. Amino acids could be one such breakers in the chain of events associated with glycation that can leads to diabetic complications such as DR or cataract. In the present study the effect of amino acid mixture on preventing / ameliorating diabetic retina changes in the retina of male Sprague dawley (SD) rats.

8.2 MATERIALS AND METHODS

8.2.1 Animals

Male SD rats of same breed were used in these experiments. All protocols abided by the Association for Research in Vision and Ophthalmology (ARVO) statement on the Use of Animals in Ophthalmology and Vision Research and were approved by the Animal Care and Use Committee of the Madras veterinary college, Chennai. The animals were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12-hour light-dark cycle. Animals were marked with picric acid indicating as body, head and tail. Husks were changed on daily basis **(Fig 8.1)**. All the animals were euthanized using carbon-di-oxide.

8.2.2 Induction of Diabetes

After 12 hours of fasting, the animals received a single 55 mg/kg intraperitoneal injection of streptozotocin (Sigma, St.Louis, MO) in 10 mmol/L of sodium citrate buffer, pH 4.5. Control non-diabetic animals were fasted and received citrate buffer alone. After 72 hours later, animals with blood glucose levels were checked and levels > 250 mg/dl were considered diabetic. The diabetic state was confirmed a second time before analysis. Insulin dose of 2 U twice a week is given (Gung, 2006).

8.2.3 Experimental animal groups

1. Group I (A): From 2 weeks after induced diabetes supplemented with amino acids SNAAM1 (n= 10).
2. Group II (B): From 2 weeks after induced diabetes supplemented with amino acids SNAAM2 (n= 10).
3. Group III (C): From 2 weeks, after induced diabetes supplemented with known antiglycating agent –Aminoguanidine-HCL, 50 mg/Kg body weight (D-AG) n= 10).
4. Group IV (D): Streptozotocin (STZ) induced diabetic group (n= 10).
5. Group V (E): Healthy Male sprague dawley non-diabetic rats- (Control group) (n= 10).

8.2.4 Amino acid composition

SNAAM1 & SNAAM2 – Composition not revealed, to be filed for patent.

SNAAM1 & SNAAM2 were given orally to the rats on daily by using feeding needle.

8.2.5 Sample processing

The animals grouped were maintained for 8 weeks and then sacrificed. The blood samples were collected by cardiopuncture using 21 guaze needle and the eyes were removed for the collection of retina. The kidney was also collected.

Half the kidney was fixed in formalin, the other half frozen. The retinas were collected and one eye was put in formalin, the other frozen. The lens were collected and stored at -80°C. The serum & lens were processed for total AGE estimation. The sectioned retinas were utilized for immunohistochemistry staining for CML-AGE, cytokines (IL-1, IL-6, IL-10) and cell adhesion molecule ICAM.

8.2.6 Blood glucose, urea, creatinine, HbA1c & body weight

Estimation of Blood glucose: By accu check strip - blood sugar-testing devices (Accu-check sensor comfort glucose meters), urea was estimated in terms of blood urea nitrogen (before diabetes was induced and after 8 weeks of diabetes), creatinine by picric acid method, HbA1c using Nycocard reader. HbA1c reference range for is 4.0 – 6.0 %. Urea, creatinine & HbA1c was done before diabetes was induced and after 8 weeks of diabetes. Blood sugar and body weight was done every 15 days.

8.2.7 Measurement of protein concentration

Protein concentrations of these samples were determined by the method of Biuret method for serum samples with the use of bovine serum albumin (BSA) as a standard.

8.2.8 Preparation of BSA–AGE

BSA–AGE was prepared by rapid thermal method (Bhatwadekar and Ghole 2005). AGEs were produced by incubation of BSA (Fraction V, Sigma Chemical Co., St Louis, MO) at a concentration of 30 mg/ml with 0.5 M glucose in 0.2 M phosphate-buffered saline (PBS) containing 0.5 mM EDTA, pH 7.4, at 50°C for 4 days. The density of brown color, which is the typical physical appearance of AGEs, was quantified by spectroflourimeter (Excitation: 370 nm, Emission – 440nm) and the AGE adducts formed are visualized using Polyacrylamide Gel electrophoresis (PAGE).

8.2.9 Estimation of advanced glycation levels

Serum (1:10), lens (1:5), retina (1:5) in phosphate-buffered saline, and the intrinsic AGE-specific fluorescence was monitored spectrofluorimetrically at Ex370 nm and Em440 nm. To ascertain the excitation and emission characteristics of AGEs measured in serum, AGE adduct (bovine serum albumin + glucose in phosphate buffer for 4 days at 50°C) was made in vitro and tested for fluorescence spectra. Fluorescence intensities of AGE adduct measured at excitation and emission wavelengths 370 and 440 nm respectively, were abolished with aminoguanidine (co-treatment during the AGE adduct formation) while lysine was added as positive control in increasing the AGE-adduct formation, thereby indicating a relative and reliable measurement of AGEs in serum. One AGE fluorescent unit is equal to the fluorescence detected by 100 µg/mL AGE-BSA standard. The final serum concentrations of AGE were normalized to total protein (Bhatwadekar and Ghole 2005; Sampathkumar, Balasubramanyam et al. 2005).

8.2.10 Immuno histochemistry for CML-AGE, IL-1β & IL-2 in retinal section

IHC for CML-AGE, IL-1β & IL-2 in rat retina were processed for sectioning after fixing in buffered formalin. IHC was done on tissue section of 5 µm. The deparaffinized sections were incubated with trypsin-EDTA and stained with Novolink™min polymer detection system. Primary antibodies to CML-AGE (1:200), IL-1β (1:50), IL-2 (1:50) were incubated overnight. The sections were counterstained with Mayer's haematoxylin for 15 sec, rinsed in tap water and air-dried.

8.2.11 ELISA for eNOS/NO/sICAM

Rat retinas were homogenized in mPER protein extraction reagent and centrifuged at 10,000 g for 10 min. Aliquots (25 µl) of the supernatant were used for eNOS & NO and sICAM (1in 50). eNOS was assayed by a sandwich enzyme immunoassay specific for rat. Retinal eNOS, NO and sICAM concentrations were normalized to total proteins.

8.2.12 Tunel assay (terminal dUTP nick-end labeling to detect apoptotic cells)

The IHC sections were processed for the terminal transferase dUTP nick-end labeling (TUNEL) reaction to detect apoptosis in the retina (in situ cell death detection kit; Roche, Mannheim, Germany). In each assay, a positive control was set up by treatment with DNase (50 U/100 µL) for 10 minutes, to fragment DNA on an additional slide. The green fluorescence of TUNEL-positive and the blue fluorescence for nuclei was observed under a Zeiss fluorescence microscope. The number of TUNEL positive green cells were counted the entire retina.

8.2.12 Statistical analysis

The results are expressed as means ± SD. Individual comparisons between control and diabetic rats or mice were made using the unpaired two-tailed Student's t-test. Significance was defined at $p \leq 0.05$. Comparison of ELISA results between control and diabetic and between diabetic and treated groups were done by non-parametric Mann Witney test.

8.3 RESULTS

Mixture of amino acids was found to be beneficial in terms glucose uptake by augmenting insulin signaling cascade as seen in CHO-K1 cells along with increased uptake by GLUT4 translocation as seen in adipocytes. The study in the CHO-K1 cells also hinted on the possible anti-angiogenic effect of AAM as seen by decrease in VEGF with increase in glucose. Therefore in order to study the beneficial effects of amino acids at the level of retina, STZ induced diabetic retinopathy SD rat model was used.

8.3.1 Biochemical parameters in diabetic rats supplemented with AAM (SNAAM1 & SNAAM2)

Sprague dawley male rats aged 2 weeks were taken for the study **(Fig 8.1)** were monitored for body weight, blood glucose, urea, creatinine, HbA1C.

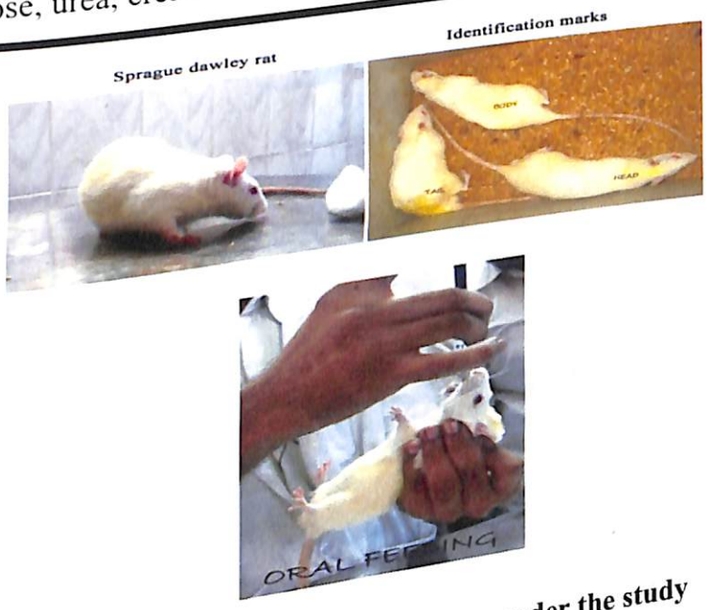


Fig 8.1: Sprague dawley male rats under the study

a) **Body weight:** Body weight was observed once every week until sacrificed. After diabetic induction with and without drug supplemented for 8 weeks, the diabetic group showed no gain of weight. Compared to aminoguanidine and SNAAM2 group, SNAAM1 showed lower weight loss **(Fig 8.2A)**.

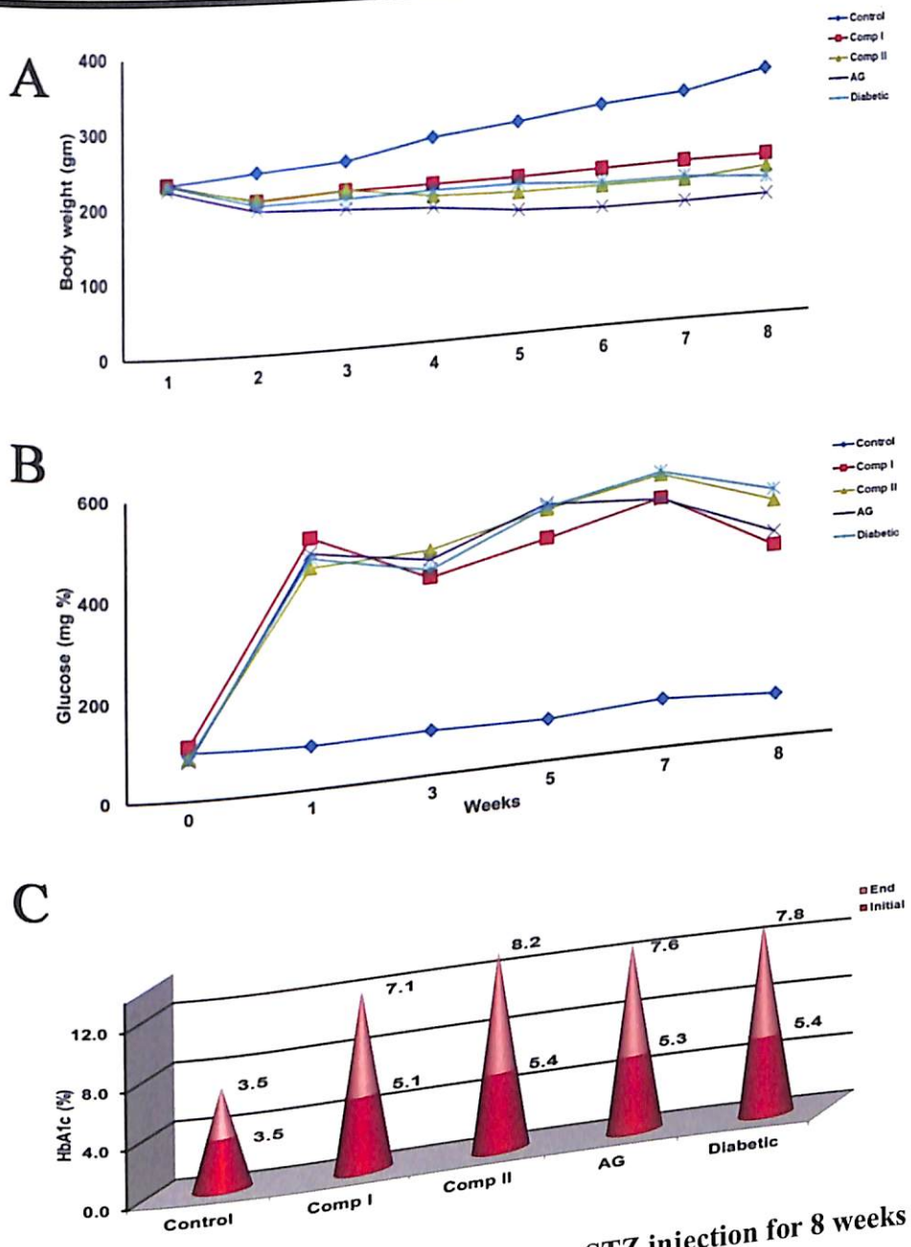


Fig 8.2 A: Body weight before and after STZ injection for 8 weeks
B: Blood glucose level at 15 days for 8 weeks.
C: HbA1c levels before diabetes induced and after 8 weeks.

b) **Blood Glucose:** Blood glucose was estimated once every 2 weeks until sacrificed. Initially the glucose was in normal range with a mean of 83 ± 15 mg/dl. After diabetic induction and AAM supplemented for 8 weeks, the diabetic group showed an increase in glucose level with a mean level of 511 ± 60 mg/dl. Compared to amino acid SNAAM2 and aminoguanidine group, the amino acid SNAAM1 showed the least increase (392 ± 151 mg/dl). A maximum of 30 % fall

in glucose level was observed in the SNAAM1 group when compared to diabetic group as seen at the end of 8 weeks, closely followed by AG group which showed 20 % decrease **(Fig 8.2B)**.

c) **HbA1c:** HbA1c was estimated initially and at the end of 8 weeks, before sacrifice. Initially the glycated protein is normal. After diabetic induction and drug supplemented for 8 weeks, the diabetic group showed an increase in HbA1c (From 5.4 % to 7.8 % increase). Compared to amino acid SNAAM2 and aminoguanidine group, the amino acid SNAAM1 showed the least increase (From 5.1 % to 7.1 %) **(Fig 8.2C)**.

d) **Urea/creatinine:** The creatinine levels were found to be normal in all the rats and BUN levels are found to be maximum in diabetes followed by AG, SNAAM2, SNAAM1 & control **(Table 8.1)**.

Table 8.1: Blood urea nitrogen & Creatinine mean ± SD value of various rats during sacrifice.

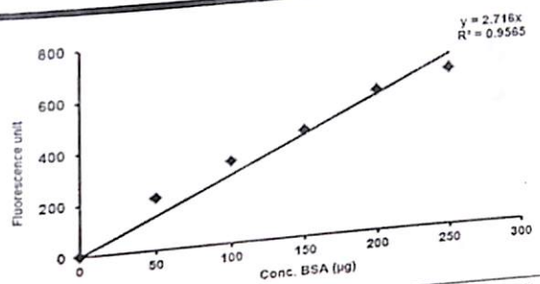
S.No.	Groups	Blood urea nitrogen (mg %)	Creatinine (mg %)
1	Control	20 ± 4	0.2 ± 0.1
2	SNAAM1	33 ± 8	0.3 ± 0.1
3	SNAAM2	30 ± 14	0.5 ± 0
4	Aminoguanidine	36 ± 8	0.3 ± 0.2
5	Diabetes	41 ± 7	0.4 ± 0.2

Note: Values are expressed as Mean ± SD. n =10 in each group with around 50% mortality.

Reference range: BUN - 15 -21 mg %
Creatinine – 0.2 – 0.8 mg %

8.3.2 Advanced glycation end products levels in serum/lens/retina of diabetic induced rats

a) **Standards:** AGEs were prepared by thermal rapid method and measured spectrofluorimetrically by exciting the samples at 370 nm and collecting the emission readouts at 440 nm **(Fig 8.3A)** and the fluorescent values are listed in **Table 8.2**.



Volume (µl)	Concentration (µg)	Fluorescence units
10	50	207
20	100	326
30	150	421
40	200	558
50	250	623

Fig 8.3A: AGE standard fluorescence value graph prepared by BSA treated with glucose at 50°C for 4 days and as analyzed by spectrofluorimetrically.

b) AGE in presence of AG & lysine (Positive controls): Amino guanidine, known to have anti-glycating property was added along with (BSA + glucose) during the preparation of AGE. AGs decreased the AGE formation at both the concentration (0.1 & 0.01 M) dose dependently, confirming AGE formation similar to that of incubation at 37°C for 6 months as done by routine method **(Fig 8.3B)**.

ε-amine group of Lysine is known to form an adduct with the glucose, thereby increasing the AGEs was too done as positive control. The addition of lysine to the reaction mixture intensifies the formation of AGEs by reacts with aldehyde groups of glucose through their amino groups, and forms glycosyl amino acid adducts at physiological pH and temperature. Lysine in confirmation of this property showed an increase in fluorescence when the AGEs were incubated with lysine at 50°C **(Fig 8.3B)**.

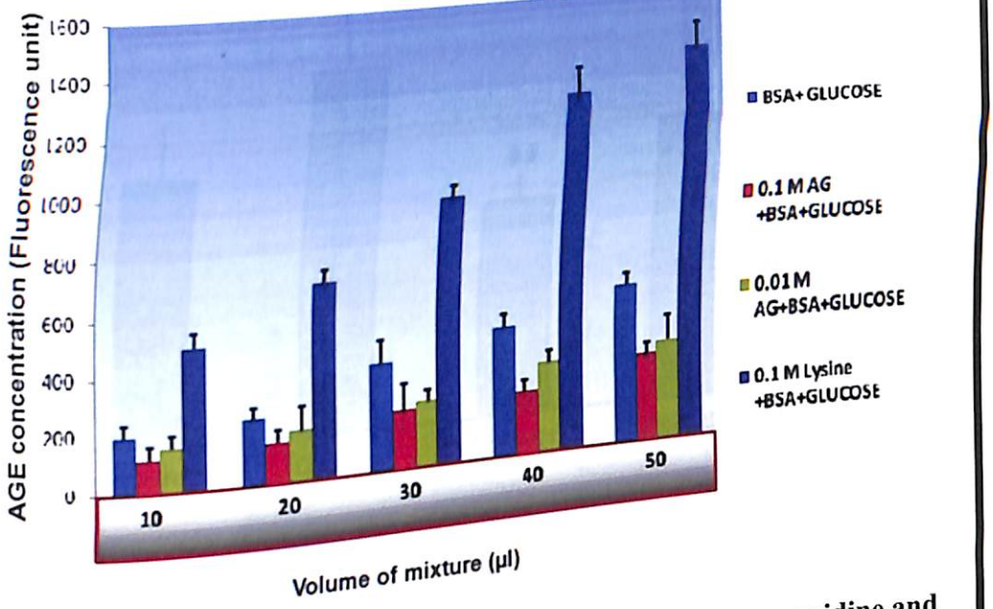


Fig 8.3B: Comparison of the effect of 0.01 M & 0.1 M aminoguanidine and 0.1 M lysine on AGE standard prepared: Lysine as positive control with glucose and BSA shows increased AGE formation and aminoguanidine shows the decrease in AGE formation as shown in fluorescence unit.

c) **AGE level in serum:** As part of in vivo study the rat serum and lens samples were analyzed for AGE levels. A significant increase in AGE level were observed when compared to control as expected *($p<0.05$)*. A significant fall in AGE levels in the SNAAM1 *($p<0.008$)* and amino guanidine group *($p<0.04$)* group was seen, whereas SNAAM2 did not show any significant decrease **(Fig 8.4)**.

d) **AGE level in lens:** Increased levels of AGEs are reported in cataractous lenses of diabetic rats. This study also shows increased AGEs compared to control ($p<0.001$). AGEs were significantly decreased in the lens of rats treated with SNAAM1 *($p<0.01$)* and aminoguanidine *($p<0.01$)* whereas SNAAM2 did not show significant decrease **(Fig 8.5)**.

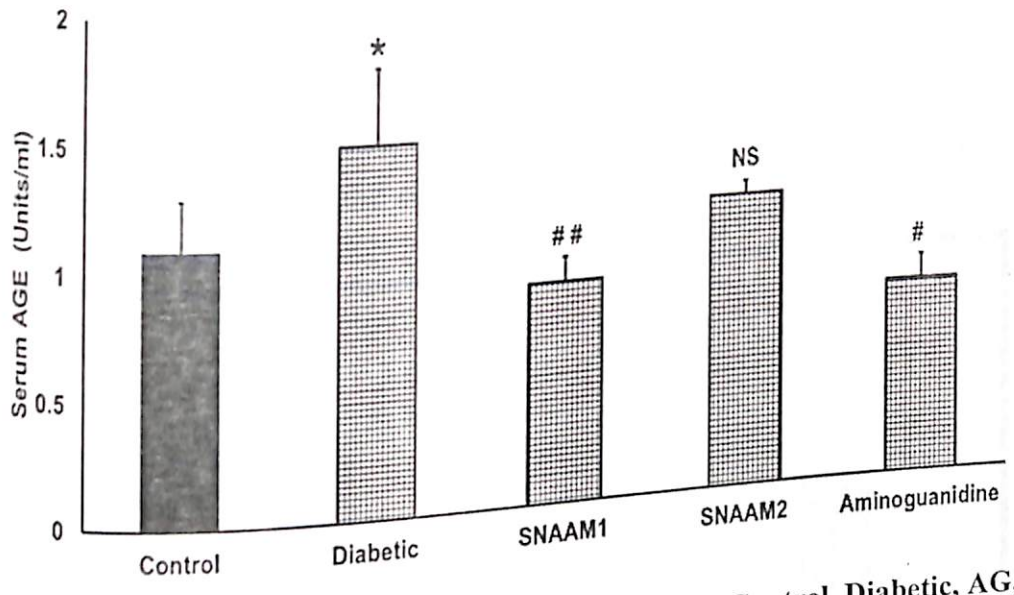


Fig 8.4: Comparison of serum AGE concentration in Control, Diabetic, AG, SNAAM1 and SNAAM2 treated rats with n=6 in each group. * $p<0.05$ when diabetic (Control Vs Diabetic) ; # $p<0.01$ (Diabetic Vs SNAAM1); # $p<0.05$ (Diabetic Vs SNAAM2).

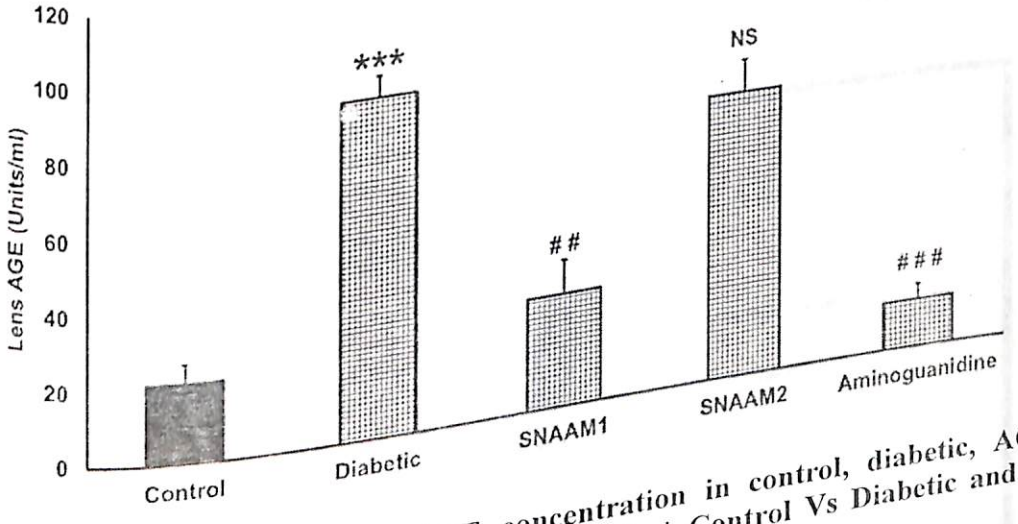


Fig 8.5: Comparison of lens AGE concentration in control, diabetic, AG, SNAAM1 and SNAAM2 treated rats with n=6. * Control Vs Diabetic and # Diabetic Vs SNAAM1, SNAAM2, AG.

e) **AGE level in retina:** This study shows increased AGE level in diabetic retina compared to control though not significant. AGE levels were significantly decreased in the retina of rats treated with SNAAM1 *($p<0.05$)* and SNAAM2 *($p<0.05$)* and aminoguanidine decreased the AGE levels to the levels seen in the control though the decrease is not statistically significance (Fig 8.6).

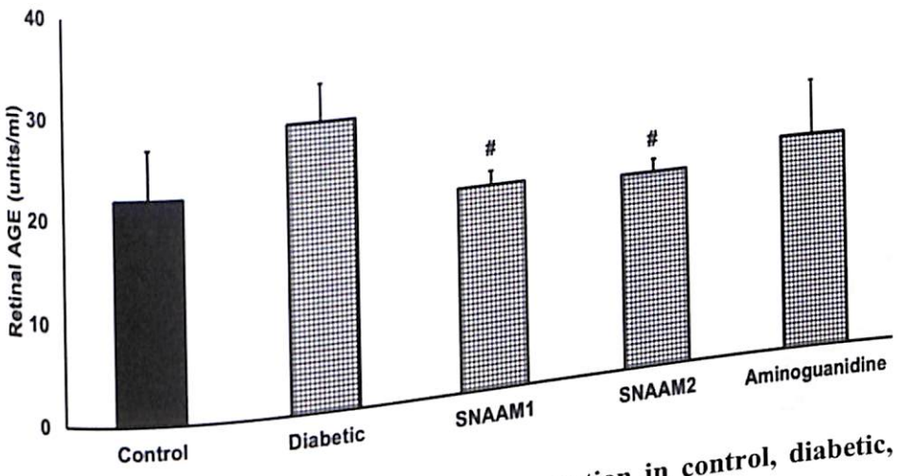


Fig 8.6: Comparison of retinal AGE concentration in control, diabetic, AG, SNAAM1 and SNAAM2 treated rats with n=5. * Control Vs Diabetic and # Diabetic Vs SNAAM1, SNAAM2, AG.

8.3.3 CML-AGE, interleukins & cell adhesion molecule levels in diabetic rat retina

The retinas were stained by H & E for visualizing the various molecules which are up regulated and down regulated during the diabetic course thus affecting the retina. The various layers normally seen are shown in **Fig 8.7)**.

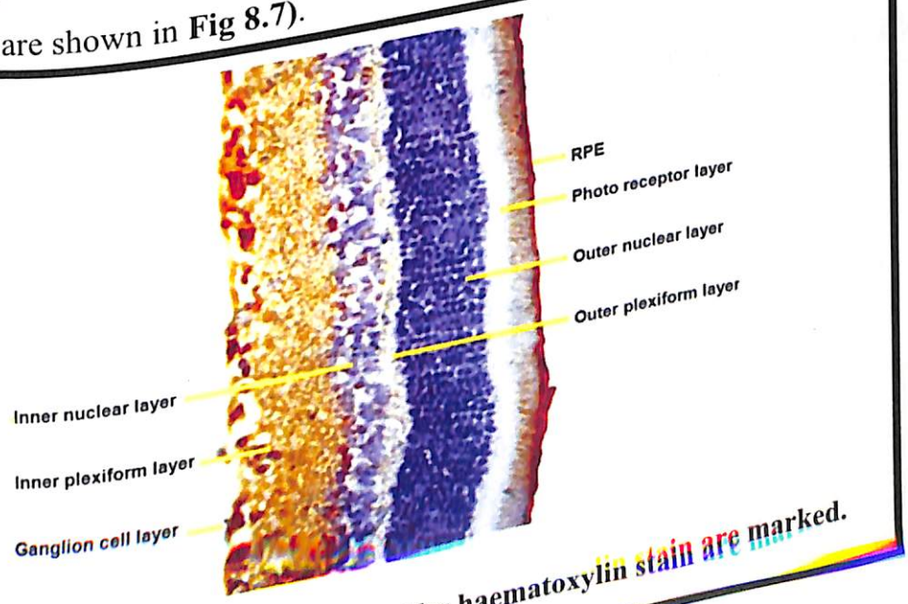


Fig8.7: The layers of rat retina stained by haematoxylin stain are marked.

a) **Carboxy methyl lysine AGE:** A strong positivity of CML-AGE was seen at ganglion, inner nuclear and outer nuclear retinal layers in diabetic retina compared to the control. While the diabetic group shows a strong overall positivity for CML-AGE, the treated group SNAAM1 and SNAAM2 and the Aminoguanidine group was found to be comparable to the control **(Fig 8.8)**.

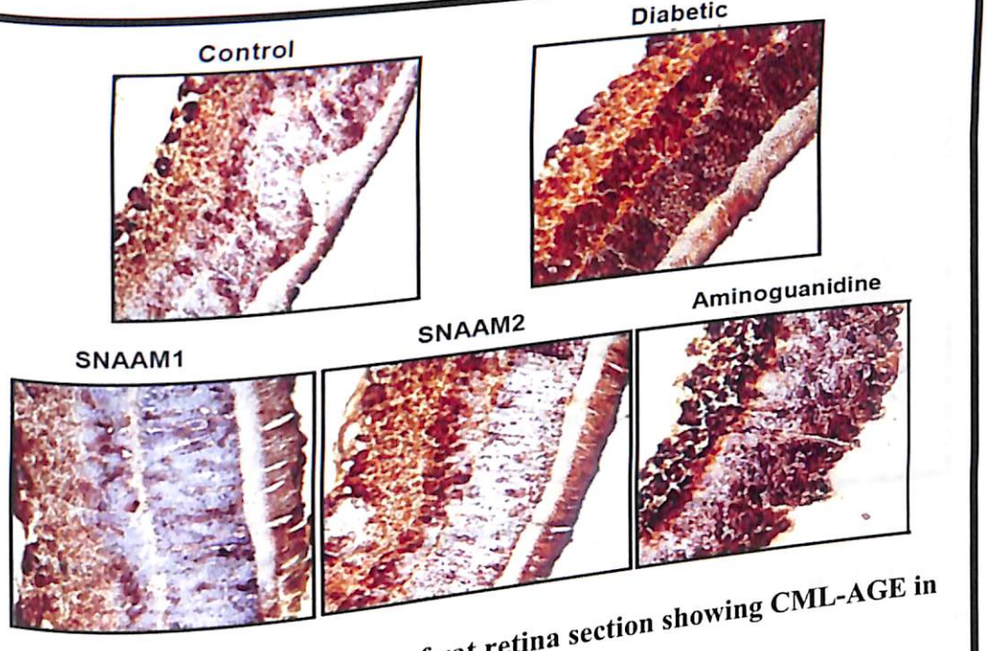


Fig 8.8: Immunohistochemistry of rat retina section showing CML-AGE in the retinal layers.

b) **Interleukin 1 beta:** A strong positivity of IL-1 β, an inflammatory cytokine was seen at ganglion, inner nuclear and outer nuclear retinal layers in diabetic group compared to the control. SNAAM1 showed a prominant decrease compared to SNAAM2 that showed a mild positivity in the inner nuclear layer while aminoguanidine treated was comparable to the untreated control group **(Fig 8.9)**.

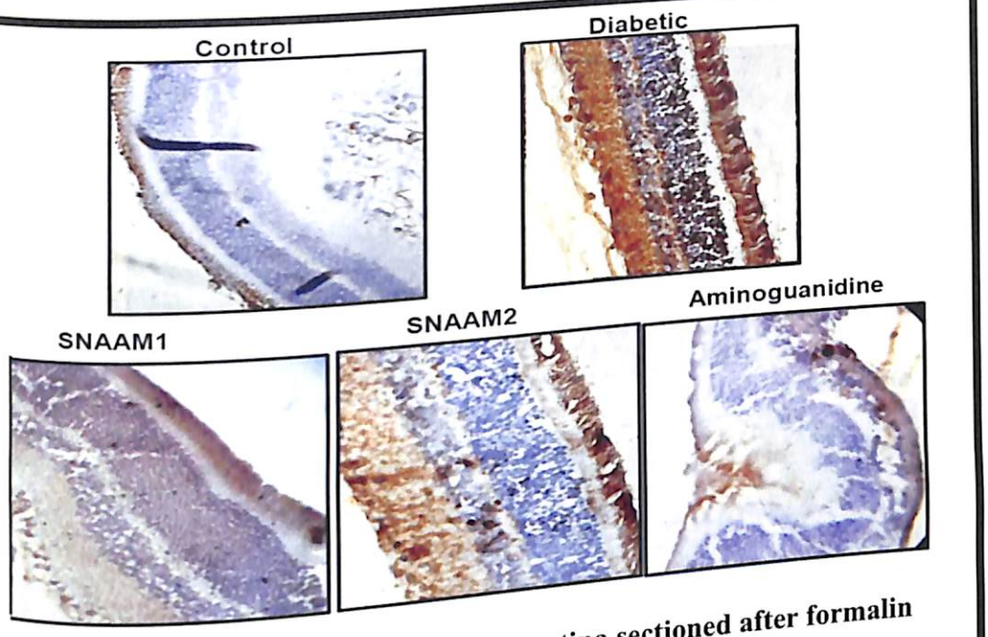


Fig 8.9: Immunohistochemistry of rat retina sectioned after formalin fixation stained for IL-1 β .

c) **Interleukin-2:** A strong positivity of IL-2, an inflammatory cytokine was seen in ganglion, inner nuclear and outer nuclear retinal layers characteristically in the diabetic group. SNAAM1 showed a prominant decrease compared to SNAAM2 that showed a mild positivity in the inner nuclear layer while aminoguanidine treated was comparable to the untreated control group **(Fig 8.10)**.

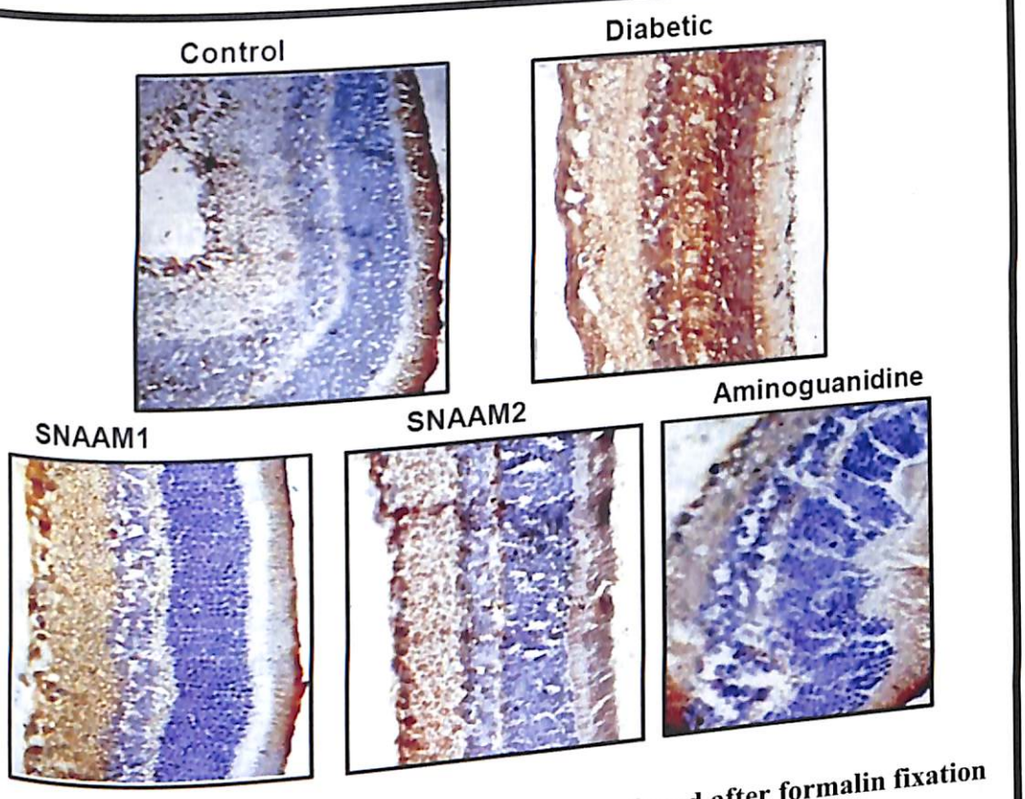


Fig 8.10: Immunohistochemistry of rat retina sectioned after formalin fixation was stained for IL-2 antibody.

8.3.4 Estimation of eNOS/NO

Rat retinas were homogenized and early biochemical markers namely eNOS and NO were estimated.

a) **Nitric oxide synthase:** A significant increase in eNOS was observed in the retinal homogenate of the diabetic rat when compared to the controls *($p<0.01$)*. The treated groups namely SNAAM1 and SNAAM2 showed a significant fall in the eNOS levels when compared to diabetic group *($p<0.01$ & $p<0.05$* respectively). The positive control group of AG treated also showed a significant fall in the eNOS *($p<0.05$)* **(Table 8.2)**.

Table 8.2: eNOS levels in rat retinal lysate by ELISA

Groups	eNOS (pg/mg protein)
Control (n=7)	65 ± 27
Diabetic (n=6)	237 ± 195 (*p=0.006)
SNAAM1 (n=6)	68 ± 21 (#p=0.01)
SNAAM2 (n=4)	39 ± 13
Aminoguanidine (n=4)	61 ± 6 (#p=0.05)

All values are expressed as mean ± SD * p value is comparison between control and diabetic. # comparison between diabetic and SNAAM1, SNAAM2 and AG groups respectively.

b) **Nitric oxide:** A significant increase in NO was seen in the untreated STZ induced diabetic rat when compared to the control *($p<0.05$)*. The treated groups with SNAAM1 showed a significant fall in the NO levels when compared to diabetic group *($p<0.05$)*. The positive control group of AG treated too showed a significant fall in the NO *($p<0.05$)* **(Table 8.3)**.

Table 8.3: NO level in rat retinal lysate by Griess method.

Groups	Nitric oxide (μM)
Control (n=7)	63 ± 42
Diabetic (n=6)	164 ± 85 (*p<0.05)
SNAAM1 (n=6)	58 ± 31 (#p<0.05)
Aminoguanidine (n=4)	22 ± 10 (#p=0.05)

* p value comparison between control and diabetic. # comparison between diabetic and SNAAM1 and AG groups.

8.3.5 Estimation of soluble Intercellular adhesion molecule-1 (sICAM)

Increase in sICAM was observed in the untreated STZ induced diabetic rat when compared to the control significantly *($p<0.05$)*. The treated groups with SNAAM1 showed a significant fall in the sICAM levels when compared to diabetic group (50 % decrease) and SNAAM2 showed no decrease. The positive control group of AG treated too showed a significant fall in the sICAM *($p<0.05$)* **(Table 8.4)**.

Table 8.4: sICAM levels in rat retinal lysate by ELISA.

Groups	sICAM (ng/mg of protein)
Control (n=7)	11 ± 9
Diabetic (n=6)	48 ± 28 *(*$p<0.05$)*
SNAAM1 (n=6)	25 ± 19 *(50 % ↓NS)*
SNAAM2 (n=5)	55 ± 17
Aminoguanidine (n=4)	24 ± 10 *(#$p<0.05$)*

* p value comparison between control and diabetic. # comparison between diabetic and SNAAM1, SNAAM2 and AG groups.

8.3.6 Early Apoptotic levels in diabetic rats

Diabetic retinopathy can result in apoptotic cell death of retinal neurons, and therefore significant visual loss. We tested the hypothesis that AA treatment could inhibit death of neuroretinal cells in diabetic rats by examining the expression of proapoptotic markers. In diabetic rat retina, number of TUNEL-immunoreactive cells were found in inner nuclear and ganglion cell layer, whereas in SNAAM1 and control group it is reduced compared to diabetic **(Fig 8.11A)** A negative control with buffer alone and positive control with 3000 KU of DNase were done **(Fig 8.11B)**. The number of $TUNEL^+$ cells as in the graphical representation **(Fig 8.12)**, shows significant increase in diabetic retina as observed compared to control *($p<0.01$)* and decrease in SNAAM1 *($p< 0.01$)*, SNAAM2 *($p< 0.05$)*and aminoguanidine compared to diabetic.

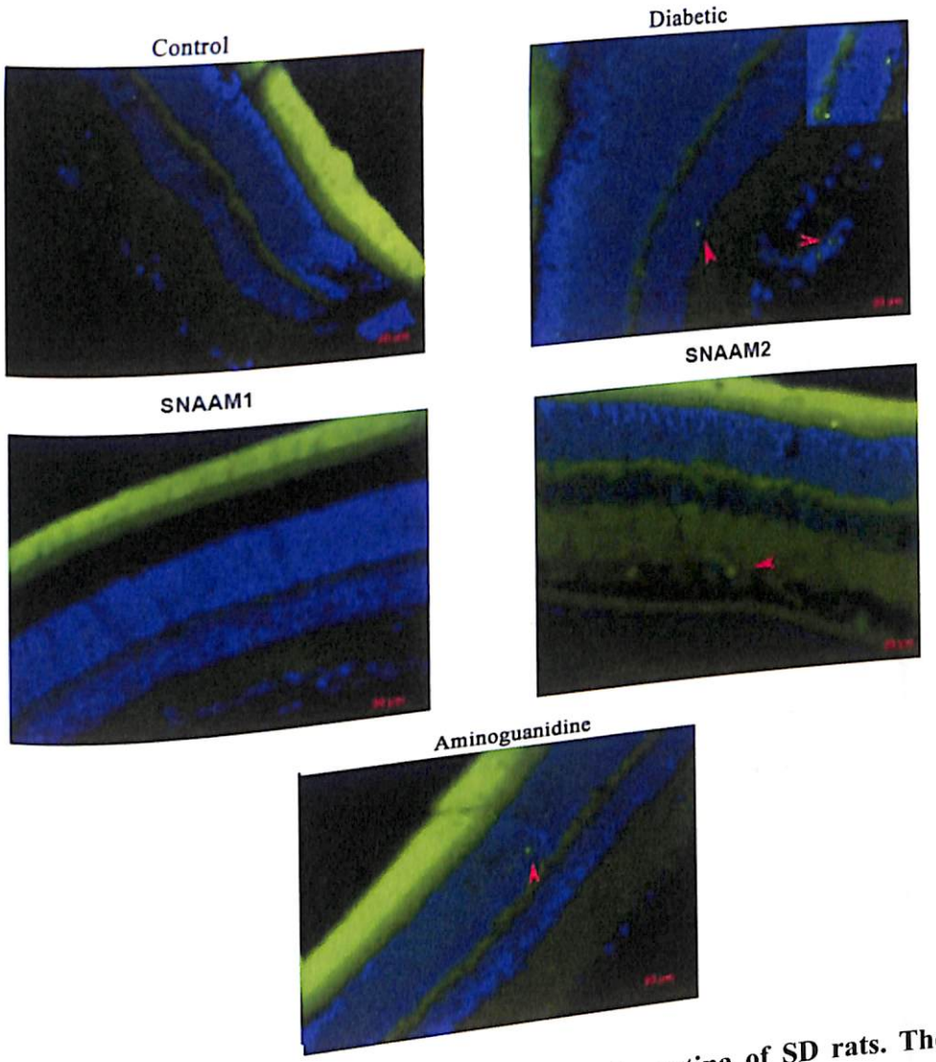


Fig 8.11A: Apoptotic cells as stained by TUNEL in the retina of SD rats. The green fluorescence indicates the positivity for DNA break.

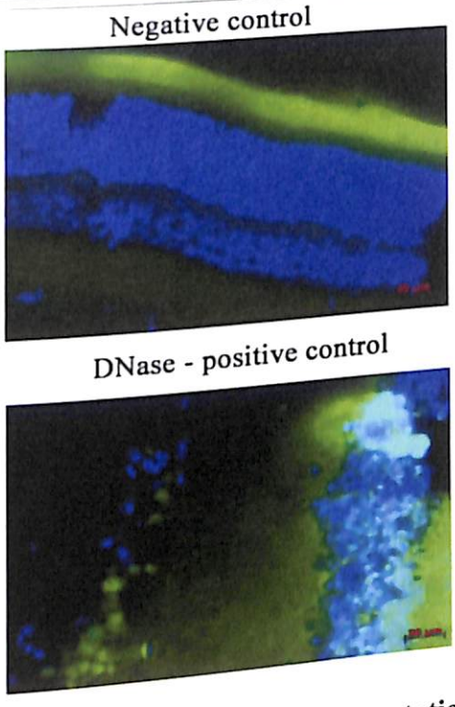


Fig 8.11B: Positive and negative control of apoptotic cells as stained by TUNEL in the retina of SD rats. The DNA break is confirmed by the negative one without enzyme solution and with label solution alone and positive staining with DNase treated. The green fluorescence due to FITC indicates the positivity for DNA break while blue is due to DAPI stain.

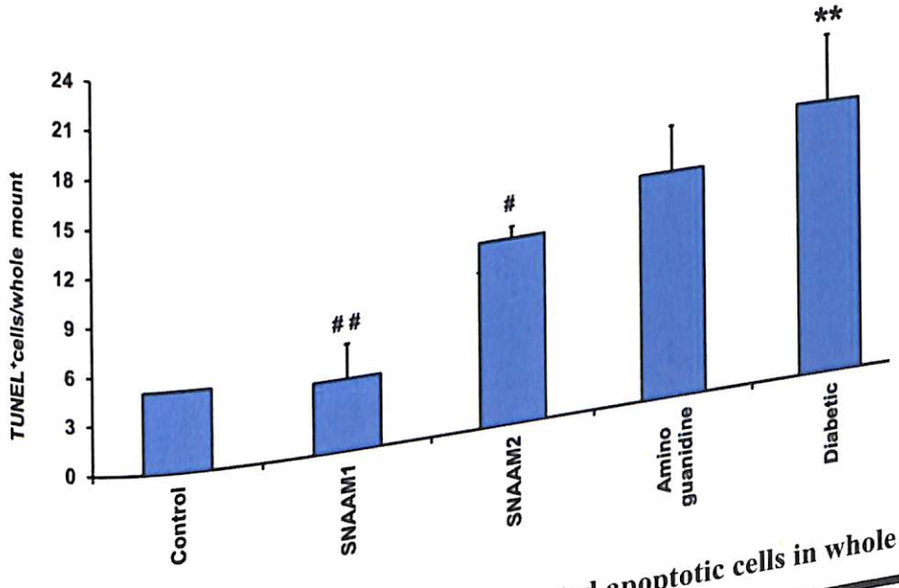


Fig 8.12: Graphical representation of counted apoptotic cells in whole

Thus amino acids in specific mixture form seems he an anti-glycating property which could be beneficial in DR cases by decreasing the apoptosis, cytokines and oxidative stress.

8.4 CONCLUSION

Animal model of diabetes mellitus in streptozotocin induced diabetic Sprague Dawley rats maintained for long duration of 2 months to induce retinal changes, when supplemented with a novel amino acid mixture (2 different compositions based on the study data and data from other studies in the laboratory), showed lower levels of AGE in serum apart from the lens and the retinal levels, indicating **anti-glycating** property of the amino acids mixture studied. AAM also showed decreased expression of CML-AGEs in retina sections. The amino acids mixture also showed decreased expression of cytokines IL-1 beta, IL-2 in retina sections which are inflammatory marker of DR indicative of **anti-inflammatory** property. AAM showed significant decrease in apoptosis which associated with diabetic retinopathy. This shows AAM has **anti-apoptotic** property that can be beneficial in DM at the level of retina.

CHAPTER 9: DISCUSSION

Type 2 diabetes is the most common metabolic disease in the world, affecting >250 million people, and is characterized by hyperglycemia, insulin resistance and dyslipidemia. It causes multi organ defects affecting eye, kidney and nervous system. The defects could be at several steps of the insulin signaling pathway. One approach to increase glucose disposal is to improve the insulin signaling. Hyperglycemia, free fatty acids induced overproduction of superoxide at the mitochondrial level and nitric oxide overproduction through NOS, favoring the formation of the strong oxidant peroxynitrite leads to altered signaling resulting in changes in expression of molecules such as VEGF, cytokines and cell adhesion molecules. The thesis work is focused on addressing the question as to whether amino acids are beneficial in the disease management of diabetes mellitus. Studies have associated the AA levels to the pathology of DM while there are also reports on anti-diabetic effect of specific amino acids in terms of improved glucose uptake or in sensitizing the specific insulin signaling molecules. Earlier in the laboratory it was observed that amino acids was able to mitigate cataractogenesis in diabetic rats and decreased the blood glucose level. However the mechanism of this anti-diabetic activity and prevention of diabetic complications was to be elucidated. Therefore this work is focused on the possible mechanism involved by addressing several questions.

Amino acids are beneficial to people with type 2 diabetes by three mechanisms namely, a) scavenging the excess glucose in the blood (Ramakrishnan and Sulochana 1993; Ramakrishnan, Sulochana et al. 1996; Ramakrishnan, Sulochana et al. 1997); b) Stimulating the pancreas for insulin secretion (Dixon, Nolan et al. 2003) and c) up-regulation of the insulin receptor tyrosine kinase (Sulochana, Rajesh et al. 2001). In this study, using CHO-K1 cell line, this study demonstrates that the amino acids, in mixture form constituted by alanine, arginine, leucine, isoleucine, aspartic acid, glutamic acid and lysine are able to significantly increase the glucose uptake as well as increase the GLUT4 recruitment when the cells are exposed to high glucose in CHO-K1 and adipocyte cells. Thus the anti-diabetic effect seen in terms of fall in the blood glucose level by 30 % in the amino acid supplemented rat model of DM reported earlier from previous studies in the lab can be attributed to the increased cellular entry as supported by the GLUT4 translocation in high glucose condition in CHO-K1 and adipocyte cells observed in this

study. In the present study, it was found that the addition of 5 & 10 mM amino acids mixture was beneficial in improving the viability of CHO cells at high glucose concentrations (12, 17 & 27 mM). Addition of the amino acids improved the viability by as much as 20 %. A 2 fold increase in the glucose uptake was seen dose dependently when 5 and 10 mM AAM was used. The addition of amino acids to the growth media of CHO cell cultures has been reported to mitigate the negative effects of ammonium stress and improved the CHO cell growth as well as important metabolic parameters, including glucose consumption while growing CHO-K1 cells for industrial applications (Chen and Harcum 2005). This study has revealed that AAM concentration at 5 mM, which was non-cytotoxic, was found to be beneficial in terms of improving glucose uptake in the CHO-K1 cells. Reports have shown that free amino acids such as the isoleucine and leucine do promote glucose uptake both based on in vitro and in vivo studies. Amino acids such as leucine and isoleucine have been shown to promote glucose uptake in rat skeletal muscle. Leucine promotes glucose uptake by the promotion of glycogen synthesis via activation of mTOR signaling in rat muscle (Nishitani,2008). Isoleucine has been shown to promote glucose uptake in C2C12 myotubes through PI3K and PKC pathway. Isoleucine reportedly promotes glucose uptake in both the presence and absence of insulin in cultured muscle cells (Doi, Yamaoka et al. 2003). In the present study, among BCAA, isoleucine showed increased glucose uptake and GLUT4 translocation when CHO-K1 and adipocyte cells were exposed to high glucose. Novelty, in this study instead of individual amino acids, a mixture is suggested, since supplementing a specific amino acid may derange the amino acids uptake as well as metabolism .

The amino acids in the mixture were relevantly chosen based on the previous experimental data in the laboratory and from the reports. This was based on the antiglycating property of amino acids namely alanine, aspartic acid, glutamic acid, and lysine studied in vitro (Ramakrishnan, Sulochana et al. 1996; Ramakrishnan, Sulochana et al. 1997; Sulochana, Punitham et al. 1998). In addition, the branched chain amino acids leucine and isoleucine namely have been known to promote glucose uptake, by regulating the AMP:ATP ratio (Doi, Yamaoka et al. 2005; Nishitani, Takehana et al. 2005). Arginine, through NO generation, has also been shown to promote glucose uptake (McConell and Wadley,2008). Amongst the free amino acids in the mixture, Alanine, arginine, lysine showed significantly increased glucose entry at 12 mM glucose and leucine at 2.5 mM and glutamic acid at 10 mM. Alanine, arginine, lysine, isoleucine (2.5 and 5

mM) significantly increased the glucose entry at 17 mM. At 10 mM concentration of the free amino acids, only alanine and arginine showed significant glucose entry. Thus the effect seems to vary with the concentration of the free amino acid and the glucose. Amino acids in mixture form showed significant increase in glucose entry at all concentrations of AAM as seen by increased glucose uptake and GLUT4 translocation in both cells taken for study.

Free amino acids have been shown to have independent effect such as in anti-glycation. Lysine, glycine, alanine, glutamic acid and aspartic acid reduced the glycation of proteins in human lens, *in vitro* (Ramakrishnan, Sulochana et al. 1997). Lysine as well as mixture of AAM (L-leucine, isoleucine, lysine, phenyl alanine, threonine, valine, tryptophan, methionine) showed anti-cataract effect as seen in experimental studies in rats. This study reveals that amino acid mixture, rather than the free amino acids, increased the glucose uptake in CHO-K1 cells as mediated by increased GLUT4 translocation (Selvi, Angayarkanni et al.). Bogan et al showed a mixture of 12 amino acids increased the glucose uptake through rapamycin sensitive pathway (Bogan,2001), while Bernard et al showed that a mixture of 5 amino acids augmented insulin signaling with respect to AS160 phosphorylation and glycogen synthase phosphorylation thereby glucose uptake in the skeletal muscle in the sprague dawley rats (Bernard, Liao et al.). However, there had been controversial results on the effect of amino acids on the glucose uptake. Krebs et al, shows that the branched-chain amino acids, inhibits glucose uptake in the human skeletal muscles (Krebs, Krssak et al. 2002). Recently Bassil et al states that elevated levels of amino acids do not modulate glucose metabolism in T2DM (Bassil, Burgos et al, 2011). The metabolite profiling in plasma that included amino acids showed that five of the amino acids to be significantly associated with predictive diabetes (Wang, Larson et al.). Traxinger et al, reported that amino acids modulate insulin sensitivity and the glucose transport system, and there were varied effects depending on the combinations and concentrations (Traxinger and Marshall 1989). *Tremblay et al,* observed that amino acids speeds up time-dependent deactivation of PI3K activity via mTOR signaling, a cause for insulin resistance (Tremblay and Marette 2001). Therefore, this study was further probed into looking at whether amino acids have potentiating or desensitizing effects on insulin signaling.

In order to assess the potentiating effect of amino acids on the key insulin signaling molecules , the effect of a specific amino acids mixture on the entire insulin signaling cascade namely

IRTK/PI3K/Akt/GLUT4 and Glycogen synthase under normal and high glucose conditions was studied. This study showed a significant increase in the IRTK, PI3K, Akt, GLUT4 and GS activity in the presence of 5 mM AAM under normal glucose condition. At high glucose addition of 5 & 20 mM AAM increased the IRTK and PI3K activity as well as the GLUT4 protein expression. In addition GS activity showed significant increase showing augmented glycogen synthesis at 5 mM indicating increased intracellular glucose availability. However, these signaling molecules seem to be modulated by the glucose level apart from the concentration of AAM as seen in the CHO-K1 cells in vitro. It is possible that depending on the type of the ligand and the type of receptor activation the downstream signaling can vary. IGF-II is reported to induce a higher p70S6kinase/Akt ratio than insulin and IGF I (Kido, Nakae et al. 2001; Sacco, Morcavallo et al. 2009). Limited information is available on the other possible ligands. Lum *et al* has shown that urea analogues are capable of influencing the structure function relationship of IRTK which influences the glucose uptake positively as studied in 3T3 adipocytes (Lum, Cheng et al. 2008). Whether amino acids can intercept as a ligand is yet to be looked into.

Insulin receptor protein is reported to be decreased in T2DM owing to both qualitative and quantitative defects in the insulin receptors in circulating cells (Chou, Dull et al. 1987). Kim et al observed decreased insulin-induced PI3K activation in T2DM (Kim, Nikoulina et al. 1999). A study by Khoanski *et al* showed polylysine induced insulin receptor tyrosine kinase activity and also its phosphorylation (Kohanski 1989). PI3K is another candidate suitable for therapeutic intervention to improve insulin action (Frojdo, Vidal et al. 2009). In this study, it was observed that the PI3k activity is increased at high glucose conditions. At 27 mM glucose AAM increase the PI3K activity which was significant at 20 mM AAM. Thus PI3K activity is augmented in the presence of AAM, similar to IRTK activity. Amino acids mediate mTOR activation by signaling through PI3K (Nobukuni, Joaquin et al. 2005). This results in the augmented protein synthesis rather than augmenting energy metabolism. *Flati et al* state that by unknown mechanism amino acids increase the GLUT4 translocation (Flati, Pasini et al. 2008). It is possible that the AAM mediate the increased GLUT4 via activation of IRTK and PI3K. The primary downstream mediator, AKT transmits the PI3K signal to a large number of molecules thereby diversifying the PI3K signal into various functional outcomes induced by high glucose and ROS (Renner 2008). In this study, Akt activity increased in response to the high glucose as reported implying that the

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cells enter a survival pathway (Song, Ouyang et al. 2005). At 27 mM high glucose, addition of 20 mM AAM decreased the Akt activation implying that the cell is not in a stress to go for the survival pathway via Akt. Meanwhile, the GLUT4 expression was increased which was associated with increased glucose uptake (Selvi, Angayarkanni et al.). This implies that activation of IRTK and PI3K, independent of Akt, can result in GLUT4 mediated glucose uptake. This is possible because there are other mechanisms wherein IRTK activation proceeds to GLUT4 translocation. Friedrichsen *et al* has shown that AKT mediated glucose uptake can be independent of IRTK (Friedrichsen, Poulsen et al.). GLUT4 is known to be translocated not only by Akt, but also by PKC-ζ (Liu, Yang et al. 2007), adiponectin (Ceddia, Somwar et al. 2005), CIP 4/2, and rho family members such as TC10 (Hou and Pessin 2007). Akt activation is shown to be increased in insulin resistance conditions in experimental animals (Liu, Hong et al. 2009).

At the cellular level, glucose uptake results from the insulin-stimulated translocation of the glucose transporter 4 (GLUT4) from intracellular storage sites to the plasma membrane (Bryant, Govers et al. 2002; Watson, Kanzaki et al. 2004; Martin, Lee et al. 2006; Muretta, Romenskaia et al. 2008). Insulin sensitizers augment the signaling resulting in increased glucose uptake (Jiang and Zhang 2005). Recent studies show that in addition to insulin, a heterogenous set of factors (insulin, amino acids) are capable of mobilizing GLUT4 via alternate molecular pathways (Flati, Pasini et al. 2008). This study result shows that the presence of 5 & 10 mM AAM significantly increased the GLUT 4 translocation with nearly 2 to 4 fold increase in CHO-K1 cells as seen by the FACS analysis. AAM alone was found to be not sufficient in the GLUT4 translocation. The presence of insulin along with AAM, showed increased GLUT4 translocation, showing an additive effect. Therefore, AAM seems to be beneficial in increasing the glucose uptake both at normal glucose (7 mM) as well as high glucose (17 & 27 mM) along with insulin. Bogan et al, reported that amino acids at sufficient concentration accumulated GLUT4 in CHO cells at the plasma membrane and at low amino acid concentrations, much of GLUT4 remained in a perinuclear or trans-Golgi location (Bogan, McKee et al. 2001).

Further, AAM increased the glycogen synthesis and the glycogen synthase activity which is promoted by insulin and decreased by high glucose. Addition of the amino acids mixture showed 2 to 4 fold increase in GS activity in the high glucose conditions, depending on the glucose

concentration. Glycogen synthase activity is increased infer that the improved glucose availability in the cell.

Exposure to free amino acid, alanine, isoleucine, glutamic acid and lysine increased uptake significantly. Similarly, AAM was also found to increase it significantly. This could be due to either increase in the AT1 expression, a transcription factor or the changes at the level of the GLUT4 vesicles. However at the level of expression of GLUT4 as seen by mRNA levels only alanine was found to increase at 33 mM glucose and alanine, aspartic acid and leucine at 5 mM glucose. All types of signaling involved in increased glucose uptake is mediated through the GLUT4 translocation rather than increase in the translation of GLUT4 proteins. However in adipocytes, the GLUT4 changes at mRNA levels associated with high glucose has been reported (Kampmann, Christensen et al.; Shan, Chen et al.). Thus, in this study few amino acids help in translocation and few in translation probably reveals that amino acids could be beneficial in both ways. Thus the effect of amino acids was found to have a similar effect in the human cell line such as that of the adipocytes in augmenting the GLUT4 translocation. Lysine was found to show a significant increase in glucose uptake in adipocytes by as much as 40 %. AAM showed a 20 % increase though not significant. Thus, lysine, AAM, isoleucine, glutamic acid showed increased uptake, while alanine showed as much as that seen in high glucose (33 mM) in tandem with glut 4 translocation observed. Whether AAM and these amino acid would be beneficial in insulin resistance conditions needs to be addressed.

Hyperglycemia is known to deplete cellular antioxidants and cause an increase in free radicals. ROS has the ability to directly oxidize and damage DNA, protein and lipid. It plays a direct role in the pathogenesis of late diabetic complications (Rosen, Nawroth et al. 2001; Houstis, Rosen et al. 2006). One of the mechanism for diabetic retinopathy is mediated by increased ROS seen in uncontrolled diabetes of long duration (Nishikawa, Edelstein et al. 2000; Evans, Goldfine et al. 2003; Brownlee 2005). DR involves VEGF mediated neovascularization and therefore the current treatment involves anti-VEGF factors. In this study, the effect of AAM on the ROS signaling to VEGF was evaluated by looking at the ROS/NO/SOD/VEGF axis. Activation of the ROS/PI3K/Akt/eNOS signaling pathway in early phase exerts protective effects against the induction of apoptosis by high glucose (Ho, Liu et al. 2000). The same activation could lead to over expression of VEGF, a downstream molecule of Akt (Shiojima and Walsh 2002). This

study has shown that AAM could decrease the VEGF by decreasing the ROS/NO when exposed to high glucose. The present study shows a fall in ROS levels (which includes peroxynitrite) when 5 mM AAM was added to high glucose (15 to 35 % decrease) depending on the glucose concentration. This could be beneficial by preventing the further signaling which induces VEGF. Peroxynitrite serves as a second messenger to activate multiple signaling pathways leading to endothelial migration, proliferation, and tube formation in vitro and angiogenesis in vivo (El-Remessy, Al-Shabrawey et al. 2007).

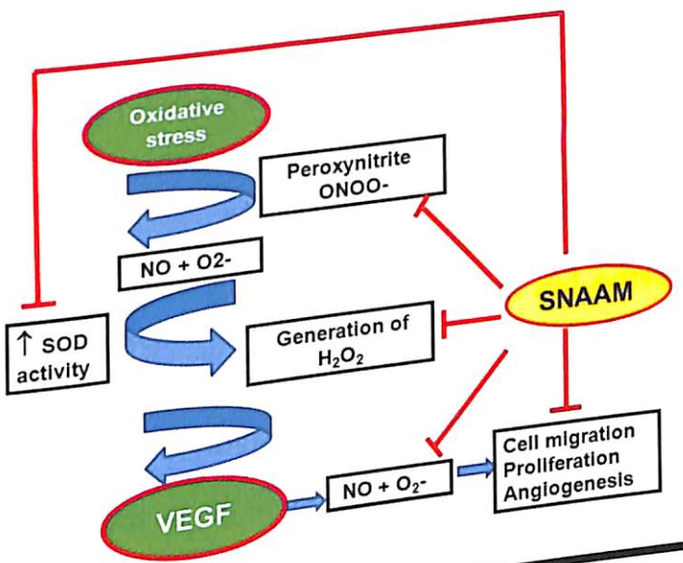
During oxidative stress insult, the formed peroxynitrite is converted to NO. The NO can upregulate VEGF and VEGF in turn produce NO and there by cell migration and proliferation (Murohara, Witzenbichler et al. 1999; Cooke and Losordo 2002). AAM decreased NO levels significantly rather than free form by 55 % with 17 mM G & 20 % with 27 mM G). Interestingly, lysine was found to increase the NO levels significantly. While arginine as a free amino acid is known to influence vasodilatation through increased NO production (Boger, Bode-Boger et al. 1996). This is the first study to show that lysine has much more potential in increasing NO levels. In diabetes, hyperproduction of free radicals is responsible for endothelial dysfunction, *via* a decrease in NO (nitric oxide) production, thus decreasing vasorelaxation of smooth muscle cells. AGEs quench the NO, and thus contribute to defective vasodilation. In such cases lysine could be beneficial in increasing the NO for the requirements of vasorelaxation. However more studies are required to evaluate the beneficial effect of lysine in terms of vasodilation.

Hyperglycemia could induce an increase in the intracellular antioxidant enzyme levels as defense mechanism (Ceriello, Giacomello et al. 1995; Sechi, Ceriello et al. 1997). The formed NO along with superoxide is removed from system by the enzyme SOD. The present study showed a maximal increase with a 60 % in 17 mM glucose and with addition of AAM this was found to be reduced by 60 % with respect to 17 & 40 % with 27 mM glucose. Present study shows decreased SOD enzyme activity by the addition of AAM irrespective of the glucose levels. But individual AA shows varied effect. The fall in SOD activity could be due to decrease in the ROS levels.

VEGF is universally accepted as the primary regulator of blood vessels throughout the body, including the retina. In this study, VEGF at protein level was found to be decreased significantly when AAM was added to CHO-K1 cells in high glucose. Alanine, arginine, isoleucine, leucine,

lysine as free amino acids showed similar effect. But this effect was varied depending on glucose. With 17 mM G only alanine, isoleucine, leucine showed the significant fall (56, 80 & 50 % respectively). Alanine, leucine, isoleucine, arginine, lysine showed fall in VEGF by 20, 35, 47, 75 & 80 % respectively when added to 27 mM G. The maximal lowering effect was seen by isoleucine, arginine and lysine wherein there is more than 75 % decrease. AAM was able to decrease the VEGF levels at all glucose concentrations though moderately compared to the isoleucine/arginine/lysine. (7 mM G = 40 %, 17 mM = 50 % & 27 mM G = 20 %). Thus, AAM seems to decrease VEGF by decreasing at various ROS signaling as shown in **Fig 9.1.**

Fig 9.1: Postulated hypothetical pathway inducing VEGF and possible AAM preventing VEGF increase.



Akt a cell survival protein leads to increase in VEGF and leads to cell proliferation. AAM decreases the Akt in high glucose and decreases the VEGF as seen in the present study.. The data on apoptosis by FACS analysis for annexin V binding shows that AAM decreases the apoptosis significantly while propidium iodide binding analyzed by FACS shows no significant change in proliferation. Thus AAM is proposed to be anti-apoptotic in nature with no proliferation inducing property as supported by VEGF and Akt expression. Long term diabetic condition leads to vascular changes where in cells undergo proliferation leading to angiogenesis.

The data on cell proliferation and apoptosis gives a clue that AAM could augment cell survival pathway but not angiogenic.

Hyperglycemia was shown to produce an increase in ROS and lipid peroxidation while also increasing the accumulation of advanced glycation end (AGE) products in bovine endothelial cells (Giardino, Edelstein et al. 1996). Lysine has epsilon amino group, which are known to bind with glucose and decrease glycation. It is a basic amino acid, which has two NH_2 group (Sulochana, Punitham et al. 1998). Alanine, aspartic acid and glutamic acid are present in large amount in the lens, which undergo non-enzymatic glycation at physiological pH and temperature. Therefore, amino acids may remove intracellular glucose and decrease the glycation of tissue proteins (Sulochana, Ramakrishnan et al. 1996). In the present study, it was found that the mixture of amino acids decreased the glycation as seen by reduced levels of AGE in serum, lens and retina in the diabetic rat model after 8 weeks induction of diabetes mellitus as supported by immunohistochemistry. Though neovascularization is not seen in animal model for DR (Rakoczy, Ali Rahman et al.), retinal changes at the level of various molecules such as growth factors, cytokines and cell adhesion molecules are known. The changes depend on the duration of the diabetes which is as early as 6 weeks -8 weeks to as long as 20- 40 weeks (Qaum, Xu et al. 2001; Joussen, Poulaki et al. 2002).

Interaction of AGEs with RAGE causes oxidative stress and activation of nuclear factor-kB (NF-kB) (Yan, Schmidt et al. 1994; Nakamura, Hasegawa et al. 2003) which modulates generation of pro-inflammatory cytokines such as IL-1α, IL-6 and tumour necrosis factor- a (TNF-α) (Wang, Vom Hagen et al. 2008). There is also enhanced expression of adhesion molecules including VCAM-1 and ICAM-1, in addition to other effects such as increased vascular permeability (Joussen, Poulaki et al. 2002). This study with AAM of 2 different composition given to diabetic SD rats showed a maximum of 30 % fall in glucose level in the AAM1 group when compared to unsupplemented diabetic group as seen at the end of 8 weeks. The AGE one of the initiator of signaling in DR was found to be significantly decreased in AAM1 composition when compared to diabetic group. The downstream of AGE, namely cytokines (IL-1, IL-2), eNOS, NO, cell adhesion (ICAM) were also significantly decreased in AAM1 treated group compared to diabetic. Diabetic retinopathy can result in apoptotic cell death of retinal neurons, and therefore significant visual loss. Determination of the number of $TUNEL^+$ cells in diabetic retina shows significant increase as compared to control *($p<0.01$)* and a

significant decrease in AAM supplemented group especially in SNAAM1 *($p<0.01$)*.Thus the animal study proved that the amino acids mixture in the study especially composition-I can significantly decrease AGE, eNOS, NO, IL-1, Il-2, ICAM in the diabetic rat retina, thereby reducing the complications of diabetes in terms of diabetic retinopathy.

Thus, the beneficial role as anti-diabetic agents by the amino acids mixture is shown by 1) Increased glucose uptake. 2) Increased GLUT4 translocation. 3) Potentiation of insulin signaling via IRTK/PI3K activation. 4) By decreasing ROS/NOS. 5) VEGF lowering effect. 6) Anti-apoptotic activity. 5) Molecular changes seen as lowering of AGE, cytokines, adhesion molecule in diabetic rat retina.

CHAPTER 10: FUTURE SCOPE

1. The effect of amino acids on insulin resistance model needs to be verified.
2. Pharmacologic activation of AMPK (AMP-activated protein kinase (AMPK) increases glucose transport in muscle, independent of the actions of insulin. Does AA increase AMPK activity? This can be addressed in the adipocytes.
3. Mechanism through which AA increases GLUT4 translocation and expression needs to be looked in adipocytes. Effect of amino acids on the Adiponectin metabolism is to be studied further.
4. Animal studies for longer duration needs to be done to see if amino acids can ameliorate the retinal changes at the level of retinal barrier changes / permeability changes
5. To establish the anti-angiogenic effect of AAM, in terms of neovascularization , oxygen induced retinopathy model of rats has to be done
6. Based on the above pre –clinical studies Phase I and phase II clinical studies needs to be done on amino acids supplementation that may prevent or postpone diabetic retinopathy and other complications of diabetes.
7. Finally a product can be arrived which shall be a mixture of amino acids that can be supplemented.

APPENDIX I

S. No	Chemicals	Company
1	CHO-K1 cell line	NCCS, Pune
2	Nutrient mixture F12 Ham Kaighn's medium (F-12K1	Sigma, USA
3	FBS	GIBCO, India
4	25 cm^2 tissue culture flask and 24 well plates	Greiner, India
5	Trypsin	Himedia, India
6	EDTA	Himedia, India
7	$NaHCO_3$	Himedia, India
8	Phosphate buffered saline	Himedia, India
9	MTT (3-(4,5-dimethyl thiazol-2-yl)2,5 diphenyl tetrazolium bromide	Invitrogen
10	DMSO	Sigma
11	L-Amino acids (Alanine, Arginine, Leucine, Isoleucine, Aspartic acid, Glutamic acid and Lysine)	Sigma
12	Insulin (human recombinant)	Sigma,USA
13	$U^{14}C$ glucose, $U^{14}C$ 2-deoxyglucose	BRIT, Mumbai
14	GLUT4 goat polyclonal IgG & Rabbit anti-goat IgG - FITC conjugate	Bangalore Genei, India
15	Anti phospho-Akt Ser 473 raised in rabbit	Cell signaling, USA
16	Anti-VEGF	Santa cruz
17	Bradford reagent	Pierce, USA
18	glycogen	Himedia, India

19	SPE cartridges (C-18)	Cayman chemicals, USA
20	Glucose oxidase/peroxidase assay kit	CPC, Spain
21	Clinical chemistry system RA50	Bayer, Germany
22	D-glucose	Sigma
23	2',7' Dichlorodihydrofluroescein Diacetate,	Sigma
24	Epinephrine	Sigma
25	eNOS elisa kit	USCN, China
26	Nitric oxide colorimetric kit	Bio vision
27	Rat sICAM elisa kit	Bi Biotech, India
28	Human Adipocyte cell	LONZA. USA
29	*Annexin-V-Alexa fluor 488*	Invitrogen
30	Propidium iodide	Sigma
31	Oil red O	Sigma
32	Preadipocyte Growth Medium, Preadipocyte differentiation Medium	LONZA. USA
32	DNase free RNase A	Bio gene, India
33	Tri reagent	Sigma
34	iScript RT-PCR Kit	BioRad
35	GLUT4 primers	Invitrogen
36	Streptozotocin	Sigma
37	Antibodies to CML-AGE, IL-1β, IL-2	Bi Biotech, India
38	Novalink polymer mini kit	Leica biosystems, UK

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LIST OF PUBLICATIONS

Articles published

1. **Selvi R**, BhuvanaSundar R , SaiJyothi A V, Sulochana K N and Angayarkanni N. Amino acids potentiate the insulin signaling in CHO-K1 at high glucose conditions. *Arch Med Res* 43;173-182:2012.
2. Ramakrishnan S, **Selvi R**, Saijyothi A V, Biswas J, Bharat Selvi M and Angayarkanni N. Clinical and Biochemical benefits of administration of vitamins E & C in patients with Eale's Disease. *Biomedicine* 32: 2012.
3. **Selvi R**, Angayarkanni N, Biswas J and Ramakrishnan S. Total antioxidant capacity in Eales disease, Uveitis and cataract. *Ind J of Med Res* 134;83-90:2011.
4. **Selvi R**, Angayarkanni N, Asma B, Seethalakshmi T and Vidhya S. Amino acids influence the glucose uptake through GLUT4 in CHO-K1 cells under high glucose conditions. *Molecular and cellular biochemistry* 344(1-2);43-53:2010.
5. Verma A, Biswas J, **Selvi R**, Angayarkanni N. Intra-ocular expression of vascular endothelial growth factor (VEGF) and pigment epithelial-derived factor (PEDF) in a case of Eales' disease by immunohistochemical analysis: a case report. *Int Ophthalmol* 30;429–434: 2010.
6. Angayarkanni N, **Selvi R**, Pukraj R, Biswas J, Bhavesh SJ, Tombran-Tink J, Barnstable CJ. Ratio of the vitreous Vascular Endothelial Growth Factor and Pigment Epithelial-Derived Factor in Eales Disease. *Journal of ocular biology diseases and informatics* 2;20–28:2009.
7. **Selvi R,** Thomas A, Angayarkanni N, Bharat selvi M, Biswas J, Ramakrishnana S and Vasanthi SB. Increase in Fe3+/Fe2+ Ratio and Iron-Induced Oxidative Stress in Eales Disease and Presence of Ferrous Iron in Circulating Transferrin. *Current Eye Research* 32;677-683: 2007.
8. Ramakrishnan S, Sulochana KN, Lakshmi S, **Selvi R,** Angayarkanni N. Biochemistry of homocysteine and diseases. *Indian J Biochem Biophys* 43;275-283: 2006.
9. Angayarkanni N, **Selvi R**, S. Ramakrishnan. Measurement of Total antioxidant capacity in Eales' disease, Uveitis and Cataract. *Insight* XXII(1);11-16:2004.

Articles under revision

1. Novel Formula Of Amino Acids : SNAAM A Potent VEGF Lowering Agent.

Patents

1. Amino acid lysine increases nitric oxide production.
2. The amino acids proline, hydroxyl proline and phenylalanine increases the secretion of adiponectin.

Patent applied

1. Amino acid composition for the treatment of diabetic retinopathy.

Articles under preparation

1. Amino acids mixture promotes glucose uptake in human adipocytes under high glucose condition.

LIST OF PAPERS PRESENTED IN CONFERENCES

NATIONAL

1. Amino acids mixture promotes GLUT4 recruitment via PI3K/AKT pathway in CHO-K1 cell line. **SBC(I), 2009, NCCS, Pune.**
2. Glucose uptake is promoted by amino acids mixture in high glucose environment – An in-vitro study on CHO cells using $U^{14}C$ deoxy glucose. **SBC(I) 75th Annual meeting,2006, JNU, New Delhi.**
3. Effect of Amino acids mixture on the CHO-K1 cell morphology, viability and glycation. **SBC(I), Annamalai chapter, 2006, Chidambaram.**
4. Homocysteinylation of plasma proteins in hyperhomocyteinemia cases of Eales' disease – a retinal vascular disease. **SBC(I) 74th Annual meeting , Nov. 7 -10, 2005, CDRI, Lucknow.**
5. Homocysteine in ocular inflammation disease – Uveitis, **SBC(I) 73rd Annual meeting , Nov. 21-24, 2004, Pant university, Uttranchal.**
6. Protein homocysteinylation in Uveitis an ocular inflammatory disease. Exclusive meet on "Opthalmic Research" in connection with annual meeting of **IERG, Aug 20th -22nd 2004, Chennai.**
7. Measurement of Total antioxidant capacity in various ocular diseases. 12th Annual meeting of the **IERG, Jul 26th – 27th 2003 LVPEI, Hyderabad.**
8. Establishment of human retinal epithelial and endothelial cell lines - **Apojee 2002, All India Academic week meeting at BITS, Pilani.**

INTERNATIONAL

1. Novel Formula Of Amino Acids : SNAAM1 A Potent VEGF Lowering Agent. **Asia-ARVO, Singapore, 2011.**
2. Glucose Uptake In CHO-K1 Cells In Presence Of Free And Mixture Of Amino Acids, **Bajaj International Conference Sep 2008 at Hotel Park Sheraton, Chennai,**

AWARDS

1. **Swarrnalatha Punshi award** for the best researcher for the year 2011-2012.
2. **Asia-ARVO travel fellowship award**, Singapore, Jan 20-23, 2011.
3. **Rajagopal Endowment award for best paper**, SBC(I) Annamalai chapter, 2006.
4. Awarded "**CCLRU travel grant**" to participate in the 12th Annual meeting of IERG held in Hyderabad from 25-27 July 2003.
5. Awarded "**Himedia Laboratories Pvt. Ltd. Endowment award**" for the year 2002 for Clinical Microbiology.

BRIEF BIOGRAPHY OF THE CANDIDATE

Ms. R. SELVI obtained her B.Sc Biochemistry degree from Jaya College, University of Madras, Chennai in 1996. She did her PG in medical laboratory technology at Loyola College, Chennai during 1996-1997. She joined Sankara Nethralaya as Lab technician in Hematology, Histopathology & Biochemistry during 1998. Then she obtained her M.S. (Medical Laboratory Technology) degree of Birla Institute of Technology and Science, Pilani in 2002 with course work at Medical Research Foundation, Chennai. Then she joined the biochemistry research department, Sankara Nethralaya as Junior Scientist and worked as junior scientist in Sankara netharalaya for 3 years and was recruited into project in 2005 project funded by Department of Science and Technology. She registered for Ph. D in Jan 2006 in Birla Institute of technology & Science, Pilani. She has made presentations in 8 national and 2 international conferences, comprising 7 poster presentations and 3 oral presentation. She has 7 publications comprising cell biology work and clinicaly in Eales' disease. 1 paper has been published and 3 papers are in review related to thesis. 2 more papers are under preparation. She has 1 Patent application been sent and 1 application to be sent. She has handled retinal pigment epithelial cell, Chinese hamster ovarian cell, 3T3L1, adipocytes. She has worked in a 2 year clinical study on "Beneficial effect of vitamin E&C in Eales' disease". She has received an endowment award in clinical microbiology during her MSMLT course, Rajagopal Endowment award for best paper, for paper "Effect of Amino acids mixture on the CHO-K1 cell morphology, viability and glycation" at Society of Biological chemist (India). She has received the travel grant award for IERG (Hyderabad, 2003) and Asia ARVO (Singapore,2011) conference. She is also involved in teaching, analyzing patients samples and is an internal auditor. Her research interests are in ocular diseases like Eales'disease, Retinopathy of premature and diabetic retinopathy.

BRIEF BIOGRAPHY OF THE SUPERVISOR

Dr. N. ANGAYARKANNI is currently the Reader in the department 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 5 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.